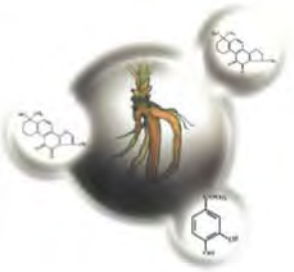
Novel Compounds from Natural Products in the New Millennium

Potential and Challenges



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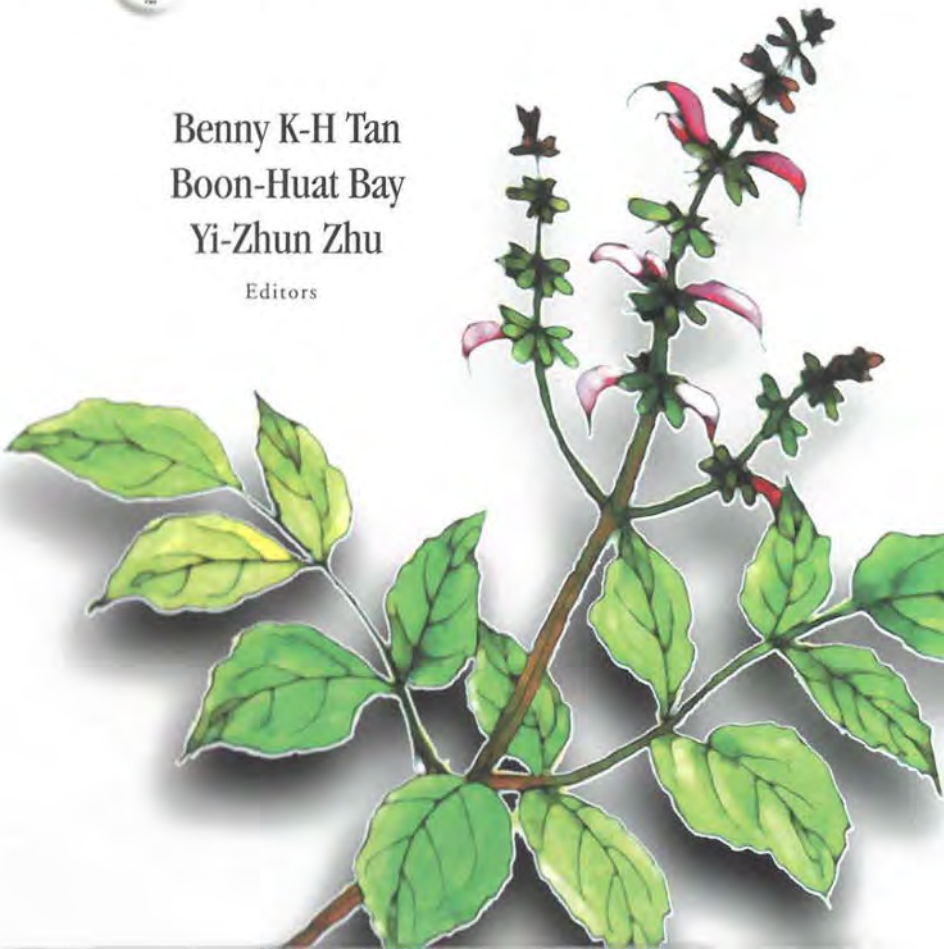

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In Appreciation to Our spouses,

Brenda Tan, Alice Bay and Sherry Zhu

for their constant help and encouragement in all our endeavours

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FOREWORD

This book is timely given the intense on-going efforts in drug discovery. The new millennium has given rise to new technology, and with it the promise for better target identification and validation. Investigators have focused this new technology on natural products as many of them hint at substantial activity if only they can be identified and further developed.

Drs. Tan, Bay, and Zhu are to be congratulated for editing this book, much of which was presented at the 2nd International Conference of the International Society for the Development of Natural Products which was co-hosted by the Department of Pharmacology, The National University of Singapore in 2002. Its comprehensive range of topics, starting with bioinformatics and structural biology, with substantial focus on cancer and cardiovascular diseases, and ending with discussions on the role of venture capital and intellectual property will be an excellent reference for all those interested in this field.

Professor John Wong
Dean
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FOREWORD

Despite the spectacular results obtained during the past years in the development of new drugs through biotechnology, genetics and genomics, natural products have still a very important role to play at the beginning of this New Millennium. In fact, only about 10% of the existing 350,000 plant species have been investigated from a phytochemical and pharmacological point of view. Among recently discovered new molecules from Nature are the antitumor agent paclitaxel from yew (*Taxus* species) and camptothecin from the Chinese plant *Camptotheca acuminata* Decne (Nyssaceae), for the treatment of ovarian and breast cancer and colorectal cancer, respectively. The aging of the population is causing new problems and challenges for scientists such as the fight against Alzheimer's disease. Two new drugs from plants, both inhibitors of acetylcholinesterase, are now used in therapy, namely galanthamine from the snowdrop (*Galanthus* species, Amaryllidaceae) and huperzine A from the Chinese clubmoss *Huperzia serrata* (Lycopdiaceae). These few examples show the existing potential of plants as a source of new drugs. In fact, Nature's architecture (not only plants but also marine organisms, insects and even animals) provides such an unpredictable range of skeletal types and novel substances that it is of immense value to evaluate as many natural products as possible in order to find sources of new drugs, new lead compounds, new pesticides and new compounds for animal health. With the introduction of high-throughput screening protocols, the capacity is present to pass large numbers of samples through a wide variety of bioassays. But for a given disease, it is not always easy to define and choose the right target for bioassays. The present book contains a couple of chapters dealing with this important topic. The whole volume gives an excellent overview of the actual trends in natural product research.

My congratulations go to the Editors of the book for putting together very diverse topics on on-going research in a still unexploited field and to the Authors for their excellent contributions showing new approaches in the discovery of bioactive molecules from Nature.

Professor Kurt Hostettmann
University of Lausanne
Switzerland

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PREFACE

There is continuing interest in natural products as sources of potentially new and exciting chemical compounds. Parallel with this growing interest is the increase in the availability of new and sophisticated technology in the area of laboratory equipment and also the emergence of intellectual areas of scientific knowledge, including bioinformatics and combinatorial libraries. Such advances enhance the research capabilities of scientists and provide them with better tools to explore further the potential of natural products.

This book seeks to support scientists in their endeavours in the field of natural product research. It brings together the knowledge, perspectives and research findings of a varied group of scientists on a wide range of topics, from microarrays, genetics and bioinformatics to yeast-based technologies and enzyme studies. The reader will find interesting the results of a range of laboratory investigations into a wide range of natural products for their biological activities in a variety of human diseases. The later chapters awaken the would-be entrepreneur to the opportunities and challenges of research and development in the natural product industry, with the concluding chapter providing helpful insights into the Intellectual Property Law.

The Editors wish to thank all the authors for their contributions and are grateful to Professor John Wong Eu Li, the Dean of Medicine, National University of Singapore and Professor Kurt Hostettmann, renowned Swiss phytochemist, for their insights in the Forewords.

The Editors

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Plants in Drug Discovery — Creating a New Vision

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INTRODUCTION

Recognizing the recent relationship between humankind and Earth, it is essential that we begin to consider, in a very broad manner, what must be the role for natural products in the future of global health care. In order to achieve this, we will survey some current perspectives of natural products, review some aspects of the contemporary practices of drug discovery and approach the question — What of the future for natural products?

It is important to acknowledge with gratefulness that plant extracts and products contribute substantially in four major areas to human health and welfare: (i) as foodstuffs, (ii) as flavoring agents and spices, (iii) as perfumes and cosmetics, and (iv) as pharmaceutical and biological agents. In the latter area we know that there are at least 120 compounds from over 90 different plant materials which are available globally as single entity prescription products [1]. In addition, because of widely divergent views of what constitutes a medicinal agent in a particular culture, there are many thousands of plant extracts and plant materials which are employed commercially in various parts of the world. For approximately 85% of the world's population (5.2 billion of 6.1 billion people), it is these plant materials which are a primary source of health care [2].

A significant evolution in both regulated and non-regulated health care in the developed countries of the North has been the rapid and dramatic increase in the availability and use of phytotherapeutic agents derived from several systems of traditional medicine in developing countries. In the United States in 1999 approximately $4.2 billion were spent on phytotherapeutic products for life-style conditions and

prevention treatments [3]. Regulation of these products differs substantially in various countries, yet consumers world-wide acquiring these products in their various forms are demanding the same assurances. Most important of these is safety, which is often reflected in plant authentication — is the correct plant (genus and species) and plant part being used? Next is whether the plant material is safe from a cytotoxic, mutagenic, and therapeutic perspective, and whether it is free of potentially toxic insecticides, pesticides and heavy metals. Is the plant material free from fungal and insect infestation, and free of radiation contamination? Adulteration of plants through the addition of biologically active ingredients, particularly with supplementation by synthetic or natural additives, is also a significant issue. Batch to batch standardization, from both a chemical and biological perspective, following a demonstration of efficacy, is also of widely increasing concern [4]. Finally, consumers are anxious that, like traditional prescription products, stability and shelf-life are established and assured for phytotherapeutic products. As natural product scientists, it is our societal responsibility to see that these minimal standards are met for consumers.

From the commercial perspective, there are four groups of companies which are involved in the future development of natural products as medicinal agents. They include the global pharmaceutical companies, the small to medium size biotechnology companies, the botanical supplement companies, and those companies, mostly in the food industry, who wish to develop natural products, either individual components or extracts, as nutraceutical agents for their beneficent effects in foods. The goals for plant natural products within each of these companies are quite different.

At this point in time, most of the large pharmaceutical companies have terminated those aspects of their drug discovery programs which are based on plant-derived natural products. The reasons for this have been discussed elsewhere [5, 6] and will be briefly mentioned subsequently. The small number of pharmaceutical companies who do continue to work in the plant area are dedicated to the evaluation and development of plant-derived natural products as templates for chemical and biological potentiation. The samples, either extracts or compounds, are evaluated as a part of a fully automated screening

program in which sample preparation, bioassay evaluation and data analysis are fully automated [6]. Philosophically, these programs are dedicated to bringing as much chemical diversity as possible to the biological screening interface. There is no consideration given to the origin of the plant-derived materials. Consequently, those plants that are evaluated are effectively a random collection with no contemplation of the chemodiversity of structural types, the functional diversity of the constituents nor any ethnomedical association of the plants.

Pharmaceutical companies recognize two significant issues with the inclusion of plant natural products in discovery programs. The first is that plant extracts are complex matrices of structurally diverse constituents which frequently provide a significant challenge for the isolation of the active principle(s). This deconvolution step may yield a known, rather than a novel, active constituent. The second consideration is that the recollection of a plant may be a time-consuming process, require extensive re-negotiations related to access (*vide infra*) and may not be biologically active. The establishment of a novel active compound as a "hit" for consideration in a timely manner compared with other "hits" therefore has become a significant issue for the involvement of natural product extracts in basic discovery programs [6]. But, since natural product extracts provide less than 3% of the chemical library to be tested (*vide infra*), the harsh realization is that pharmaceutical companies do not need natural products in their programs of primary bioevaluation.

For the biotechnology companies, there are several substantial opportunities for the involvement of natural products in a discovery or a development mode. One of these is that of translocating the genes for the production of established medicinal agents or their precursors from slow-growing to fast-growing, large biomass plants or other organisms. Another opportunity is the area of combinatorial biosynthesis, and a third is the use of tissue culture systems in order to examine the ability of plant calli or cell-free systems to produce metabolites which are not present in field-grown plants.

The botanical supplement companies are scrambling to enhance their level of plant science in a newly demanding and more competitive market place. The focus on plant identity and safety is frequently matched with the development of analytical systems for marker

compounds, active principles or adulterants. In the more sophisticated companies, these efforts have led to the evolution of specific programs dedicated to the establishment of the biologically active principles. Finally, the nutraceutical companies are beginning to explore whether there are compounds, such as cancer chemopreventive or cholesterol-lowering agents, which can be added to high-volume foods which might already be present in other dietary sources.

NATURAL PRODUCTS IN DRUG DISCOVERY — THE FUTURE

With this very brief background regarding the present role of natural products in discovery programs, it is appropriate to consider what must be the role of natural products in health care systems globally for the next fifty years. For the past fifty years we have forgotten an important axiom: that what we do for drug discovery and natural products is of prime significance for our descendants, not for us. We must be sure to leave these generations the tools for their health care. Chemicals and chemical reagents are typically a non-renewable resource, and their use depletes our future resources. Consequently, a fundamental precept for all drug discovery programs, be they synthetic or natural, must now be the concept of sustainability.

Considering our future situation with respect to plant drugs, we can observe some striking polarities. There is a global population which is anticipated to reach at least 9 billion by 2050, and a rapidly increasing technology base in the areas of automation and biological assessment. By contrast, bio- (and therefore chemo)-diversity in the world's hardwood forests is being irreversibly degraded at an alarming rate. And finally, oil stocks, a staple for the production of synthetic drugs, are projected to last only another 70-75 years at current rates of usage.

In exploring facets of the future focus of natural products in the drug discovery process in particular, there are six aspects which I would like to present: (i) access to the biome, (ii) acquisition and analysis of traditional knowledge and on-going research, (iii) biotechnology development, (iv) safety and efficacy of plant medicinal

agents, (v) dereplication studies and (vi) natural product structure diversification.

ACCESS TO THE BIOME

Article 15.2 of the Convention on Biological Diversity (CBD) proposes that signatory countries facilitate access to their biome in exchange for present and future considerations [7]. Articles 15.5 and 15.6 indicate that all collections of biological material should occur with prior consent and with the accompaniment of local scientists. Since the CBD, a number of developments have occurred which relate to the establishment of protocols and systems within countries for the approval and collection of biological materials. In some instances indigenous groups have established protocols for approval to access across national boundaries.

However, if the approval processes, or the requirements to negotiate the associated considerations are too onerous, developed country academic and/or industrial laboratories will not invest in either the people or the places, when more amenable choices (based on bureaucracy, cost and time) are available. Two situations will occur as a result. Firstly, the bio-rich developing country will not be in a position to collaborate in programs designed to evaluate its biome, and will be unable to continue to develop enhanced taxonomic, chemical and biological capabilities. Secondly, in the long term, local pharmaceutical development will be inhibited and reliance on externally acquired pharmaceutical and medicinal agents will be at least maintained, and even increased.

Simply stated, companies and academic institutions in developed countries have become frustrated by the numerous different procedures, protocols and costs being instituted by various countries and the protracted time necessary to obtain approval for access. Such bureaucracies are not acting for the betterment of natural products research either locally or internationally. Without access to biological materials, the discovery of new biologically active natural products will be diminished and advances in health care may be impeded in that country and elsewhere.

Prior negotiated agreements are an essential aspect of plant collection programs for drug discovery. In our own work in this area, we are continuously evolving new types of agreement which will satisfy the changing environment of compensation and reciprocity for a particular country or a particular region of the world. Our present model agreements establish a trust fund for royalty streams in the event that a product results. Under this plan, the country of origin of the biological material receives more than 50% of the royalty stream received by the University [8]. We are also in the process of implementing several other forms of reciprocity which can provide a more immediate form of compensation.

An alternative proposal for access rights is the development of a two-tier approval process for the collection of biological materials. One agreement would serve for the periods of time where academic/pre-toxicological research is being undertaken on limited size samples, and the other where scale-up for more advanced pharmacological and clinical studies are involved, and sustainable development as a crop is needed.

ACQUISITION AND ANALYSIS OF TRADITIONAL KNOWLEDGE AND ON-GOING RESEARCH

We are in an era of burgeoning information in science and technology, and of increasingly facile, global access to that information. Underlying such ease of access are some very serious issues regarding how knowledge of the use of indigenous plants, acquired over many years and generations, can and should be accumulated, organized and disseminated. Substantial information is already available, collected by ethnobotanists and medical anthropologists, from data acquired prior to the CBD through numerous published articles, books, reviews, etc. Unfortunately, there is no central, impartial, broadly funded location where this information could be catalogued and made available internationally for the benefit of all interested parties. Such a resource facility, perhaps supported by a group of United Nations agencies and available globally in real time, is urgently needed. In addition to ethnomedical information, there is a need to examine how data on the biological evaluation of plant extracts and their

constituents, as well as the chemistry of natural sources, and the clinical evaluation of plant extracts, can be assembled and made accessible globally.

At the same time, there are now very substantial concerns being expressed about the ethical issues involved with the future collection and analysis of information concerning indigenous systems of medicine and the intellectual property rights involved. The CBD values this knowledge in the same way as biological plant material. Substantial concerns about unrestricted and unaccountable access to ethnomedical and biological information which is already collected are also being expressed. However, for rational and ecologically-based drug discovery from plant sources all over the world, it is critically important to evaluate all of the available information in order to prioritize collection plans, to avoid the unnecessary duplication of effort and reduce the consumption of precious (fiscal, personnel and oil-based) resources [9].

BIOTECHNOLOGY DEVELOPMENT

With the completion of the human genome project, it is expected that there will be a very large number of potential new drug targets generated. For reasons which will become clear subsequently, it is absolutely essential that natural products be an integral part of the chemical diversity employed for the primary screening of target systems. Indeed, since these targets are disease-related, there may be some very specific opportunities for the evaluation of selected ethnomedical preparations. However, in order to facilitate the assessment of plant materials on a wider basis, we must reverse the paradigm of bringing plant materials from the collection site to the laboratory for extraction and bioassay. There is an urgent need for the development of genomically-based, bioassay systems which can be conducted, in the field, directly on the locally available plant extracts. This approach would permit the focus of plant collection to be shifted to those plants which have a demonstrated biological activity, and at the same time will markedly reduce the failure of plant extracts to reconfirm activity on recollection.

For over 170 years, it was tacitly accepted that the secondary metabolites isolated from a plant at a moment in time represent the biosynthetic capability of that plant. Recent work with tissue culture and cell-free systems, particularly the use of elicitor molecules such as methyl jasmonate, has demonstrated that this paradigm is inappropriate [10]. As the efforts to disclose the intricacies of plant biosynthesis, and its genetics and its diversity continue, it will be possible to assess whether secondary metabolite biosynthesis can be enhanced in order that a given organism may be induced to produce its full spectrum of metabolites. Consequently, establishing the molecular switches which modulate the activity of biosynthetic genes must be a high priority.

There are numerous aspects of biotechnology which impinge on natural product drug discovery in addition to biosynthesis. These include the development of specifically modified bioassay systems and the translocation of whole biosynthetic gene systems for medicinally useful compounds from one, slow-growing, organism to another, faster-growing, organism of high biomass. For example, the ability to produce vaccines in staple food crops in order to provide or enhance immunity to disease in a population, will be crucial for the future improvement of global health care for certain disease states [11].

SAFETY AND EFFICACY OF PLANT MEDICINAL AGENTS

The past ten years have witnessed an extremely dramatic increase in the number of phytotherapeutical products from various traditional medicine systems around the world which are entering the commercial markets in developed countries. Although the very varied regulatory requirements being applied to these products in various countries are of great concern, generally, the quality of the science (botany, pharmacognosy, chemistry, and biology) being conducted on them is steadily increasing. However, because of the absence of clear and harmonious regulations regarding quality control and marketing, and issues of safety and efficacy are being both understated and neglected. Concurrently, the whole area of the clinical interactions of prescription

and over-the-counter products with phytotherapeutical agents is substantially underdeveloped.

One of the most important inferences from the burgeoning science of phytotherapeutical products is that the literature is expanding correspondingly. Consequently, continuous evaluation of the literature relating to analytical procedures, to biological information, and to clinical trials becomes a very important facet. It is pertinent to contrast this situation with that of a regulated product where typically, at the time of marketing, there is relatively little scientific information available in the primary literature, and may originate from only one or two sources.

Concerted attempts are now being made to establish the active principles of several of the major phytotherapeutical products. In the meantime, substantial compromises are being made, in which marker compounds, which may or may not be the active principle, are frequently used to characterize extracts or plant materials [12]. A number of issues regarding phytotherapeutical products are presently being largely ignored, particularly in the United States. Quality control in the areas of pesticide levels, solvent residues, aflatoxins, radioactivity, chemical and biological reproducibility and stability, and the authentication of primary reference standards will need to be improved substantially in the near future, simply to meet international standards. It is axiomatic that the development of global markets for medicinal plants must be based on the principle of sustainability, which can lead to affordability.

It is also essential for integrated chemical and biological systems to be developed which can correlate the stability of the active principle(s) with biological activity over time, and thereby establish a meaningful shelf-life. As the active principles become known, efforts to enhance the bioavailability of herbal preparations will be more forthcoming through liposome preparations and other forms of controlled release formulation.

Extraction techniques may change over time as the need to optimize the active principle(s) in the preparation becomes apparent. Safety issues regarding the new extract will need to be re-established, and thus monographs for specific drug preparations and their standardization will need to be developed. Sustainability in the use of

solvents for primary extraction will become an important criterion in the future.

Overall, the present scientific and regulatory situation regarding phytotherapeutical agents is no longer acceptable from a public health perspective in either the developed or the developing countries. A more concerted effort by major international bodies, legislative groups, professional organizations and industrial consortia is needed to harmonize the regulations applying to the status and quality of phytotherapeutical preparations.

DEREPLICATION STUDIES

The efficient isolation of novel, biologically active natural products from complex matrices is important for the involvement of natural products in the drug discovery process. Being able to discern, in an active extract, the probability that the active principle is novel is therefore a highly desirable goal. Previously, we discussed an HPLC/ESMS/bioassay/database dereplication system for active natural products in biologically active matrices [13], and described its use for the identification of both new and known natural products in active extracts [14]. Refinements in this system in the future will be three-fold. Firstly, to develop software which can link the mass and biological data to the NAPRALERT database so that searches and conclusions can be achieved on-line. Secondly, continuous flow NMR will be added in order to further support the projected structural conclusions regarding the identification of active masses. Finally, the database system will be expanded and enhanced to be able to recognize carbon-13 NMR data in order to identify with a high degree of certainty what is the possible skeletal structure and functional group disposition of a molecule within the matrix. Undoubtedly, we will move closer to the ability to characterize the majority of the known constituents in an extract without isolation.

A further important use of this HPLC/ESMS/bioassay system technology will be for the development of techniques for the chemical and biological standardization of phytotherapeutical samples on a batch to batch basis, once the appropriate active principle(s) have been identified.

NATURAL PRODUCT STRUCTURE DIVERSIFICATION

Pharmaceutical companies are focused on bringing as many discrete molecular entities as possible to a particular screen for biological evaluation at a given point in time; hence the need to compile and develop libraries of synthetic and natural products and extracts. But this natural product structure diversity still represents a very small proportion (3% or so) of the samples which are biologically evaluated.

If this paradigm is to change for plant natural products, it will be important to develop new approaches to enhancing the number of natural chemical substrates from a given milieu which can be made available for biological screening. In the past, this has occurred by expanding the collection of plant extracts or by acquiring purified natural products from academic laboratories. Consideration must now be given to alternative strategies to producing large numbers of natural products. One of these approaches is that of combinatorial chemistry in which a template can be amplified through successive chemical manipulations [15, 16]. This technique is finding increasing interest as far as natural products are concerned, although mostly in academic settings [17]. Peripherally related to this strategy is the technique of applying solid phase stabilized enzymes to natural product mixtures and examining the changes in biological activity which have been induced.

In combinatorial biosynthesis, gene segments responsible for specific chemical steps in a biosynthetic pathway can be deleted, moved or added to other pathways, thereby creating the potential for the formation of new natural products, depending on the substrate specificity of the individual enzymes involved. Thus far, this work has been conducted primarily in *Streptomyces* systems producing macrocyclic polyketide metabolites [18, 19]. Unfortunately, it will some time before such a strategy can be fully applied to plant systems, since evidence to date suggests that these biosynthetic enzymes are not clustered, and indeed may be located in several parts of the cell for a given pathway, making resequencing impractical [20].

As more natural product biosynthetic pathways are examined at the enzyme level, it will be possible to isolate these enzymes, and identify, clone and express the genes responsible for these selected,

chemical steps. The question is whether these enzymes can be produced and stabilized on solid matrices such that they are available for use as chemical reagents. In addition, the opportunity exists for resequencing the enzyme order externally. Substrate specificity would then become *the* controlling factor for novel metabolite production.

Another potentially interesting route to modulating natural product chemical diversity in an extract is to conduct discrete chemical reactions directly on the extract. Surprisingly, this does not appear to have been attempted as yet. One can envisage either an active or an inactive extract being analyzed initially by the HPLC/ESMS/ bioassay system. After induced chemical modification through several carefully selected standard techniques, the modified extract is re-evaluated both biologically and chemically.

It is manifest that the full biosynthetic potential of a given organism (plant, fungus, bacteria) is unknown. Thus, it is not appropriate to think of any extract of a plant sample as comprising the sole representative constituents of that plant. The reason is that the constituents are being extracted and analyzed only at a single moment in time, ignoring diurnal metabolic flux, seasonal variation in enzyme activities, and, more importantly, the biosynthetic genes which are present, but not fully functional. Although some modifications to metabolite production are possible through media modification, at this point, the outcomes are totally unpredictable. Consequently, it will be very important in the future to be able to modulate these genes, and thus the enzymes they operate, in a full and controllable manner. Only in this way can the biosynthetic potential of a given plant system be optimized over time, such that the extracts deliver their full range of chemical diversity.

Another consideration to enhancing structural diversity from a given natural product source is that a plant is regarded as a single organism. However, it is now quite clear that typically there are numerous fungi, bacteria, and, in some cases, algae, symbiotically associated with the plant, which are also capable of independent biosynthetic production. The ability to explore this potential from a chemical perspective is an area of biology which promises substantial potential from the perspective of enhancing the range of natural product structures in a sustainable manner. Similarly, there are those microbes

present in soil which are difficult to culture, but which, through making eDNA cosmid libraries in *E. coli* and screening for viable clones, may produce biologically active secondary metabolites [21].

CREATING A NEW VISION

Our vision for natural products embraces the concept that natural products will be increasingly important as fossil-based resources are depleted while the global population increases. Our vision must therefore involve the creation of new paradigms for the conduct of the natural product sciences on a global basis, including new balances, new alliances and new values. We will begin with the creation of new balances.

Humankind probably cannot survive another century as destructive of the Earth's resources as the 20th century. We left that century with one-eighth of all plant species threatened, 50% of bird species likely to become extinct in the next 50 years, and oil resources scheduled to last about 70 years. Before we can begin to address restoration of a balance we must see this as our destruction and our restoration. Simply put, we are not separate from Earth, but an integral part of all that is nature [6].

We must be very mindful of the balance between the conservation of the existing rain forests and the destruction of these fragile and deeply interwoven ecosystems and their deforestation for crop and grazing lands. We do this for ecological, climactic and geological reasons and for reasons of maintaining bio-, and therefore chemo-, diversity. As new medicinal plants are introduced into commerce, we must be very clear that our plans for their production to fill the market niche are sustainable and that wild-crafting does not move medicinal plant populations out of balance. We must strive for a balance between intellectual property rights and the burgeoning technology of drug discovery. A balance between those who are the holders of the biodiversity and the indigenous knowledge and those who would potentiate (create value) in that biodiversity for the health and economic benefit of all parties.

International development efforts for new medicinal and biological agents will require the creation of numerous new alliances

and the substantial strengthening of those already in place. These alliances must be both local and global in their structure and involve individuals who can set aside their ego for the greater good of the whole program. There are several examples of such well-organized and funded collaborations at this time [6], including programs which, rather than being solely academic in nature, have a strong industrial partner. Many countries around the world would do well to examine the structures of these programs as a potential model for collaborative investment to potentiate the development of local medicinal plants and natural products. The need for in-country capacity necessitates that such alliances be part of the backbone of a sound health care program. In order to accomplish these goals, though, we must create value; value in places, in people and in plants.

The poignant question asked by the American naturalist philosopher Ralph Waldo Emerson still rings true today, "What is a weed?" A plant whose virtues have yet to be discovered. One of our prime responsibilities as natural product scientists to the future generations is to demonstrate the value of biodiversity through new discoveries of plant-derived medicinal agents and raise awareness of the importance of plants in our everyday lives for health purposes. Such efforts will require the establishment of solid linkages which unite the interests of environmental preservation, medicinal plant research and drug discovery, and the development of the agro-industrial enterprise. As an example, countries should be examining their imports of finished pharmaceuticals and natural products (essential oils, flavor and fragrance materials) with a view to developing or expanding the local capacity to produce these materials. Is it possible that such programs could lead to a reduction in imports and increased exports?

There are very interesting programs underway to examine the potential for various crops, including corn, rice, potato, taro, etc to be the mode of production and distribution of important vaccines and medicinal agents [11]. While there are important ecological concerns regarding these genetically modified crops, they may be the only economically effective way to bring preventative health care to hundreds of millions of people. In addition, local production of such health care agents will aid in developing the science and the technology of the country, as well improving the economic base.

Creating value in places also means the location of where the work will be conducted. It will be necessary to invest in the development of academic centers of research excellence. One approach is to identify and support key laboratories in certain areas of science or in specific physical locations. Another strategy is to choose multiple groups in various universities for investment, while an alternative is to foster collaborative relationships with academic institutions in developed countries or with pharmaceutical industry on highly targeted areas reflecting a local health care need.

Scientists with appropriate background and training to the highest levels necessary for both supervising and doing the experimental work in research and development programs are also required. Thus governmental support for more PhD and postdoctoral programs, both in country and abroad, which can provide the manpower necessary are needed. In addition, for those who received their PhD several years ago, specialised training programs may be necessary to establish specific areas of expertise in chemistry, instrumentation or bioassay technology.

CHALLENGES FOR THE FUTURE

For the natural product sciences to contribute fully to global health care 10 years from now and beyond, we have seen that there are a number of very significant challenges that we need to be considering and acting on at the present time. The first is to catalogue and preserve the bio- and chemo-diversity of the rainforests and the oceans for the benefit of future generations. There is a need to catalogue the eco- and ethno-information on plants and the chemistry and biology of their products in central locations where the information can be collated and analyzed and accessed globally in real time. In order to potentiate the benefits to be derived from the studies of the biota of the world, there must be equitable access to the biome and substantial assurances of intellectual property rights and investment commitments. In order to protect the strains of medicinal plants that are presently being used in various parts of the world, there is a need for the development of medicinal plant germbanks in selected locations throughout the world. As plants are brought to the market place for

health care purposes, there must be assurances that these materials are being developed in a sustainable manner. Natural products, both individual compounds and extracts, must be evaluated using the most current advances in drug discovery technology (automation, genomics, and bioassay targets). Rather than bringing dried (or fresh) plant materials to the laboratory for extraction and bioassay, there is a need to develop genomics-based, in-field bioassays which can evaluate plant extracts on site, so that recollection of an active plant can be made efficiently. There is a need to expand the number of natural products presented to systems for biological evaluation through chemistry, combinatorial synthesis, combinatorial biosynthesis, and other strategies. More feasibility and safety studies are needed to evaluate the potential for large scale drug and vaccine delivery through the use of genetically modified crops. The safety and efficacy, and the long-term consistency of phytotherapeutical products must be assured for consumers on a global basis. Integrated global alliances are needed both in-country and between developed and developing countries for medicinal plant product development, and the facilities, the infrastructure, and the personnel who are needed to conduct the above programs.

The missing component is: how will these goals be achieved? Our vision is that countries will have an infrastructure that will allow them to develop their own sustainable medicinal agents from natural sources based on the quality of their natural product sciences. To achieve this will require programs to assist countries to potentiate their resources, their facilities, and their scientists in order to evaluate and standardize natural product-based medicinal agents on a sustainable basis for their health care systems. It is my hope that there will evolve in the years ahead a Global Alliance for Natural Product Development. Such an alliance would be composed of international agencies (WHO, UNIDO, UNDP, NATO, EU, etc.), government agencies (NIH, NSF, NIE, SRC, DAAD, etc.), pharmaceutical companies, academic institutions, non-government organizations (WWF, WRI, CYTED, TRAMIL, IFS, TWAS, etc.), scientific societies (IUPAC, RSC, ASP, PSE, GA, JSPS, etc.), and major foundations (Ford, Gates, MacArthur, Rockefeller, etc.). It

will be an important coordinating component in the development of natural product-based drugs for health care.

CONCLUSION

Natural products in the form of purified active principles and plant extracts are recognized as the cornerstone of primary health globally, and in developed countries for the amelioration of lifestyle conditions.

The challenges for health care in the future remain significant. For the major lethal diseases in both the developed and the developing worlds there are no truly effective drug treatments. Drug resistance to existing chemotherapeutic regimens for fungal and bacterial infections, for AIDS, for cancer, and for malaria, is increasing in an unabated manner. Overall health care is, however, improving, life expectancy is being enhanced, and more children are surviving. All are contributing factors to the anticipated continuing dramatic rise in the global population. At the present time, it appears unlikely that we will have adequate resources of Northern-style medicinal agents for a population of 9 billion by the year 2050. Consequently, medicinal plants must be an essential element in any global health care strategy.

This overview has briefly illuminated what might be required in order to enhance the efficiency of natural products in the discovery process and improve the level of chemical diversity which is presented for biological evaluation on a sustainable basis. What is required for future global health care is to enhance the natural product sciences, and the plant sciences in particular, on an international basis. For those developments to be achieved globally, there is a profound need at the international level for multi-centered research partnerships to develop the chemical and biological sciences. Such development must take place through investment in both facilities and training programs which focus on the development of local natural products which can enhance health care.

This paper is therefore a call to critical decision-making and action by governments, international agencies and pharmaceutical companies to commit to the sustainable development of natural

products as medicinal agents for an unprecedented level of human inhabitation of Earth.

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The Bio-Extraction Industry in Jamaica: Potential and Challenges

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INTRODUCTION

A number of Jamaican plants have been harvested both from the wild and from cultivation and exported as raw materials, and in a few cases extraction facilities have been established in Jamaica. Some of the most important plants harvested and which have export potential are described below.

Some Economically Important Plants of Jamaica

Logwood *(Haematoxylum campechianum* [Caesalpiniaceae])

The use of logwood as a dye was described by Robert Hooke as early as 1650. It is believed to have been used by the Amerindians of the region long before then. It is a plant native to Mexico, hence its species name, Central America and tropical South America. The plant was introduced to Jamaica in the early 18th century and the country became an important source of the tree for the extraction of its dye in the 18th and 19th centuries [1, 2]. The south coastal town of Black River was the center of export, and because of its economic activity and development became the first town in Jamaica to have installed electricity. In the early 20th century, a large bioextraction plant was established in Kingston, processing over fifty tonnes of the wood per production day.

The heartwood contains up to 10% haematoxylin (Figure 1), a colourless to pale yellow crystalline substance extracted by hot water. This is oxidized to several oxidation products, the main one being haematein (Figure 2), a dark purple to black dye.

With the development of less expensive synthetic dyes, the demand for haematoxylin fell and the extraction plant ceased operations

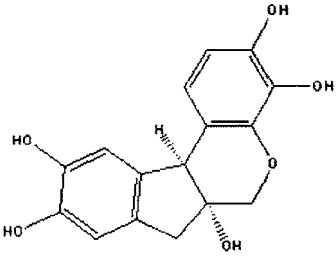

Figure 1. Haematoxylin

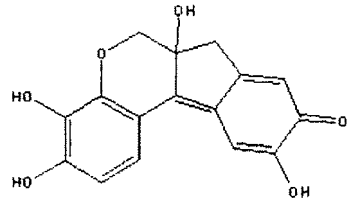

Figure 2. Haematein

in the mid 20th century. There is still some demand for the dye for use on fine fabrics such as silks and as a histological stain. Besides the use of the tree for dyes, the flowers are the source of the much sought after aromatic logwood honey. Before its closure, the bioextraction plant in Kingston also extracted another tree, *Chlorophora tinctoria* (Moraceae), a source of brilliant yellows.

Sugar cane (*Saccharum officinarum* [Graminae/Poaceae])

Sugar cane is a native plant of South East Asia that was introduced to the West Indies, and is important to the economies of a number of countries in the region. In Jamaica, there have been many calls for the further development and diversification of sugar into other products, as the market for sugar is now very competitive [1, 3].

Some of our most successfully developed products from sugar cane are our rums and rum liqueurs. The value addition and consequent economic benefits of the rum industry to Jamaica is a good example of the comparison of a successfully developed by-product, with considerable market positioning and segmentation, and sugar itself, a commodity product. Apart from sugar and rum, molasses, alcohol and vinegar are produced from sugar cane. The development of other by-products and use of the waste products in the processing of sugar canes is an area which deserves attention. In addition, there are many inefficiencies in the processing of sugar which reduce profitability. Table 1 gives data on the output of the sugar industry in the years 1999-2001.

Table 1. Sugar Industry Production Data, 1999 – 2001 (Source: STATIN [Statistical Institute of Jamaica])

Year	Product			
	Sugar	**Molasses**	**Rum**	**Alcohol**
	(Metric tonnes)	(Metric tonnes)	(000's litres)	(000's litres)
1999	201,319	85,120	73,367	86,983
2000	209,825	19,709	19,836	23,703
2001	205,128	319	253	194

Sarsaparilla and *Chainy Root*, *Smilax regelii* and *Smilax balbisiana* (Smilacaceae)

Jamaican sarsaparilla has been exported from the island as a raw material since the 19th century. It has been listed in the U.S. Pharmacopeia from as early as 1820 to 1910, and is regarded as GRAS, generally recognized as safe. It has a wide range of claims: energy restorer, tonic and aphrodisiac, antibiotic, hormone regulator, blood purifier, general health restorer for nervous system disorders, for premenstrual syndrome, and for use after childbirth [4-6].

Chainy Root, which may be a corruption of China Root, is like sarsaparilla from the Western Hemisphere. It is less used than sarsaparilla but is regarded locally as a tonic and aphrodisiac and prepared in tonic wines and other extracts. Recent research on some Chinese species confirm that these are antibiotics, effective in the treatment of venereal diseases and leptospirosis, anti-cancerous, and also aid short-term memory [7-9].

Sarsaparilla is cultivated in Jamaica but it is reported that demand exceeds supply. STATIN reported exports of approximately 7 tonnes in 2001 and one of our national export agencies (JETCO, the Jamaica Export Trading Company Ltd.) reported a demand for 50 tonnes of the dried roots.

There are several commercial opportunities for *Smilax spp.* Cultivation could be increased to satisfy demand but a better route would be to provide an extract, and even better to isolate the active principles or develop products from the extracts of the roots. *Smilax*

spp. are known to contain a number of plant sterols and saponins. The local species appear to be good candidates for chemical isolation, analyses and characterisation of the active principles. This would afford value addition with increased economic benefits.

Annatto (*Bixa orellana* [Bixaceae])

Annatto is native to the Western Hemisphere and is the source of an edible red dye. It is used as colouring for a number of foods eg. yellow colouring for butter and margarine; orange colouring for cheeses and red colouring of the flesh of some fish. It is also used in cosmetics. The active principle is bixin and nor-bixin (Figure 3), which are extracted from the crushed seeds with hot water. Bixin is the cis form of a mono-methyl ether of a carotenoid, carboxylic acid.

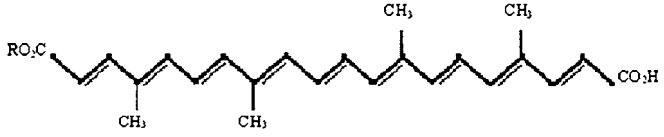

Figure 3. R=CH_3 in Bixin; R=H in Nor-bixin

The average yield of the active principles is about 2% but high yielding varieties produce up to 3%. Present world trade is about 7,000 to 9,000 tonnes of seeds (Table 2). The main producer is Peru, followed by Kenya, where it was introduced. Jamaica's production has fallen significantly in recent years and exports are presently negligible.

Table 2. Present world suppliers of Annatto
(Source: Natural Resources Institute [NRI])

Supplier	**Quantity (tonnes)**
Peru	4,000
Kenya	1,500
Others	1,500

Some other potential uses for annatto are as insect repellant and as a good antidote for prussic acid (HCN) produced from improperly prepared foods such as cassava, *Manihot esculenta* (Euphorbiaceae). A possible alternative market to explore is that for of bixin and nor-bixin, instead of the unprocessed seeds.

Cola Nuts (*Cola acuminata* [Sterculiaceae])

Cola nut is a native of West Africa, which has been introduced to the West Indies. It is used in large quantities in the soft drink industry. The active principles are caffeine (Figure 4) and theobromine (Figure 5), which are both stimulants. The active principles are also contained in a related plant, native to the New World, *Theobroma cacao* (Sterculiaceae), the source of cocoa and chocolate.

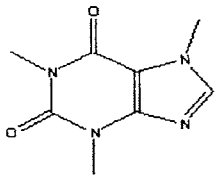

Figure 4. Caffeine

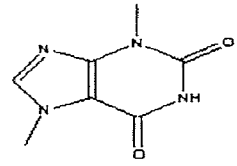

Figure 5. Theobromine

Jamaica is an exporter of cola nuts to the USA for use in soft drinks. Exports for 2001 were approximately 0.5 tonnes at a value of J$27M (Rate US1$ = J$48 approx).Other reported uses for cola nuts are anti- diarrhea and as an antidote for poisons.

Pimento leaf oil (*Pimenta dioica* [Myrtaceae])

Pimento is a native of Jamaica and Central America. Jamaica is the world's leading supplier of pimento, the spice, and pimento leaf oil, which is used in foods and as an essential oil in the fragrance industry. In order to protect our position in the market Jamaica should be value adding this industry more than we are presently.

The main ingredient (95% –96%) in pimento leaf oil, which constitutes 0.8% of the leaves, is eugenol (Figure 6), with a small amount of methyl-eugenol. Eugenol is also the main principle of a related plant, cloves (*Syzygium aromaticum* [Myrtaceae]).

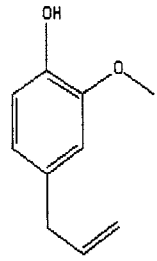

Figure 6. Eugenol

Exports of pimento leaf oil in 2001 were approximately 25,000 Kg at a value of US$1M. Jamaica produces a range of pimento products, such as spice mixtures, liqueurs and soaps. Some of the issues facing increased production are the need for policies relating to conservation and sustainability. More cultivation is needed while production, being seasonal, is available only for about six months of the year.

Some Other Plants:

Bitterwood (*Picrasma excelsa* [Simaroubaceae])

Another plant exported as a raw material is bitterwood. This plant has an extract used in the beverage industry, and is another candidate for possible extraction and value addition.

Ginger (*Zingiber officinale* [Zingiberaceae])

Jamaican ginger has had a reputation for its rich aroma and provides the ginger oil used in the original Canada Dry ginger ale. Recently production has fallen dramatically despite considerable export demand. The crop is subject to fungal attack in cultivation and the dried root, which is exported, is subject to infestation by insects. The problems need to be solved to increase our production and satisfy export demand.

Some Other Areas:

The Natural Products Institute is undertaking a detailed investigation of a number of plants and later animals with potential for an economically feasible bioextraction. Our research has been in a number of specific areas, and can be classified as the following:

Functional Foods. There are a number of plants which have the potential to enhance a number of foods and are under investigation.
Nutraceuticals/Pharmaceuticals. Some areas of investigation in this area are as anti-hypertensives, anti-diabetics and anti-arteriosclerotic agents.

Botanical Pesticides. These are proven to be environmentally more favourable than synthetic pesticides in that they generally degrade more readily and into less harmful end products. Our investigations also indicate that these will be less expensive to farmers and would be available primarily from locally obtained raw materials. Some of the pests targeted are the coffee berry borer, cattle ticks and cruciferous crop pests.

Essential Oils. There are a number of plants which offer considerable potential for extraction and development of essential oils. These could enhance the profitability of the pimento leaf oil extractors in the island. With the seasonality of the pimento leaf oil-bearing tree, which lasts only 6 – 7 months of the year, the plants tend to be "idle" during the off-season period, and other essential oils would be a welcome source of additional revenue, if extracted.

Some Issues and Challenges

Need for value addition and product development

There are still many plants which are exported from Jamaica as raw materials. The economic benefits would increase if these were extracted and more so if the active principles were isolated and marketed and further products developed from these. From such processing, our foreign exchange earnings would increase, agro-industries would develop, employment would be created and some of the problems related to rural migration to our cities could be addressed.

The marketing of commodity-type products offers little protection from competitors with more arable lands and more efficient production. The Jamaican rum is one example of a commodity which provides considerable value addition and is protected by market positioning.

Need to search for and develop new crops and candidates for bioextraction

Many of our major crops, e.g. sugar, bananas and coffee were introduced in colonial times. We need to be more pro-active in the search for potentially new crops and candidates for bioextraction. We have developed new crops in the past. The ortanique, as an example, was developed in Jamaica from a cross between an orange and a tangerine. It is a successfully exported crop.

Conservation and Sustainability

For many of the trees harvested for export, little conservation is practiced and there is a real danger that these could be over-exploited. Policies and programs need to be stated for sustainability of these crops. A part of the problem is the lack of an inventory of the stocks of the trees and their distribution and location. The Natural Products Institute together with the Institute of Jamaica are jointly attempting the establishment of a National Plants Biodiversity Data Base to address this problem.

A policy document for bio-prospecting in Jamaica is being developed by the Government with contribution from the Natural Products Institute. Indeed, with its rich biodiversity, Jamaica is attractive to multinationals in their search for new pharmaceuticals and other extracts.

The rationalisation of land use also needs attention, and issue of houses being constructed in primary forests and agricultural lands need attention.

Jamaica has been developing aquaculture as an economic activity. Red and Silver Tilapia are pond-raised for local consumption and for export. The island recently had two marine shrimp and one fresh water shrimp hatcheries. One of the marine hatcheries was closed recently but the others are successful and are considering expansion. One of our more profitable exports is conch, *Strombus gigas.* These are presently harvested from the wild. While the wild population may be presently sufficient, research into farm rearing could be of extreme value in the future when wild stocks become threatened. The process of farm-rearing conch has the prospect of producing large numbers to meet increasing demand for conch. A company in the Turk and

Caicos Islands has been successful in hatching the larvae in farms. A joint venture with Jamaican companies is a possibility.

Education

As a country Jamaica needs to invest more in education in general and science and technology education in particular. Not only will trained personnel be required to equip factories, they will also be the conceivers and implementors of new ideas and ventures.

Infrastructure Development

Most of the plants with potential for bioextraction come from the rural areas. Infrastructure such as roads, water, electricity and telephones are required for the development of these areas. Like many other countries, Jamaica suffers from an urban drift where large numbers of especially the young migrate to the towns to find employment, since this is not available in the country. As a country, we suffer from a relatively high crime rate. Rural development and employment are two possible important solutions to this problem.

Economic Policies for Development

Recently there has been a reduction of productive enterprises in favour of trading. We have a widening trade deficit with imports exceeding exports. Services and similar net inflows have evened the equation but more foreign exchange earnings are required. There is a need for investments into manufacturing and production and we need incentives and policies to encourage these. The cost of capital and interest rates are high and are disincentives to investment in manufacturing and production industries. Similarly, the cost of utilities is presently skewed against large consumers such as factories. These problems need to be examined and addressed.

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Structural Studies of Drug Metabolising Enzymes and the Relevance to Natural Product Development

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INTRODUCTION

The exposure to a vast array of xenobiotics (foreign chemicals from sources such as drugs, food additives, cigarette smoke, cosmetics and various natural products) is frequent and inevitable and depending on their toxicity, xenobiotic elimination from the body system is crucial. Since many xenobiotics that penetrate cell membranes are lipophilic, chemical modifications that render them more hydrophilic are necessary for effective removal via the urine, for example. For toxic hydrophilic compounds, modifications that make them more inert are equally important. The biological catalysts responsible for these chemical modifying processes are known as the drug metabolising enzymes (DMEs).

These enzymes have activities with characteristics like a double-edged sword, catalyzing pathways that make xenobiotics more active in addition to deactivation processes described above. A generalized scheme representing this dual role is illustrated in Figure 1. The formation of reactive intermediates [1] although rare, has led to DNA binding and has been linked with various carcinomas of the bladder [2,3] and colon [4], among others. Biotransformations catalysed by the DMEs are also responsible for the generation of metabolites for the needed pharmacology in the case of pro-drugs. Each pathway is subject to variations in dietary, genetic and environmental impacts which govern the efficacy and toxicity of xenobiotics. It is well accepted that the study of xenobiotic interaction with these DMEs is the prudent approach to gaining a mechanistic insight into and avoiding adverse effects associated with numerous xenobiotics.

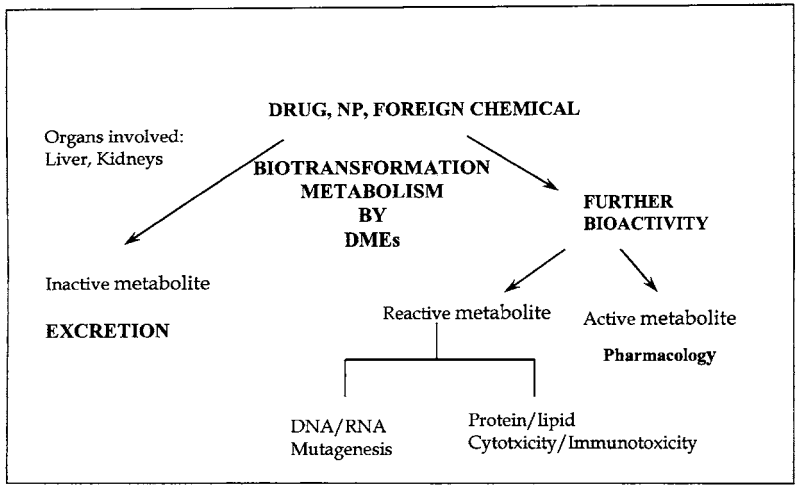

Figure 1. Schematic diagram illustrating the dual role of drug metabolizing enzymes in drug toxicity and efficacy.
(**NP** = Natural product).

To help classify the plethora of reactions, the DMEs are broadly classified as phase I and phase II enzymes, where the latter usually (but not always) act upon a substrate which has been first chemically functionalised by the phase I enzymes. The cytochrome P450 superfamily of enzymes are an example of phase I enzymes while arylamine N-acetyltransferase is an example of the phase II enzymes. The studies of both these enzymes will be discussed in this paper.

THE IMPORTANCE OF DRUG METABOLISM IN DRUG DEVELOPMENT

Drug development is a costly and time-consuming process. In the year 2001, the pharmaceutical industry spent US$44 billion on research and development with an average expenditure of US$800m to get a drug to market (from a report by Tufts Centre for the study of drug development) [5]. However, 70% of the cost of development is lost in drugs that do not make it to the market, with roughly 3/5 of drugs in clinical development faltering mainly due to toxicity or other safety issues. It is needless to state that predicting these toxic effects early and preventing many failures at clinical trials would benefit the

industry tremendously. A sensible approach then would be to align the study of drug metabolism and pharmacokinetics with the process of drug discovery [6].

The aspirations of efficient medicinal product development lie in being able to predict the human pharmacokinetic properties associated with drug absorption, clearance, distribution, metabolism and elimination. The ability to provide useful clinical results from computer models based on the structure-activity relationship data is still in its infancy. However, there are many expectations from pursuing this route, as three dimensional models of various drug metabolising enzymes emerge, along with advances in *in-vitro* techniques that can provide physiologically relevant information to validate these models. Hepatocytes, microsomes and recombinant proteins have, in combination, provided means for studying drug clearance *in vitro* (see [7] for details). Advances in DNA technology which have paved the way for generating sufficient quantities of recombinant proteins have been pivotal in enabling biophysical studies to provide the structure-activity relationship (SAR) data. This paper will highlight some such studies on the recombinant proteins, cytochrome P450 and arlyamine N-acetyltransferase.

CYTOCHROME P450 (CYP) ENZYMES

Named after the absorption maximum at 450nm of its ferrous-CO complex (8), the heme containing P450 family of enzyme has been extensively studied due to the key role these enzymes play in the activation and deactivation of a vast array of xenobiotics of toxicological and pharmacological importance. More than 500 distinct P450 genes have been identified in all species and it has been estimated that humans may possess as many as 60 distinct genes [9]. This multiplicity has given rise to a standard nomenclature based on sequence similarity of the resultant proteins [10]. In general, CYP1, CYP2 and CYP3 families code for enzymes which are primarily responsible for the reactions that lead to drug, natural product, promutagen, and procarcinogen metabolism. CYP3A4, the largest constituent of P450s in the liver, is responsible for metabolising over 90% of the known drugs in the market [11].

The relevance of studying Cytochrome P450s (CYPs) in the natural product industry

CYPs are involved in the metabolism of almost all drugs in the market today and have been shown to be crucial in the metabolism of a variety of natural products. They are linked to numerous drug-drug interactions [12]; of particular interest has been the issue of drug-natural product interactions which has received much attention both in the scientific and non-scientific communities, given the global trend of increasing consumption of natural products. Table 1 illustrates a few examples, the involvement of specific CYPs, their toxicological and pharmacological impact and the mechanisms responsible for such interactions.

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Structural Studies of Drug Metabolising Enzymes and the Relevance to Natural Product Development

Rupika Delgoda
Natural Products Institute, University of the West Indies, Mona, Jamaica, West Indies

INTRODUCTION

The exposure to a vast array of xenobiotics (foreign chemicals from sources such as drugs, food additives, cigarette smoke, cosmetics and various natural products) is frequent and inevitable and depending on their toxicity, xenobiotic elimination from the body system is crucial. Since many xenobiotics that penetrate cell membranes are lipophilic, chemical modifications that render them more hydrophilic are necessary for effective removal via the urine, for example. For toxic hydrophilic compounds, modifications that make them more inert are equally important. The biological catalysts responsible for these chemical modifying processes are known as the drug metabolising enzymes (DMEs).

These enzymes have activities with characteristics like a double-edged sword, catalyzing pathways that make xenobiotics more active in addition to deactivation processes described above. A generalized scheme representing this dual role is illustrated in Figure 1. The formation of reactive intermediates [1] although rare, has led to DNA binding and has been linked with various carcinomas of the bladder [2,3] and colon [4], among others. Biotransformations catalysed by the DMEs are also responsible for the generation of metabolites for the needed pharmacology in the case of pro-drugs. Each pathway is subject to variations in dietary, genetic and environmental impacts which govern the efficacy and toxicity of xenobiotics. It is well accepted that the study of xenobiotic interaction with these DMEs is the prudent approach to gaining a mechanistic insight into and avoiding adverse effects associated with numerous xenobiotics.

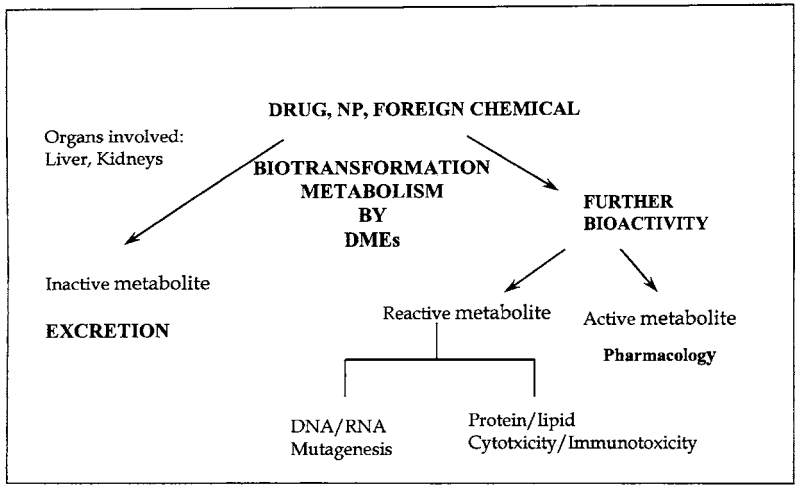

Figure 1. Schematic diagram illustrating the dual role of drug metabolizing enzymes in drug toxicity and efficacy.
(**NP** = Natural product).

To help classify the plethora of reactions, the DMEs are broadly classified as phase I and phase II enzymes, where the latter usually (but not always) act upon a substrate which has been first chemically functionalised by the phase I enzymes. The cytochrome P450 superfamily of enzymes are an example of phase I enzymes while arylamine N-acetyltransferase is an example of the phase II enzymes. The studies of both these enzymes will be discussed in this paper.

THE IMPORTANCE OF DRUG METABOLISM IN DRUG DEVELOPMENT

Drug development is a costly and time-consuming process. In the year 2001, the pharmaceutical industry spent US$44 billion on research and development with an average expenditure of US$800m to get a drug to market (from a report by Tufts Centre for the study of drug development) [5]. However, 70% of the cost of development is lost in drugs that do not make it to the market, with roughly 3/5 of drugs in clinical development faltering mainly due to toxicity or other safety issues. It is needless to state that predicting these toxic effects early and preventing many failures at clinical trials would benefit the

industry tremendously. A sensible approach then would be to align the study of drug metabolism and pharmacokinetics with the process of drug discovery [6].

The aspirations of efficient medicinal product development lie in being able to predict the human pharmacokinetic properties associated with drug absorption, clearance, distribution, metabolism and elimination. The ability to provide useful clinical results from computer models based on the structure-activity relationship data is still in its infancy. However, there are many expectations from pursuing this route, as three dimensional models of various drug metabolising enzymes emerge, along with advances in *in-vitro* techniques that can provide physiologically relevant information to validate these models. Hepatocytes, microsomes and recombinant proteins have, in combination, provided means for studying drug clearance *in vitro* (see [7] for details). Advances in DNA technology which have paved the way for generating sufficient quantities of recombinant proteins have been pivotal in enabling biophysical studies to provide the structure-activity relationship (SAR) data. This paper will highlight some such studies on the recombinant proteins, cytochrome P450 and arlyamine N-acetyltransferase.

CYTOCHROME P450 (CYP) ENZYMES

Named after the absorption maximum at 450nm of its ferrous-CO complex (8), the heme containing P450 family of enzyme has been extensively studied due to the key role these enzymes play in the activation and deactivation of a vast array of xenobiotics of toxicological and pharmacological importance. More than 500 distinct P450 genes have been identified in all species and it has been estimated that humans may possess as many as 60 distinct genes [9]. This multiplicity has given rise to a standard nomenclature based on sequence similarity of the resultant proteins [10]. In general, CYP1, CYP2 and CYP3 families code for enzymes which are primarily responsible for the reactions that lead to drug, natural product, promutagen, and procarcinogen metabolism. CYP3A4, the largest constituent of P450s in the liver, is responsible for metabolising over 90% of the known drugs in the market [11].

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Bioinformatics — Species Diversity Databases: Tools for Facilitating Natural Product Research

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INTRODUCTION

Molecular Bioinformatics has acquired great importance due to its recent application in vast amount of data generated in the Human Genome sequence projects. The nature and priorities of bioinformatics research and applications are changing in several ways, viz. comparative genomics, functional genomics, proteomics and structural genomics.

Bioinformatics tools can be used to obtain sequences of genes or proteins of interest, either from material obtained, labeled, prepared and examined in electric fields by individual researchers/groups or from repositories of sequences from previously investigated material. The collecting, organizing and indexing of sequence information into a database, a challenging task in itself, provides the scientist with a wealth of information, albeit of limited use. The power of a database comes not from the collection of information, but in its analysis. A sequence of DNA does not necessarily constitute a gene. It may constitute only a fragment of a gene or alternatively, it may contain several genes. PCAPSS (Protein Classification through the Assessment of Predicted Secondary Structure), BLAST Filter, SeqCheck and SeqMake are a few notable tools of Molecular Bioinformatics.

About 6 billion people on this planet consume 40% of the planet's annual biological productivity. By the middle of 2020-25, about two thirds of living organisms will face the danger of extinction. In this era of maximum destruction, it has become a global priority to organize information by using modern bioinformatics tools, while at the same time continuing in efforts to preserving the biodiversity and increase the pace of studies and development of medicinal plants.

Natural products continue to provide the most productive source of leads for new medicines, including the statins, immunosuppressants and anticancer agents. However, less than ten percent of this vast resource has been tested for biological activity, yet nearly half of the top 20 selling drugs owe their origins to natural products, as do many drugs now in development. Besides molecular and species bioinformatics and natural/herbal products, biotech process and products are other important areas where bioinformatics can play an important role.

What is the status of global biodiversity?

Our world with 267 nations is now thought to be 4.55 billion years old, i.e. about 1/3 of the 13-billion year age estimated for the universe. The total area is approximately 510.072 million sq. km. of which 148.94 million sq. km. is land and 361.132 million sq. km. is water.

On our planet there are ca. 13–14 million taxa, the diversity of which ranges from microbes to man. Of these only 1.75 million species have so far been described [1]. Many of them are still poorly known in biological terms due to lack of a comprehensive catalogue. Studies have indicated that a disappearing plant takes with it another 15 or even more species of plants and animals from the earth and the situation is even worse in rich biodiversity areas.

The scale of human enterprise on Earth has become so great — we are now nearly 6 billion strong and consume about 40 percent of the planet's annual biological productivity — that we are eroding the very ecological foundations of plant biodiversity and losing unique gene pools species and even entire communities of species forever. Though human beings evolved several thousand years ago, the cultivation of crops was developed at many centers only about 10,000 years ago. With crop development, the world has witnessed a rapid growth in human population — it has grown from 1 billion people about 2000 years ago to almost 2 billion people in 1930, 2.5 billion in 1950, 6 billion in 1999 and, in the last 12 years, the last billion people have been added. It is estimated that by the middle of the next century, about 2.9 billion people more will be added to this planet [2]. Of this population, about 80% are in developing countries with 20% in

industrialized countries. For such a large number of people, 90% of the food is obtained from only 20 plant species, with the world dependent on 60% of its food on just three species — wheat, rice and maize.

It is said that if the duration of the life of earth is 24 hours, the life of the human species is ¼ second. Yet this one species has caused several alarming changes such as disturbing the forests, depleting the groundwater, increasing the rate of extinction and reducing the tropical flora to half. By the middle of 2020-25 about two thirds of today's living organisms will face danger of extinction [3]. Thus, in this era of maximum destruction, the conservation of biodiversity is fundamental to the success of the development process.

We now have a first and foremost obligation to organize information in a readily available manner by using modern Information Technology (IT) tools, besides preserving our biodiversity and doing more work on medicinal plants.

40% of all prescriptions written today are based on or synthesized from natural compounds from different species. Not only do these species save lives, they contribute to a booming pharmaceutical industry worth over $40 billion annually.

Unfortunately, only 5% of known plant species have been screened for their medicinal values, although we continue to lose up to 100 species daily. The Pacific Yew, a slow-growing tree found in the ancient forests of the Pacific Northwest, was historically considered a "trash tree", which was burned after clear-cutting. Yet, recently, a substance in its bark, taxol, was identified as one of the most promising treatments for ovarian and breast cancer. More than 3 million American heart disease sufferers would find their lives cut short within 72 hours without digitalis, a drug derived from the purple foxglove.

Species biodiversity also make up the fabric of healthy ecosystems such as coastal estuaries, prairie grasslands and ancient forests which we depend on to purify our air, clean our water and supply us with food. When species become endangered, it is an indicator that the health of these vital ecosystems is beginning to decline. The US Fish and Wildlife Service estimates that losing one plant species can trigger the loss of up to 30 other insect, plant and higher animal species.

The Convention on Biological Diversity (CBD) has, in unequivocal terms, asserted the sovereign rights of nations over their biodiversity and also makes provision for empowering nations for conservation, sustainable utilization, and equitable sharing of benefits with stakeholders arising from the use of biological diversity and associated knowledge systems. Plant diversity can be securely maintained only by protecting the native habitats and ecosystems where plants have evolved. Countries have safeguarded wild lands primarily through establishing national parks, forest reserves and other formally protected areas. Governments have steadily increased protected area networks, and they now encompass nearly 12 million square kilometers, or about 8 percent of the Earth's land surface.

The realization that nature is the largest and the best combinatorial library and the recent development of powerful techniques like High Throughput Screening bioassay-guided isolation of active principles and identification and isolation of relevant genes and its horizontal transfer to desired organisms, to make super hybrids of crop plants of farm animals or super micro-organisms for commercial manufacture of products of great pharmaceutical or industrial value, poses great challenges and opportunities to biodiversity-rich nations. The first and foremost responsibility in this context is to protect our rights over our own biodiversity, particularly over the genes and the traditional knowledge systems. Preparation of a digitized database on Internet about the Bioresource with information on taxonomy, identity, intraspecies variability, their potential use, distribution maps and availability and the associated indigenous knowledge systems, are urgently required to prevent any possible piracy and to establish the IPRs on these plants at the associated knowledge systems.

Gene prospecting and drug hunting from bioresources have emerged as the best selling technology of the 21st Century. These are indeed the most challenging areas that hold the key to human welfare through industrial programs and economic prospecting. The efficient use of biological resources in concert with innovative genetic engineering and biotechnology can go a long way in generating wealth and prosperity, in a sustainable manner from the hitherto untapped wealth of our rich biogenetic resources.

There is a revival of interest in herbal drugs world over. The global herbal drug trade is a fast growing industry and the annual turnover is expected to be around US$60 billion by 2000. This resurgence of interest in herbal drugs is mainly due to realization of the harmful side affects of many drugs of modern medicine. The preventive and primitive aspects of the traditional medicine particularly prevailing in the oriental system of medicine such as Ayurveda, Siddha, Unani of India and traditional medicines of China are finding increasing popularity in the world over. Also, as a part of the strategy to reduce the financial burden of the developing countries that spend 40–50 percent of their total health budget on modern drugs, World Health Organization (WHO) is encouraging these countries to receive and promote traditional remedies in their national heath care programme. The WHO estimates that 3.5 billion people in developing countries rely on plant-based medicines for their primary health care.

Ayurvedic and other traditional healers in South Asia use at least 1,800 different plant species in treatments and are regularly consulted by some 800 million people. In China, where medicinal plant use goes back at least four millennia, healers employ more than 5,000 plant species. At least 89 plant-derived commercial drugs used in industrial countries were originally discovered by folk healers, many of whom are women. Traditional medicine is particularly important for poor and rural residents, who typically are not well served by formal health care systems. Recent evidence suggests that when economic woes and structural adjustment programs restrict governments' abilities to provide health care, urban and even middle-class residents of developing countries also turn to more affordable traditional medicinal experts.

The increasing popularity of herbal drugs in the Western countries is evident from the publication of volumes of pharmacopoeia by UK in 1990 and the sale of health literature of herbal product to the tune of US$33 million in 1998 in USA alone. Sensing the great market potential of herbal drugs, many developed countries, particularly Germany, UK, France, Switzerland and Japan, have started active research programmes in oriental medicine and begun to produce the standardized extracts of herbal drugs. Many Ayurvedic physicians/ scholars and medical plants experts from India including Kerala are

right now in Japan helping the Japanese to build a strong herbal drug industry.

Natural products continue to provide the most productive source of leads for new medicines, including the statins, immunosuppressants and anticancer agents. However, less than ten percent of this vast resource has been tested for biological activity, yet nearly half of the top twenty selling drugs owe their origins to natural products, as do many drugs now in development.

The success of natural products derives from their structural diversity, with higher plants producing secondary metabolites of greater molecular diversity than most other classes of organisms. Natural products provide greater structural diversity than any combinatorial or other practicable synthetic approach. Drug discovery has largely overcome the restrictions of working with extracts of natural products by integrating novel solid phase fractionation and chromatography technologies with screening assays specifically optimized for natural products. These are linked to mass spectrometry and nuclear magnetic resonance techniques for rapid identification of the structures of active compounds.

Currently, much of the data sources required by biologists and chemists are in different forms. The difficulty users face is in organizing and filtering these data.

WHAT IS BIOINFORMATICS?

The term "bioinformatics" encompasses almost all computer applications in biological sciences. It was originally coined in the mid-1980s for the analysis of biological sequence data [4]. In practice, bioinformatics is used as a synonym for "computational molecular biology" i.e. the use of computers to characterize the molecular components of living things. Fred Tekaia of the Pasteur Institute defines bioinformatics as "The mathematical, statistical and computing methods that aim to solve biological problems using DNA and amino acid sequences and related information."

Bioinformatics as a subject is a merger of computing and biology. David Searls of Glaxo Smith-Kline likes to call this emerging area "the midwife of the post-genomic era". There are as many

definitions of the word "bioinformatics" as there are people who are willing to give one. The following are some views on bioinformatics for understanding its meaning and scope:

1. Bioinformatics is the application of computer technologies to the biological sciences, particularly genomics, with the object of discovering knowledge.
2. Bioinformatics is an application of computation to the field of biology, including data management, algorithm development and data mining.
3. Bioinformatics is related particularly to the biological entities involved in the drug discovery process, covering genomics and proteomics.
4. Bioinformatics is a set of tools that allows the scientist to see cause-and-effect relationships between disease and polymorphism, or differences in the DNA sequence among individuals.
5. Bioinformatics is the use of computers in assigning function to proteins and in comparing protein – protein interaction in different protein families.
6. Bioinformatics is the application of software or information technology to biology and experimental medicine with the objective of discovering knowledge.
7. Bioinformatics is simply the management of biological information.
8. Bioinformatics includes the technology needed to bring together groups of researchers from academia and industry so they can collaborate more effectively.
9. Bioinformatics includes the tools to bridge the gap between different types of data so that scientists and managers have access to as much information as possible in making decisions on which paths of research to pursue or kill.

Bioinformatics is storage, retrieval and analysis of computer-stored information in biological research. Bioinformatics should help scientists and companies to access and analyze their growing databases

of experimental results and to exploit public data from genome programmes and other sources.

Bioinformatics has become an essential component of biotechnology-based product and process development. The process of drug design and development is expensive and time-consuming. The application of the tools and techniques of Bioinformatics has resulted in the reduction in cost and the development cycle of the drugs. This aspect has a tremendous impact on the society. If a newly discovered drug is a life saving one, then the resulting gains are not only in terms of financial savings but also in saving the lives of several million people. Major pharmaceutical and biotechnology companies have set up large research and development groups in Bioinformatics.

WHAT ARE THE SUB-DISCIPLINES?

Bioinformatics is a multi disciplinary subject. Though only about a decade old, it has become very important for the growth of biosciences, biotechnology, and the economic prosperity of nations. The following are the well-identified subdivisions of Bioinformatics:

a) Molecular Bioinformatics,
b) Cellular and sub-cellular Bioinformatics including epigenetics, and neuro Bioinformatics,
c) Orgasmic and community Bioinformatics,
d) Medical Bioinformatics including metabolic pathways,
e) Species Diversity Bioinformatics including behaviour, evolution and the effect of pollutants on higher as well lower species.

Bioinformatics may therefore be defined as the area/ branch of information technology that deals with all aspects of biological systems and associated scientific and technological information/data, including the traditional knowledge systems, with the aim of conservation and sustainable utilisation that converts bioresources into economic wealth. It thus involves information pertaining to biological resources, inventory documentation, acquisition, processing, storage, distribution, analysis and interpretation, combining the tools and techniques of mathematics,

computer science and biology with the aim of understanding the biological significance of a variety of data.

Protein analysis, cell metabolism, biodiversity, biotechnology, downstream processing in chemical engineering, genetic engineering and vaccine designs and diagnostic kits are some of the important areas in which Bioinformatics constitutes an integral component. Bioinformatics relates itself with different entities. It may be classified on the basis of the level of investigations, species under study and different body conditions. Thus one can talk about DNA bioinformatics, mRNA bioinformatics, protein bioinformatics, cellular bioinformatics, microbial bioinformatics, parasite bioinformatics, human bioinformatics, physiological bioinformatics, pathological bioinformatics and developmental bioinformatics.

Current research has identified biotechnology to be the fastest growing sector of production technology. Further advances in this sector will depend quite a lot upon the progress of Bioinformatics and hence there is a great emphasis on Bioinformatics the world over.

Bioinformatics has acquired great importance due to its recent applications in the vast mount of data generated in the Genome sequence projects. The greatest achievement of Bioinformatics methods, the Human Genome Project, is currently being completed. The target of decoding the three billion base pairs of the human DNA has become achievable only through the use of various innovative techniques and methods evolved by Bioinformatics scientists.

The nature and priorities of Bioinformatics research and applications are changing in several ways:

- Particular conclusions about species and general ones about evolution can be drawn. This kind of science is often referred to as comparative genomics.
- Large-scale ways of identifying gene functions and associations (for example yeast two-hybrid methods) are growing in significance and with them the accompanying Bioinformatics of functional genomics.
- There will soon be a general shift in emphasis (in the area of sequence analysis especially) from genes themselves to gene products, which will lead to attempts to:

- catalogue the activities and characterize interactions between all gene products (*prote*omics) and
- crystallise and or predict the structures of all proteins (structural genomics) [see http://bioinformatics.org/faq/].

The field of bioinformatics can be examined from data-centered view that serves to recapitulate the major phases of the generation of bioinformatics data, or technology-centered point of view.

DATA-CENTERED VIEW

Genome data

Expressed Sequence Tag (EST): The arrival of ESTs as a source of truly large-scale gene sequence data in 1993 was the beginning of the genome era in the pharmaceutical industry. Many privately held EST libraries (Incyte Pharmaceuticals, Millennium Pharmaceuticals) are being established for pharmaceutical applications.

Genomic Sequence Data: This area is growing very rapidly and some of the important sequencing projects are: Microbial Genomes, Model Organisms, Plant Genome, Pathogenic Protozoan Genome and the Human genome. The completion of the first draft of the human genome sequence, by a consortium of 16 public laboratories, is a major achievement in biology. The number of protein coding regions in the human genome is estimated to be around 31000.

Single-nucleotide polymorphism (SNP) data

It has become evident that there is substantial variation in the DNA sequences between two individuals at many points throughout the genome. Most commonly, sequence variation occurs at discrete, single-nucleotide positions referred to as SNPs, which are estimated to occur at a frequency of approximately one per 1000 nucleotides. A wide variety of approaches to genotyping SNPs have been developed in recent years; amongst the most promising technologies being developed is matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS).

TECHNOLOGY-CENTERED VIEW

Databases and search tools

(A) Database organisations

The National Centre for Biotechnology Information (NCBI), USA, is one of the three main life science servers. It maintains reliable databases and analytical software that serve as valuable tools for the scientific community.

The following are the seven main databases and analysis tools supported by the NCBI server at their website.

PubMed : Search service of the National Library of Medicine (NLM). MEDLINE — It allows the subscriber to gain access to bibliographic citations and biological data from a variety of databases, with over 99 million biomedical articles.

BLAST : Basic Local Alignment Search Tool — It is useful for identifying the classification and potential homologues for a given sequence.

Entrez : It is a search and retrieval system that integrates information from databases at NCBI. These databases include nucleotide sequences, protein sequences, macromolecular structures, whole genomes, and MEDLINE, through PubMed.

BankIt : It is the GenBank's sequence submission server.

OMIM : A database of human genes and gene disorders.

Taxonomy : Phylogeny — Organisimic databases with the scientific and common names of organisms for which some sequence information is known.

Structure : It supports the Molecular Modeling database (MMDB) and a variety of software tools relevant tool structural analysis.

THE European Bioinformatics Institute (EBI) supports the following databases:

EMBL	:	Database of nucleotide sequences
SWISS-PROT	:	Protein sequence database
PDB	:	A collection of all known public domain protein 3-D structure
dbEST	:	Mirror of NCBI

Genome Net, Japan-GenomeNet provides the following main services:

DBGET/LinkDB:		An Integrated Database Retrieval System
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
LIGAND	:	Chemical Database for Enzyme Reactions
BRITE	:	Bio-molecular Relation in Information Transmission and Expression

(B) Database-mining tools

Bioinformatics tools can be used to obtain sequences of genes or proteins of interest, either from material obtained, labeled, prepared and examined in electric fields by individual researchers/groups or from repositories of sequences from previously investigated material.

The most pressing tasks in bioinformatics involve the analysis of sequence information. **Computational Biology** is the name given to this process, and involves the following:

- finding the genes in the DNA sequences of various organisms
- developing methods to predict the structure and/or function of newly discovered proteins and structural RNA sequences.
- clustering protein sequences into families of related sequences and the development of protein models.
- aligning similar proteins and generating phylogenetic trees to examine evolutionary relationships.

Most biological databases consist of long strings of nucleotides (guanine, adenine, thymine, cytosine and uracil) and/or amino acids (threonine, serine, glycine, etc.). Each sequence of nucleotides or amino acids represents a particular gene or protein (or section thereof), respectively. Sequences are represented in shorthand, using single

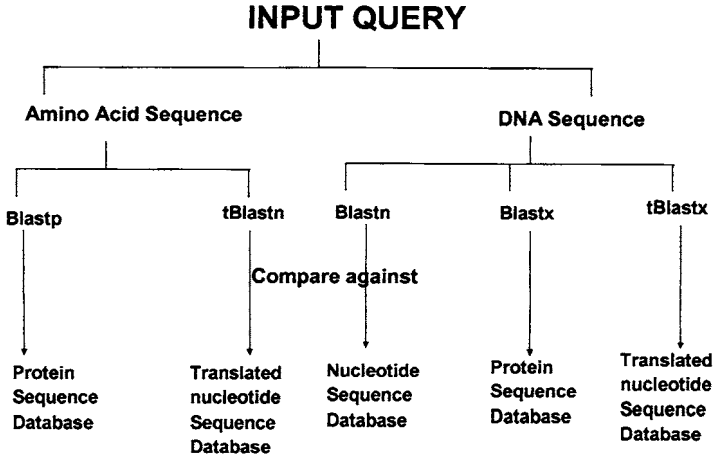
letter designations. This decreases the space necessary to store information and increases processing speed for analysis.

The collecting, organizing and indexing of sequence information into a database, a challenging task in itself, provides the scientist with a wealth of information, albeit of limited use. The power of a database comes not from the collection of information, but in its analysis. A sequence of DNA does not necessarily constitute a gene. It may constitute only a fragment of a gene or alternatively, may contain several genes (see http:/biotech.icmb.utexas.edu/pages/bioinform).

The two most popular and user-friendly sequence similarity search tools on the web are:

BLAST - supported by NCBI, USA, and
FASTA - maintained by EBI, UK.

Sequence of steps when using a search tool:



GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. GenBank (at NCBI), together with the DNA DataBank of Japan (DDBJ) and the European Molecular Biology Laboratory (EMBL) comprise the International Nucleotide Sequence Database Collaboration. These

three organizations exchange data on a daily basis. GenBank grows at an exponential rate, with the number of nucleotide bases doubling approximately every 14 months. Genomes of over 800 organisms can be found in this database representing both completely sequenced organisms and those for which sequencing is in progress.

PCAPSS (Protein Classification through the Assessment of Predicted Secondary Structure): A new fold-recognition tool for helping identify hypothetical protein sequences. From a single query protein sequence, it builds a hidden Markov model of predicted secondary structure to search the PDB for proteins of similar structure.

BLAST Filter: Builds a set of related sequences by running BLAST 2.0 on your DNA or protein query sequence, filtering the matches through a set of rules, and returning the complete sequences of the BLAST matches that pass all rules.

SeqCheck: Provides several utilities for formatting and analyzing a file of one or more sequences, including line formatting, alphabet checker, and composition analysis.

SeqMake: Generates up to 10,000 random DNA, RNA, protein or user-defined sequences (http://www.swbic.org/products/bioinfo/bioinfo.php).

Gene finders and other sequence analysis programs are:

- **Glimmer** is a system that uses Interpolated Markov Models (IMMs) to identify coding regions in microbial DNA. A version of the system built for the malaria parasite, GlimmerM is also available.
- **GENSCAN** is a program designed to predict complete gene structures, including exons, introns, promoter and poly-adenylation signals, in genomic sequences.
- **MORGAN** is an integrated system for finding genes in vertebrate DNA sequences.
- **Genie**, a gene finder based on generalized Hidden Markov Models.

- **Grail, GENQUEST and The Genome Channel** provide analysis and purative annotation of DNA sequences both interactively and through the use of automated computation.
- The **FGENE** family of programs finds splice sites, genes, promoters, and ply-A recognition regions in eukaryotic sequence data
- **The GeneID** server contains the GeneID system for finding genes in eukaryotes.
- **GeneParser** identifies protein-coding regions in eukaryotin DNA sequences.
- **GenLang** is a syntactic pattern recognition system that uses the tools and techniques of computational linguistics to find genes and other higher-order features in biological sequence data.
- **GeneMark** is a system for finding genes in bacterial DNA sequences.
- **Promoter Prediction by Neural Network (NNPP)** is a method that finds eukaryotic and prokaryotic promoters in a DNA sequence.
- **PROSITE** Search Form allows us to rapidly compare a protein sequence against all patterns stored in the PROSITE pattern database.
- **Motifs** in protein database program determine if a protein motif is present in a database of protein sequences.

A few important Protein Structure Prediction programmes are:

- **THREADER2** — a program for predicting protein tertiary structure by recognizing the correct fold from a library of alternatives.
- **PredictProtein** — a service for sequence analysis and structure prediction.
- **NNPREDICT (Protein Secondary Structure Prediction)** — A program that predict the secondary structure type for each residue in an amino acid sequence. The basis of the prediction is a two layer, feed-forward neural network. In "classical" drug research, the research was for new compounds with specific biological or clinical effects. The biological research is producing increasingly large amount of data, which is reflected in an expansion of biological literature by at least 250,000 articles every year. In

this new era of drug discovery, the critical impasse is no longer data generation, but data interpretation.

WHAT UNIFYING FRAMEWORKS CAN BE FOUND FOR BIOLOGICAL DATASETS?

Bioinformatics is in a period of growth characterized by rapid proliferation both of electronically stored factual datasets and of software to analyze and model biological features. The ever-increasing array of datasets reflects both the complexity of biological functions — the functions of biomes, species, populations, crops, individuals, organs, tissues, cells, organelles, molecules — and the very essence of biology that this set of functions, replicates and evolves by biological processes of inheriting information.

A challenge for Bioinformatics is to find a unifying framework in which to organize this multiplicity of datasets. Nearly all biological data refer to the organism in question — in which species do the data occur? There are a number of frameworks to address the following questions:

- The taxonomic dimension — in which species, cultivar or population does the data occur?
- The homology dimension — in what organelle, cell, tissue or organ is the data found?

The most useful feature of a taxonomic or eventually a species diversity framework is its ability to link different datasets electronically to allow combined presentation or searching and analysis. This opens us to not only the potential for more compatible information provision, but also the possibilities for discovering new associations, imposing knowledge "rules" for improving data integrity and generating automatically higher level knowledge.

International Legume Database and Information Service (ILDIS), UK has created a species diversity database of over 19000 legumes, the economically most important group of plants for man and environment. The main elements of the ILDIS project are the database, an information service and an electronic network. *LegumeWeb* (http://www.ildis.org) searches the database by species

name and is a notable example of application of Bioinformatics for digital treatment of biodiversity. A Phase I South Asia Legumes database of 2029 taxa belonging to 239 genera and 34 tribes has been developed, which will serve as a species core to link molecular data and value-added data on economic uses, images, germplasm sources and ecology, and correct taxonomic nomenclature of herbaria and germplasm.

CONCLUSION

Profound changes are occurring in the strategies that biotechnology-based industries are deploying in the search for exploitable biology and to discover new products and develop new or improved processes. There is a paradigm shift from traditional biology to bioinformatics that is revolutionising exploitable biology. This paradigm shift has been driven by a convergence of complementary technologies, exemplified by DNA sequencing and amplification, genome sequencing and annotation, proteome analysis and phenotypic inventorying, resulting in the establishment of huge databases that can be mined to generate useful knowledge [5].

Natural products continue to provide the most productive source of leads for new medicines, including the statins, immunosuppressants and anticancer agents. However, less than ten percent of this vast resource has been tested for biological activity, yet nearly half of the top twenty selling drugs owe their origins to natural products, as do many drugs now in development. With the varied applications of Bioinformatics, re-invigorated means of detecting novel organisms, novel chemical structures and novel biocatalytic activities ensure that natural products will continue to be a primary resource for developments and applications in biotechnology.

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http://bioinformatics.org/faq/

http://biotech.icmb.utexas.edu/pages/bioinform

http://www.swbic.org/products/bioinfo/bioinfo.php

http://www.bic.nus.edu.sg/admin/News/Mar98/Access3.html

From Genomes to Drugs with Bioinformatics

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INTRODUCTION

There is no doubt that bioinformatics has become an active field in recent years. But what *is* bioinformatics? According to NIH [1], bioinformatics applies the principles of information sciences and technologies to make the vast, diverse and complex life sciences data more understandable and useful. While using supercomputers to decode human genomes is an impressive bioinformatics task, analyzing your own data using a desktop PC and a spreadsheet software could also be called as bioinformatics. In any case, bioinformatics serves as a bridge linking the information technology and biology in this genomic era.

The goal of this paper is to provide an overview of the bioinformatics technologies, with emphasis on those directly applicable to genomics and drug discovery. The first several sections will introduce the basic sequence analysis technologies, including similarity comparison, database searching, gene finding, protein structures prediction and protein-ligand docking. Applications of those technologies to two biological problems — genome sequencing and the EST mining — will be discussed. Finally, the trends and the challenges of bioinformatics will be addressed.

SEQUENCE ANALYSIS

Although it is relatively easy to obtain a DNA or protein sequence in a laboratory, determining its function or structure by direct experiment remains a tedious and costly procedure. Hence, using computers to infer biological information from DNA or protein sequences are one of the most important problems of bioinformatics.

The computational method for solving those problems is called sequence analysis.

SEQUENCE COMPARISON

The type of information that can be extracted from a sequence alone is restricted compared to what can be obtained from analyzing many sequences. As a result, many efforts have been placed on the problems of sequence comparisons, which include database similarity search and sequence alignment.

Similarity searches on sequence databases are perhaps the most popular bioinformatics tasks. Given a protein or DNA sequence, similarity search reveals the other sequences that look like it, or more precisely, the sequences that might be derived from the same ancestor. The assumption is that if the sequences are similar, they probably have the same structure and a similar biological function. This assumption even works when the sequences come from very different organisms.

BLAST [2, 3] is no doubt the most widely used program to search a database. The BLAST program applies various heuristics to speed up the potentially lengthy search. Among the most important heuristics, BLAST aims at identifying core similarities for later extension. The core similarity is defined by a window with certain matches on DNA (the default is 11 nucleotides) or with an amino acid score above some threshold for proteins. Washington University has an alternative implementation of BLAST [4], which has many features that are not seen on the NCBI implementation of BLAST. Those two versions of BLAST also have different default parameters. PatternHunter [5] and BLASTZ [6] are two of the recent programs using discontinuous seeds, which were meant to improve the sensitivity of database searching.

Instead of finding similar sequences in a database, pairwise sequence alignment is a scheme of writing one sequence on top of another so that portions sharing the same evolutionary origin may become identifiable. Due to insertion or deletions in sequences, the aligned sequences may have different length, which is represented by dashes in one of the two sequences. There are two kinds of alignments:

global alignment, where the two sequences are aligned over their entire lengths, and local alignments, where only the similar portions of the two sequences are aligned. The general idea of pair-wise alignment is to assign a score to an alignment and then to minimize or maximize the scores over all possible alignments. Needleman and Wunsch first proposed a global alignment algorithm using dynamic programming in 1970 [7]. Still using dynamic programming, Smith and Waterman developed a local alignment algorithm in 1984 [8]. Although dynamic programming algorithms are guaranteed to find the optimal scoring alignment, the long execution time makes them less attractive in large scale alignment jobs. Commonly used heuristic methods include Lalign [9] (best with proteins), BLAST and MegaBLAST [10] (best with DNA), SSAHA [11] (aligning sequence reads to genome) and BLAT [12] (aligning cDNA to genome).

FINDING PARTICULAR SEQUENCE PATTERNS

Another class of sequence analysis problems involves only individual sequences. They are analyzed primarily on the basis of either the statistical signals, such as exon and intron prediction, or the chemical and physical properties of the residues, such as hydrophobicity.

Recognition of functional signals in genes is important in gene identification. Traditional methods to find functional sites are based on using consensus sequences [13] or weight matrices [14] reflecting conservative nucleotides of a signal. Most gene prediction systems combine information about functional signals and the regularities of coding and intron regions. Single gene prediction programs normally use dynamic programming to find an optimal combination of pre-selected exons [15]. GENSCAN [16] was the first algorithm to predict multiple eukaryotic genes and remains to be one of the most widely used gene prediction systems.

As the genomic data ramps up, the automated gene finding or gene structure prediction systems are of increasing importance. Large-scale bioinformatics systems are needed to manage and integrate large volumes of genomic data efficiently. A recent review by Rust [17] provides more details in this field.

PROTEIN STRUCTURE PREDICTION

The three-dimensional structure of a protein is an essential factor of the protein's function. X-ray crystallography and NMR have been the two experimental techniques for resolving protein structures. However, since both techniques are time-consuming and expensive processes, the prediction from a primary sequence to a 3D structure becomes an important and challenging computational problem.

There are three types of bioinformatics approaches towards predicting protein structures. Secondary structure prediction assigns α-helix, β-strand or loop structures to the residues of a protein. *Ab initio* methods often employ energy minimization or molecular dynamics methods to predict structure without any additional information. Sequence-structure alignment, or protein threading, constructs a model for a protein sequence after the known structure of another template protein.

Proteins are composed of regular recurring elements, called secondary structures. The practice of assigning helical or extended secondary structures to amino acids is called secondary structure prediction. The classical method of secondary structure prediction is to use a table listing the conformational parameters determined primarily from experimental measurements of the various secondary structure elements [18]. The table shows a likelihood for an amino acid to appear in a certain secondary structure element. The more powerful methods are based on neural networks, which are a class of computational structures based on the anatomy of biological nervous systems. For example, PSIPRED incorporates two feed-forward neural networks on the output obtained from PSI-BLAST and is a highly accurate secondary structure prediction method with accuracy over 75% [19].

PROTEIN-LIGAND DOCKING IN DRUG DESIGN

The first step in a drug discovery process is to find the structure of a lead. A lead is a small molecule binding to a given target protein and may be further developed into a drug. Computationally, given a target protein, the docking algorithms search for potential leads that

bind to the target. This section only covers the protein-ligand docking in which a small molecule is docked to a macromolecule. This type of docking is more important since most drugs are small molecules due to reasons such as bioavailability.

Rigid-body docking algorithms are the first approaches for screening sets of small molecules by their fit to a given protein target. Here both the protein and the small molecule are considered as hard spheres to reduce the complexity of the problem. A widely used software tool for protein-ligand docking is the DOCK program [20]. The idea of DOCK is to search all distance-compatible matches between the ligand and the target proteins. For a target protein, a set of spheres is used to represent the volume that is occupied by the ligand molecule. The ligand is also represented as spheres labeled with chemical properties.

A major limitation of the rigid-body docking algorithm is that it does not consider the conformational flexibility of the ligand molecule. Small molecules often have large conformation spaces. To solve the flexible ligand docking problem, genetic algorithms or evolutionary programming is one possible approach. Genetic algorithm (GA) is a general purpose optimization algorithm. It mimics the process of evolution. GOLD [21] is a popular GA-based molecular docking system. Two chromosomes are described in GOLD. One represents the conformation of the ligand and selected protein side-chains by defining the torsion angles. The other represents the hydrogen bond mapping between the ligand and the protein. The fitness is evaluated by the hydrogen bonds, the ligand internal energy as well as the van der Waals energy.

Simulation of m olecular dynamics is another approach for solving the molecular docking problem [22]. Here the velocity and acceleration of each atom in the docking system are calculated using Newtonian mechanics. Smaller simulation time steps usually lead to more accurate simulation. Thus, molecular dynamics simulation can be quite time consuming and is not appropriate for large sets of molecules. The AUTODOCK program [23] is based on a well-known combinatorial optimisation technique called simulated annealing. The program combines simulated annealing for conformation searching with a rapid grid-based method of energy evaluation.

The molecular docking software packages discussed in this section are-
GOLD: http://www.ccdc.cam.ac.uk/prods/gold.html;
AutoDock: http://www.scripps.edu/pub/olson-web/doc/autodock/;
DOCK: http://www.cmpharm.ucsf.edu/kuntz/dock.html.

MINING GENES FROM EXPRESSED SEQUENCE TAQ (EST)

EST represents sequences of most mRNA present in various tissues. The EST databases contain a large part of the transcriptome of many species. Thus they are probably the most abundant source of new coding sequences. This section reviews the methods that can be used to find novel genes using EST data.

The EST sequencing projects have generated many more sequences than there are expressed genes. Hence, there are many genes from which more than one EST was derived, and much of the sequence information in the EST databases is redundant. EST clustering is a process to obtain a non-redundant catalog of the genes represented by EST sequences [24]. The most widely used EST clustering information is UniGene of NCBI. Each cluster in UniGene has a unique number and a list of accession numbers of ESTs and known mRNAs or gene transcripts belonging to the cluster. Note that UniGene does not provide a contig or an assembled EST sequence; instead, the longest EST is identified as the representative sequence for a particular cluster. The Institute of Genome Research (TIGR) has created unique gene indices of clustered and assembled ESTs [25].

A common task for searching the EST database is to find novel genes that are related to a particular known gene or a family of genes, or that contain a known protein domain. A possibility is to search the query sequence against EST collections that have been clustered, such as NCBI's UniGene or TIGR's gene indices. The results of such a search will indicate the cluster number and the accession numbers of the hits, thereby enabling a quick identification of the related genes. Another possible scenario is to find new coding sequences (CDS) that are more distantly related to the query sequence. In this case,

TBLASTN may be used to search a query protein against EST databases. The more sensitive way for finding related genes in the EST databases is to use a protein motif as a query. A motif is generally extracted from a multiple alignment of the related proteins. The EST database has to be explicitly translated into protein format before being searchable. The problem of searching a protein database with an HMM (Hidden Markov Model) motif profile can be achieved by using hmmsearch, a program in the HMMER package [26].

GENOME SEQUENCING

Determination of the sequence of a genome is one of the most fundamental tasks in molecular biology. Due to the inability of the current technologies to produce DNA sequences longer than about 1000 base pairs, a complete genome sequence must be computationally assembled from a large set of overlapping sequence segments.

The sequence assembly problem is difficult for a few reasons. First, the genome contains repetitive regions, which cause ambiguities when linking overlapping sequence segments to form a contig. Secondly, the orientation of a sequence read is unknown, i.e. it is not known whether a read is from the plus or the minus of the two complementary strands. Thirdly, the error rate of a sequencing machine can range from 1% to 5%, with higher rate close to the end of a read. Contaminated sequences are also a problem where foreign DNA might be observed at the left end of a sequence read. Lastly, a read occasionally consists of two pieces that are from different parts of the DNA segment; this is the so-called chimeric read.

In the clone-based sequencing strategy, a physical map of the genome is constructed before sequencing. The entire genome will be split into many segments from 40 kb to 200 kb. Each segment carries a well-defined marker. A physical map is a collection of such DNA segments. The advantage of this approach is that the large genome is broken into smaller and manageable pieces, which would cause fewer problems in the assembly stage due to the smaller number of repetitive sequences. The disadvantage arises from the difficulty in finding markers that can order the DNA segments.

In the whole-genome shotgun sequencing strategy, multiple copies of the genome are broken into pieces. Both ends of every piece are sequenced and put back into order through intensive computation. The clear advantage of this method is that it can quickly produce a draft picture of a genome. The disadvantage is that it has trouble handling long repetitive regions.

A commonly used assembly algorithm is CAP3 [27]. It consists of three major stages: the identification of similar reads in the first stage, the connection of reads based on overlapped portions in the second stage and the construction of a consensus sequence based on multiple sequence alignment in the final stage.

The strength of CAP3 is in the assembly of ESTs, while the strength of Celera Assembler (http://www.celera.com) is on the assembly of large genomes. TIGR also provides its own assembler, called TIGR Assembler [28]. A case study of the genome-level sequence assembly can also be found [29].

TRENDS AND CHALLENGES

Since the first whole bacterial genome appeared in the mid-1990s, the attempts at genome-wide annotation with bioinformatics methods have been an active research subject. Today, the annotation of whole genomes and their comparison remain a challenge. Hypothesis-driven annotation [30], as opposed to traditional data driven annotation, appears to have some success.

As proteomics gains ground from genomics, the importance of mRNA expression data will probably be limited to specific applications, such as diagnostics, since genomics experiments are still cheaper compared with proteomics experiments. With regard to proteomics, bioinformatics has to address the challenge of interpreting the large amount of data generated by proteomics technologies like mass spectrometry, protein microarray and yeast two-hybrid methods.

Another type of data that challenges Bioinformaticians are the genetic variations. Most of the genetic variation data take the form of single nucleotide polymorphisms (SNPs). Bringing bioinformatics and statistical genetics together will form a new field called

Pharmacogenetics, where the differential effect of a drug on different patients is studied and hopefully can be predicted.

The large investments of pharmaceutical industry into genomics have certainly raised the expectation that bioinformatics and genomics will provide all solutions to biology and medicine. However, examples described in the above sections do show that Bioinformatics along with genomics have presented a paradigm shift in biology. For example, the protein-ligand docking algorithms, although not perfect, have been demonstrated to provide relevant predictions. In any case, we have to bear in mind that Bioinformatics is an instrument for suggestions. Nature will be too complex to be accurately modeled or simulated by computers.

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Yeast-Based Technologies for High Throughput Screening of Natural Anti-Viral Agents

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INTRODUCTION

During translation of mRNA, the ribosomes maintain precisely the correct translational reading frame. This is a fundamental property that provides the basis for the integrity of the protein translational machinery and eventually for cell growth and viability. This machinery operates at an extremely low error rate in frame-shifting [1-3]. Most double-stranded RNA (dsRNA) and non-segmented (+) stranded RNA viruses, including Retroviruses, use programmed -1 ribosomal frame-shifting as a fundamental process in their replication within the host cell [4]. What makes the whole process a more interesting target for anti-viral intervention is the fact that as yet there are no reports of any eukaryotic cellular mRNAs that show -1 ribosomal frame-shifting.

The basic rationale behind the development of this assay is that many viruses that cause disease in humans, animals and plants utilise programmed ribosomal frame-shifting to regulate the production of their structural and enzymatic proteins. Since the altering of this molecular phenomenon disrupts the virus life cycle and eliminates/ reduces virus production, it would serve as an excellent target for screening of compounds for their antiviral properties [5]. This phenomenon is aimed at developing a yeast-based assay system to identify compounds that may have antiviral properties [4].

In viruses that utilize programmed -1 frame-shifting, the open-reading frame (ORF) encoding the major viral structural protein (typically Gag protein) is located at the 5' end of the mRNA, whereas the ORFs encoding proteins with enzymatic function (typically Pro and Pol) are located at the 3' end of the transcript and out of frame

with the Gag ORF. It is a ribosomal frame-shift event that results in the production of the minority enzymatic proteins while the majority of the protein produced is Gag [6-8]. The importance in maintainance of this delicate yet appropriate ratio of the Gag and Pol proteins which is a direct function of the efficiency of -1 ribosomal frame-shifting has been well-established [9]. In an attempt to use this approach to screen for anti-viral agents, we have developed this assay and used it for our initial screening. Programmed -1 ribosomal frame-shifting has become an excellent target for compounds that function as anti-viral agents, because this molecular process is predominantly utilised by these viruses to regulate their gene expression. This demonstrates the great need for identifying agents that affect programmed -1 ribosomal frame-shifting and thus reduce viral titer.

The L-A helper virus-infected yeast host with M_1 super-infection was used for the described assay. A -1 ribosomal frame-shift event was responsible for the correct expression ratios of Gag and Pol, both encoded in the L-A helper virus [2, 8]. The killer trait of the L-A + M_1-infected yeast strain failed to show up when YPD and SD (synthetic dextrose) media were used at natural pH. Since the killer toxin that is produced by this infected yeast strain is stable between pH 4.6 and 4.8, buffered methylene blue medium (MBM) was used for the assay [10-12]. Phosphate-citrate buffer, made by adjusting the pH of citric acid (final concentration 1M) to 4.5 with K_2HPO_4,, was used to lower the pH of the media to 4.7. After sterilization of YPD medium in an autoclave, 100 ml of the phosphate-citrate buffer was added to 900 ml of the medium. Methylene blue, a stain for dead yeast cells, was then incorporated into the medium at a final concentration of 0.003% [13, 14].

The clear zone forming phenotype of the super-infected yeast strain was observed when a suspension of this strain was grown in YPD liquid medium, pelletted and placed over low-pH MBM plates that have previously been spread with a culture of sensitive yeast Y526 strain cells. After incubation for two days at 22°C, the super-infected yeast strain was surrounded by a zone of clearing fringed with a deep blue color indicating the death of the sensitive cells. This clear zone phenotype is indicative of the L-A virus and M_1, processing their RNA inside the yeast cell by producing exactly the right amount

of ribosomal slippage for production of the correct ratio of Gag and Pol proteins. The correct ratio of Gag and Pol results in the production of the toxin so as to kill the sensitive strain around it. This killing of the sensitive strain is what results in clearing of the lawn of cells (Figure 1).

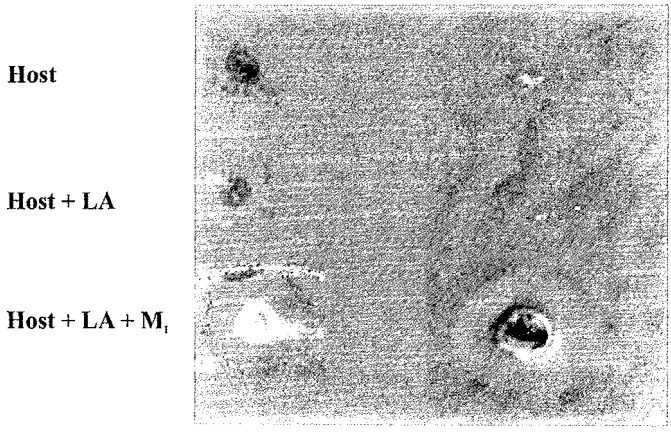

Figure 1. The development of a superinfected *S. cerevisiae* yeast strain which can harbor L-A and M_1 viruses. The zone of clearance formed can be seen clearly around the spotted superinfected strain.

To abolish the clear zone-forming phenotype of the super-infected yeast strain, various concentrations of antibiotics that disrupt -1 ribosomal frame-shifting were used with the super-infected strain. Spotting the super-infected strain after antibiotic treatment or mixing the super-infected strain with antibiotic solution did not give any positive results (data not shown). The super-infected yeast strain was then grown in the presence of the antibiotics in concentrations ranging from 50 to 200 mg/ml in YPD broth. The period of incubation and the volume of inoculation to be plated on the lawn of sensitive strains on MBM plate were then standardized. It was found that a concentration of 50 mg/ml was too low for the killer and the sensitive strains. Sparsomycin did not have any effect in the range used for the test. It was found that within the concentration range of 100 to 200 μg/ml of Anisomycin and Cycloheximide in YPD broth, when the super-infected yeast strain was grown overnight for 2 days, 25 μl of this suspension was successful in eliminating the killer phenotype, which appeared as

a loss of the zone of inhibition (Figure 2). Since these antibiotics specifically disrupt ribosomal frame-shifting, it is clear that when this assay system is used for screening of natural anti-viral agents, it will function as well and show detectable phenotype.

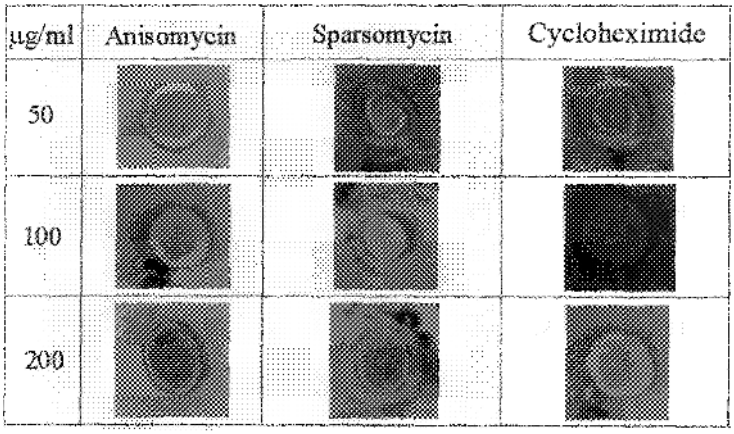

Figure 2. Effects of varying concentrations of Anisomycin, Sparsomycin and Cycloheximide on -1 ribosomal frameshifting efficiencies of the L-A and M_1 virus. Small amounts (100 µg/ml) of Anisomycin and Cycloheximide were able to show disappearance of the zone of inhibition created by the superinfected yeast strain. Similar dissapearance of zones of inhibition would be expected by the usage of this assay during anti-viral screening.

MATERIALS AND METHODS

General procedures

When intact L-A virions were infected into the yeast host strain SL321 (MATa *his(3,4), leu2, lys2-801 ade2-10 trp1-1*), super-infection of M_1 followed. Yeast strains L40a and Y526 showed good sensitivity with sharp zones of inhibition and thus were used as the sensitive strain for subsequent experiments. Sparsomycin, Anisomycin and Cycloheximide were purchased from Sigma Chemical Co., USA. These antibiotics were used in the detection assays and the exact amounts were determined per Petri dish in order to get reproducible results with the screening.

The L-A double stranded RNA virus is endogenous to yeast cells along with the M_1 satellite dsRNA virus. The M_1 satellite virus secretes a killer toxin and is encapsidated and replicated using the Gag and Gag-Pol gene products synthesized by the L-A virus inside the infected yeast cell. Yeast cells harbouring both L-A and M_1 viruses secrete the toxin and are immune to its action whereas virus-free cells are sensitive to the toxin. A ring of growth inhibition is indicative of the killer activity of the super-infected yeast cells harboring both L-A and M_1 viruses.

Phosphate-citrate buffer (PCB) was prepared by dissolving 192 g of citric acid to 1 litre of distilled water and adjusting the pH with K_2HPO_4 to 4.5. The solution was autoclaved before use.

Methylene Blue Stock Solution (MBSS) contained 5% Methylene Blue Dye. Methylene Blue Media (MBM) contained 600 ul of MBSS (0.003%), 100 ml of PCB and 900ml of liquid YPD (agar).

Assay protocol

Liquid cultures of the sensitive yeast strain Y526 were grown with shaking at 300 rpm at 30°C overnight. The following day, this strain was spread-plated on the MBM plates as described above. Another liquid culture of the super-infected yeast strain containing both the L-A and M_1 viruses was grown overnight in liquid YPD medium with shaking at 300 rpm at 30°C. [For antibiotic assay, 50/100/200 μg/ml was added to the shake tubes at this stage]. The super-infected yeast cells were harvested after centrifugation and used for the assay. These cells were spotted on the spread-plated sensitive cells on the MBM plate. The plate was incubated over-night at 22.5°C for 2 nights. The lawn of sensitive cells starts to grow and the zone of clearing begins to show after the second overnight incubation. To screen for natural anti-viral agents, the same protocol may be used, adding the test agent in place of the antibiotics used in the experiments above.

The yeast-based zone of clearing, due to toxin production by the super-infected yeast strain, has been developed as an anti-viral screening assay. Typically this assay is composed of the L-A helper virus and the M_1 satellite virus. The dsRNA genome of L-A contains

two ORFs, the 5' *Gag* gene encoding the major viral coat protein (Gag) and the 3' *Pol* gene encoding a multi-functional protein that includes the RNA-dependent RNA polymerase and a domain required for viral RNA packaging. A -1 ribosomal frame-shift event within the host cell is responsible for the production of the L-A-encoded Gag–Pol fusion protein. The M_1 satellite dsRNA genome is encapsidated and replicated inside the icosahedral 39 nm L-A viral particle. Changes in the efficiency of programmed -1 ribosomal frame-shifting along the L-A mRNA result in a rapid loss of encapsidated M_1. This phenotype is evident by replica-plating colonies of test cells on a lawn of uninfected yeast cells that are sensitive to the killer toxin. Super-infected cells maintaining the M_1 virus secrete the killer toxin, creating a characteristic ring of growth inhibition.

Figure 1 shows clearly the formation of the zone of clearing when the host yeast cells are super-infected with L-A and M_1. This zone of clearing of the sensitive yeast cells is missing when the spotted strain contains only L-A or no infection. The fact that programmed -1 ribosomal frame-shifting appears to be virus-specific makes it an attractive target to identify agents that affect the efficiency of this process and, consequently, disturb the equilibrium of viral maintenance. It is of great interest to mention that small changes in the -1 ribosomal frame-shifting efficiencies will have large effects on virus production. Increasing or decreasing minutely the efficiency of programmed -1 ribosomal frame-shifting within the yeast host cells will hence result in an amplified response showing a large inability of yeast cells to maintain the M_1 "killer" satellite virus of L-A.

Peptidyl-transferase inhibitors (anisomycin and sparsomycin) were used to further confirm the specificity of phenotype due to -1 ribosomal frame-shifting in yeast super-infected cells [13]. These drugs represent a well-studied class of small molecules that affect the protein-synthetic machinery at the step at which the -1 ribosomal frame-shifting is postulated to occur. We have shown (see Figure 2) that at concentrations as low as 100 μg/ml, there was no effect on the viability of the host cell or on the rates of protein translation (data not shown). However the efficiency of programmed -1 ribosomal frame-shifting was affected, resulting in the disappearance of the zone of clearing around the spotted super-infected host.

Upon screening for anti-viral agents, candidate compounds that change the efficiency of programmed -1 ribosomal frame-shifting efficiencies at concentrations that do not drastically inhibit the translational machinery would be the ideal candidates for further investigations. These compounds are potential agents for therapy at low drug concentrations. Since these candidate compounds will have significantly low toxic effects on the host, such candidates will carry greater potential for anti-viral drugs.

Most conventional anti-viral strategies, for example nucleoside analogues and protease inhibitor, target a virus-specific protein. Both these commonly used classes of anti-viral agents target gene products encoded by the viral pathogen. The yeast-based strategy we describe targets a host-cellular process rather than a viral gene and minimizes the ability of viruses to evolve drug-resistant mutants.

Besides all the above advantages that contribute to making this approach an important one, thousands of candidate anti-viral candidates can be quickly screened for anti-viral activity. The yeast phenotype being used in the screen for anti-viral activity is easily detectable and many candidate anti-viral agents can be screened on a single plate. This makes it a low recurring-cost yet efficient screen for thousands of candidate anti-viral compounds.

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Use of High-Density Oligonucleotide Microarrays for Gene Expression Profiling in Natural Product Research

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INTRODUCTION

Natural products have long been used by man to strengthen the body and to treat various ailments [1]. The first written records of their use came from Mesopotamia around 2600 BC, which documented the utilisation of licorice, cedar and other plant oils for treatment of coughs and parasitic infections [2]. Aspirin, a commonly-used analgesic and anti-platelet agent, was first obtained from the white willow bark over 2,500 years ago [1]. Today, natural products and their derivatives are widely used in many medical disciplines, such as cardiology, endocrinology, neurology, gastroenterology and respiratory medicine [3]. Indeed, approximately 60% of anti-neoplastic drugs in clinical trials in 2000 are either natural products or derived from them [4].

Despite the long history of their use, the mechanisms of action of many natural products are not well understood. Traditionally, researchers have adopted a 'one molecule at a time' approach, using experimental methods such as immunohistochemistry, Western blotting and generation of gene knockout animals. Although these techniques produce excellent results and have led to great advances in our knowledge, they are time- and labour-intensive and thus not suitable for high throughput studies on a genome-wide scale. The development of microarray technology in recent years has provided researchers with an important tool that enables determination and analysis of complex molecular and genetic events in biological systems to be made at the genomic level [5-8]. This paper provides an overview

on the use of high-density oligonucleotide microarrays for gene expression profiling and analysis, which can be applied towards natural product research.

HIGH-DENSITY OLIGONUCLEOTIDE MICROARRAYS

High-density oligonucleotide microarrays allow multiple gene transcript levels to be measured at the same time. This is achieved by making use of the inherent ability of nucleic acid strands to recognise and specifically bind to complementary sequences through base pairing [6]. The net effect is thus similar to that of performing multiple Northern blots [9, 10].

High-density oligonucleotide microarrays from Affymetrix (Santa Clara, CA), also known as GeneChip® Arrays, contain tens to hundreds of thousands of different oligonucleotide probes. The probes are synthesised on glass wafers in precisely-defined locations using a combination of photo-lithography and oligonucleotide chemistry [6, 1-16]. Each probe pair consists of two 25-mer oligonucleotides, desig-nated the perfect match (PM) oligonucleotide and the mismatch (MM) oligonucleotide. The PM probe is complementary to a reference sequence, and serves as a unique, sequence-specific detector. The MM oligonucleotide is identical to the PM probe except for a base change at the 13th position. This enables subtraction of background and cross-hybridisation noise from the PM signal to be made.

The present generation of GeneChip® Arrays contains 11 probe pairs per probe set. Probes exhibit probe redundancy, in which each probe set is designed with unique sequences that hybridise to different regions of a given transcript. By using one or more probe sets for the detection of each transcript, multiple independent measurements are made. This results in improved signal-to-noise ratios, greater accuracy in transcript quantification and lower frequencies of false positives [6]. Currently, the Human GeneChip® (U133 Plus 2.0 Array) enables 47,400 transcripts and variants, including 38,500 well-characterised human genes, to be measured simultaneously on a single microarray.

CHOICE OF SAMPLES

Samples for microarray experiments should be chosen after careful consideration of the aims of the experiments and the ease with which data can be analysed thereafter. Cultures of pure cell populations are easily experimented upon *in vitro*, and provide sufficient RNA for microarray studies. However, the results obtained may not be directly extrapolated to the whole organism. On the contrary, tissues or organs from *in vivo* experimentation may be a more suitable option. The drawback is that tissues and organs contain multiple cell types. Thus, microarray studies on these specimens produce complicated expression profiles, which may make subsequent analysis problematic. One solution to this is laser capture microdissection, which can be used to isolate the cells of interest from either frozen or paraffin-embedded sections [17, 18]. A separate transcription profile for each cell type can then be obtained.

TARGET PREPARATION, HYBRIDISATION AND SCANNING

In target preparation, high quality RNA is extracted from cells or tissues of interest and labelled with biotin. The labelled RNA (target) is then hybridised to a high-density oligonucleotide microarray. After extensive washings, the array is stained with a fluorescent reagent and scanned with a laser confocal fluorescence scanner.

Since the work involves handling of RNA, all the necessary precautions against RNA degradation and RNase contamination should be taken prior to completion of the hybridisation step. RNA can be extracted from eukaryotic cells and tissues using either TRIzol [19] or a silica membrane-based technique. The extracted RNA is quantified by spectrophotometry, and its purity determined using the absorbance ratio at 260 nm and 280 nm (A_{260}:A_{280}). Checking of RNA integrity can be done by gel electrophoresis using a denaturing gel or the Lab-on-a-Chip System (Agilent, Palo Alto, CA) to look for intactness of the two ribosomal RNA bands (18S and 28S). Alternatively, RNA integrity can be determined using gene-specific real-time polymerase chain reaction (PCR).

If the extraction yields 1 μg or more of total RNA (0.2 μg of mRNA), biotin-labelling for subsequent microarray studies is relatively straightforward and well-documented in the Affymetrix GeneChip® Expression Analysis Technical Manual. Briefly, double-stranded cDNA is synthesised from the extracted RNA, using reverse transcriptase and a T7-oligo(dT) primer (5'-GGCCAGTGAATTGTAATA-CGACTCACTATAGGGAGG-CGG-$(dT)_{24}$-3') for synthesis of the first cDNA strand. The T7-oligo(dT) primer binds to the mRNA poly-A tail, thus allowing T7 RNA polymerase to subsequently label cRNA by *in vitro* transcription using a biotinylated nucleotide analogue as a pseudouridine reagent.

In cases where the amount of starting material is limited, or where pooling of samples is not desirable, alternative protocols are needed in order to generate sufficient biotin-labelled cRNA for microarray hybridisation. These scenarios are often encountered when laser capture microdissection is used to isolate cells from small tissue sections. One protocol uses two rounds of T7 RNA polymerase-driven *in vitro* transcription to achieve linear RNA amplification with no reduction in overall sensitivity and only a slight effect on fidelity [20]. This technique has been used to profile renal vesicles and S-shaped bodies isolated by microdissection from murine embryonic kidneys [21]. Although the amplified RNA may show a reduction in 5' complexity, this is not a major issue because the design of GeneChip® probes are based on the 600 bases at the 3'-end of transcripts. An alternative protocol, which has been used for expression profiling of a single cell isolated from the developing pancreas, exploits logarithmic cDNA amplification by PCR [22].

After biotin-labelling, cRNA is broken into 35- to 200-base fragments to obtain optimal assay sensitivity. These fragments are hybridised to the microarray for 16 hours at 45°C. After extensive high stringency washes using the fluidics station, the array is stained with streptavidin phycoerythrin and the signal enhanced using an antibody amplification protocol. The array is then scanned at high spatial resolution at 570 nm [11-13]. The expression level of each gene is indicated by its corresponding fluorescence intensity (Figure 1).

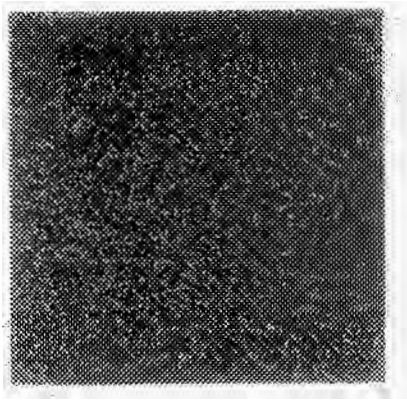

Figure 1. Example of a scanned image of a high-density oligonucleotide micro -array. Intensity of the fluorescence signal is proportional to the level of gene expression.

The eukaryotic GeneChip® arrays include probe sets for several prokaryotic genes. These function as experimental controls. Biotinylated hybridisation controls for these genes are spiked into the hybridisation cocktail prior to the overnight hybridisation step. In addition, the 3':5' signal intensity ratios of housekeeping genes, such as GAPDH and ACTB, serve as checks on the integrity of the extracted RNA, and the efficiency of the cDNA synthesis and *in vitro* cRNA transcription steps.

DATA ANALYSIS AND DATA MINING

Massive amounts of data are generated from microarray experiments, which need to be properly managed and analysed. Specialised software is available, both commercially and in the public domain, for doing this.

The GeneChip® Operating Software, which replaces the Affymetrix Microarray Suite program, controls the fluidics station and includes functions for basic analysis of microarray data. Examples of other commercially available software for data mining include the Data Mining Tool (Affymetrix), GeneSpring (Silicon Genetics, Redwood City, CA) and Resolver (Rosetta Biosoftware, Seattle, WA). Data analysis software is also available for free download for the academic community. Examples of this are dChip [23], GeneCluster [24-26], SAM [27], GenMAPP [28, 29], and Chip-Info [30].

Normalisation or scaling is necessary in order for comparisons among different microarray data sets to be made. In the majority of cases, most transcript levels do not differ among samples. Hence, the global method, which makes use of all probe sets, is used for adjustment. However, in situations where changes in expression levels are seen in a relatively large number of transcripts, it may be more appropriate to make adjustments based on selected probe sets.

The next step in data analysis involves removal of non-informative genes from the data set. Non-informative genes are those whose transcript levels either are below the threshold level of detection and hence cannot be differentiated from background noise, or show no changes in the level of expression among comparison groups. After filtering, transcript levels in the treatment group (such as those exposed to a natural product) can be compared with those in the non-treated control group to detect genes that show significant up- or down-regulation (Figure 2).

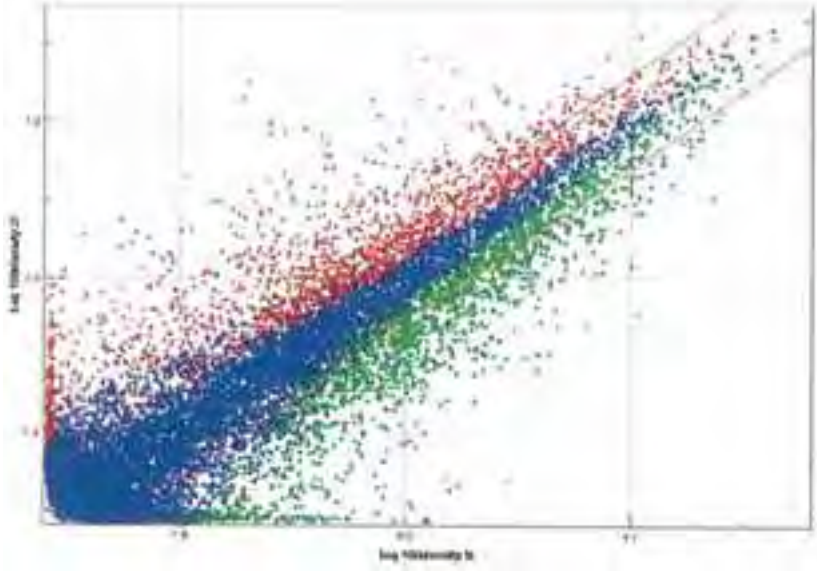

Figure 2. Correlation plot from Resolver to show log10(fluorescence intensity value) of treatment group (Intensity 2) versus control group (Intensity 1). Most gene transcripts do not show significant fold changes (blue). Genes with significant up-regulation are shown in red, while down-regulated genes are represented in green ($p < 0.001$).

Additional analyses can be made to look for patterns and relationships between genes in a data set. Some of the more commonly used methods include principal components analysis [31], hierarchical clustering [32], self-organising map [24,33] and relevance networks [34]. The value of the data set can be further enhanced by linking it to annotations and information available from other databases, such as the Gene Ontology Consortium Networks (www.geneontology.org), GenBank (www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html), and LocusLink (www.ncbi.nlm.nih.gov/LocusLink).

POST-ANALYSIS CHALLENGES

Although the tools for data analysis can whittle down the long list of genes in the microarray data set and help to focus attention on more 'interesting' items, the remaining number is often still quite substantial. Northern blotting and real-time PCR can be used in the post-analysis phase to verify the microarray analysis results. *In situ* hybridisation also helps in data verification and, in addition, provides valuable information on gene expression patterns. A major future challenge is to develop high throughput functional studies that can be used to test the many hypotheses generated from the large data sets.

CONCLUSION

The number of scientific publications on high-density oligonucleotide microarray studies has grown exponentially since 1994, when the first GeneChip® became available commercially. A recent example focuses on demethyl-asterriquinone B1, a fungal metabolite, and diabetes mellitus [35, 36]. Microarray can be used to identify targets and downstream effects of natural products and their derivatives, and help to monitor gene expression changes in response to drug administration. It is a very important and useful tool, and enables researchers to pose questions and evaluate hypotheses on a genomic scale.

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Limitations of Natural Pesticides: A Challenge to Scientists

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INTRODUCTION

Crop production and its protection have faced agriculturalists from the dawn of civilization. During the days of agroforestry, pressures on food production and plant protection were not severe enough to merit attention. Ever-increasing human population needed more food, which forced multiple cropping of selected food plants. It created pests and the need for crop protection. Natural balance between pests and its natural enemies, cultural practices and use of trap and companion crops to lure and repel pests provided satisfactory control up to a point. Later, it became necessary to introduce certain natural products for suppressing the pest populations. Thus, for the past 4,000 years and more, Indian farmers have been employing leaves and kernel of neem, *Azadirachta indica* A. Juss (Maliaceae), to repel insects from stored products [1]. In other parts of Asia, Africa and the Americas, indigenous technology involving plant and animal products were evolved for pest management over the centuries [2].

The Europeans, however, relied on inorganic compounds-sulphur arsenicals and soda, and olive oil. The Westerners continued to develop several effective inorganic pesticides such as Paris Green, Bordeaux mixture and iron sulphate between 1867 and 1890, and dinitro-orthocresol in 1923. Since the 1930s, organic chemists have synthesized thousands of toxic organophosphorous, organochlorine, carbamate, allethrin and pyrethroid pesticides, which revolutionized agriculture and vector control. The highly profitable cost-benefit ratio of using synthetic pesticides elevated their status by early 1950s, to a subculture within agriculture all over the world.

However, increased food production and effective vector control came at a high cost to environmental pollution and near extinction of several soil and river fauna and birds. Pesticide residues contaminated the environment and caused severe ecotoxicity to fish and other fauna and ecological imbalance. Pests started to develop resistance against pesticides, natural enemies of the pest were disappearing and the cost-benefit ratio of using pesticides became low. When the intangible cost of environmental contamination and ecotoxicity of residues was added to ecological problems created by the synthetic pesticides, their continued usage did not make economic or environmental sense.

Environmental and consumer interests forced governments around the world to develop strict legislative management of pesticides. Food and Agricultural Organization established limits for pesticide residues in foods. European Union Maximum Residue Levels of its own, leading to Zero Tolerance levels in the near future. Consumers demanded organically grown food, without the use of synthetic inputs. Farmers need natural pesticides for both organic and traditional farming.

Since the 1950s, scientists have refocused their attention to plants (as sources of natural pesticides) and other biocontrol rational (BR), which included natural enemies of pests, indigenous technology (IT), cultural practices, trap and companion crops and resistant natural and genetically modified plants for suppressing pest populations. The resurgence of botanical pesticides was pioneered by Dr. S. Pradhan at the Indian Agricultural Research Institute in the mid-1950s with research on pine oil, and later in the 1960s, on the antifeedant properties of neem kernel [4]. The discovery of 'paper factor' [5] in 1965 created great interest in plant-derived insect growth hormones. World-wide research has identified almost 2,500 plants with insecticidal properties. Indian scientists have developed integrated pest management (IPM) for the tropics with several BR adjuncts developed locally and made available to farmers as kits [6]. Most African farmers have integrated IT with aqueous extracts of neem for managing pests.

In spite of hundreds of research publications and successful usage of neem and other plant products, botanical pesticides have failed to replace synthetic ones. The present paper briefly reviews the progress and limitations of BR, particularly botanical pesticides, in

IPM around the world and identifies areas for research and development.

Natural pesticides

Natural pesticides may be defined as naturally occurring inorganic compounds and energies and animate beings, including microbes and genetically engineered organisms and plants. Many consumers may not accept the use of nuclear energy or genetically engineered plants in pest management but have no objection to synthesized, unmodified natural compounds. The present discussion is restricted to the role of most of these products in IPM and the challenges they offer to scientists, with special reference to botanical pesticides.

a. Microbial pesticides

Live virus, bacteria and fungi are the active ingredients in microbial pesticides. The polyhedrosis virus (NPV) and granulosis virus (GV) are much more effective pest control agents, than the cytoplasmic polyhedrosis virus (CPV). Of about a dozen commercially available microbial formulations, NPV of *H. armigera* and *S. litura* and GV of *C. infestans* have been developed in India [6]. However, the most popular microbial formulation is of *Bacillus thuringiensis* (BT), which is grown from fermentation products. BT is effective against resistant strains of pests, though prolonged usage does select resistant populations in the cabbage moth *Plutella xylostella* [4].

Marine and terrestrial fungi have not shown much promise as sources of pesticides [7]. More than 60 bioactive compounds from fungi have been chemically characterized but most of them have high mammalian toxicity and are considered unsafe for use in environment. Only avermectins (metabolites of *Streptomyces avermitilis*), milbemycins from *S. hygroscopisus* and tetranacins from *S. aurius* have been commercially dveloped [7]. It may be mentioned that marine flora is still an unexplored treasure.

b. Invertebrate pesticides

Nereistoxin, a metabolite in the shellfish *Lambriconereis hetropoda* provided the lead for the synthesis of Cartap, a popular

pesticide. Small scale farmers in Jamaica have been successfully controlling vegetable pests with extract of millipedes and centipedes. However, several bioactive compounds isolated from marine sponges, corals and annelids are not commercially viable [7].

c. Botanical pesticides

Since ancient times, leaves and stem of *Nicotina abacus*, roots of *Llanchocarpus utilis, L. urcu, Ryania speciosal* and *Schoenocaulony officinale* in the Americas, leaves and kernel of *Azadirachta indica* and dried seed powder of custard apple, *Annona squamosa* as, in India, and roots of *Derris elliptica* and *D. malaccensis* in South East Asia have provided products for pest management. African folklore recorded 106 plant species with insecticidal and molluscicidal properties [8]. However, the Chinese were the first to spray a plant formulation, tobacco decoction, on plum aphids in 1773, and Persians pioneered the use of Chrysanthemum sprays in 1818 [3].

During the past three decades, over 2,600 plants, belonging to about 200 families have been found to possess pesticidally active compounds [9]. Voluminous data has now been generated since the 1970s on chemistry, modes of action and filed trials of compounds from *A. indica* [10-15]. Neem extract does have multiple mode of action against a wide range of pests. Dev and Kaul [7] have described 324 compounds from micro-organisms, higher plants and marine organisms in proper sequence of acyclic and alicyclic, including isoprenoids, mono-sesqui-di-terpenoids, aromatic, heterocyclic and specific alkaloids, along with their structures and acute toxic effects. Hydrocarbons, ketones, acids and lactones have been arranged by their activity.

Features of plant compounds

Intensive research data on chrysanthemum, tobacco and neem provide a generalized insight into the characteristic features of pesticidal compounds from plants, which could be useful in bioprospecting for the botanicals. There are various factors which determine the manifestation of bioactivity on target organisms. These are briefly discussed as follows:

Variations in the levels of active ingredients

Most of the existing literature [10-12] on pesticidally active compounds in plants is derived from data obtained from a single part of the plant, collected at a certain time in a year and from one location. It is now becoming evident that the levels of bioactive compounds vary in different parts of the plant, and with season and location. For instance, the concentration of bioactive compounds is significantly more in roots of *L. utilis, L. urucu, S. officinalis, D. elliptica* and *D. malaccensis*, in the kernel of *A. indica,* and pyrethrins in the flowers of chrysanthemum than in any other part of the plant [7].

Ecotype has a great effect on the chemical content in plants. Pyrethrin is much higher in Yugoslavian chrysanthemum than from African or Asian plants [7]. The yield of crude extract varied by about 20%, and azadirachtin (Az) content from 1.5 to 6.5% from neem in different countries and continents [10-12]. Even tree to tree variations were reported from neem in Australia [14]. Little is known about seasonal and environmental influences on chemical content in trees but high levels of nimicinolide and isonimocinilide are found in neem leaves during winter than summer [12].

Stability of bioactive compounds

Most natural products undergo rapid photolysis. About 75% of Az is degraded under UV light within 14 hours and 80% of salannin in 10 minutes; under field conditions, 50% of Az is lost within 7 days. Temperature and humidity also influence degradation; at 65°C and 100% relative humidity, 30% of Az is lost in one day and 90% in three weeks [12]. Indeed, low persistence of nicotine and Az is one single factor against total dependence of farmers on botanical pesticides.

Extraction technology

The quality of appropriate solvents and the extraction method employed are important for obtaining standard plant extracts. Cold, steam or soxhlet extraction methods are commonly used. Water, methanol, methyl-ethyl-ketone and a mixture of methyl-tertiary-butyl ether and methanol were found to be the best for extracting Az [11, 12].

Evaluation criteria: Significant differences in the responses of different target species to a natural compound have been demonstrated. For instance, neem extract is active against 413 species of insects including grasshoppers and locusts but is ineffective against North American grasshoppers [18].

Pesticidal properties of plant extracts have been evaluated mainly on mortality of test organisms, though their impact may range from retardation of growth, development, oogenesis and embryogenesis to death. The discovery of 'paper factor' [5] had led to commercial development of several insect hormone mimics. Most plant extracts have little acute toxic effects on cattle ticks but almost all retard oogenesis and embryogenesis significantly. In fact tobacco, *Hibiscus* and neem extracts inflict high mortality while *Simarauba glauca* extract almost completely blocks oogenesis and embryogenesis [21].

Structure-Activity relationship

Elucidation of structure-activity relationship is useful in exploring plant species and genera for pesticidally active compounds, which are allied to chemically defined plants. Jacobson [12] and Dev and Koul [7] have provided an overview of isolation and identification of antifeedant phenols, quinines, alkaloids and other phenolic compounds, acids and lactones from various plants. Generally, the bioactivity of compounds depends upon the side groups, isomers and length of carbon side chains.

Neem has 55 bioactive Az compounds, which are complex molecules that are difficult to synthesise [16]. Rembold *et al.* [11, 12] have concluded that bioactivity of Az A and B and their nine derivatives is generally dependent upon the (a) presence of epoxide structure (C-13/C-14 oxirane ring), (b) hydrogenation of the dihydrofurane ring, (c) addition of alcohol, and (d) hydroxyl group at C-1 and C-3. Chiu [11] had demonstrated that polar fractions of neem and chinaberry extracts had systemic action, non-polar and polar fractions had antifeedant properties, and Az and triterpenoid toosendanin in chinaberry exerted toxic action.

Formulations and synergism

Bioactivity of plant compounds can be enhanced by extracts of other plants and adjuvants. Piperonyl butoxide has been a choice synergist of both natural and synthetic organic pesticides. Vegetable oils and mineral oil synergize Az activity; just as Az synergizes BT formulation [12]. Even spreaders and surfactants such as dimethyl sulfoxide, Gel-spray surfactant and Tritox X synergize the action of a number of plant extracts [17].

Compatibility with synthetic insecticides

Several workers have found neem formulations to be compatible with and synergistic to many synthetic insecticides such as fenvalerate, quinalphos, carbofuran, pyrethroids, endosulphan, malathion, profenfos, chlorpyrifos and monocrotophos [13,14].

Friendliness to natural enemies

Plant extracts have no adverse effect on several parasites and predators of crop pests; on the contrary, parasitism of rice leaf-folder is increased by neem extracts. In fact, most parasites are repelled by plant extracts [10-15]. This is in contrast to the devastating effects of synthetic pesticides on the natural enemies, which has created ecological imbalance in field crops, resulting in resurgence of pest populations.

Environmental, occupational and consumer friendliness

Of various plant compounds, nicotine has the highest mammalian toxicity (oral LD_{50} of 60 mg/kg rat), followed by that of pyrethrin (260–400/kg rat). Az, which is consumed as medicine in India is very safe, the oral LD_{50} to mice being 6760 to 8700 mg/kg. Similarly, *Hibiscus* is another plant with medicinal and insecticidal properties. It may be pointed out that Az at 0.5 mg/kg reversibly reduced sperm count and motility, weight and muscle tone in rats [13, 14]. Az used for mosquito control did not have any adverse effects on various species of fish. The LD_{50} of Az for chicken is 39.9 mg/kg and 7,000 mg/kg for quail. Neem extracts and cake increase earthworm population and reproduction, while crustaceans are adversely affected [13, 14] Urea-coated neem cake enhances nitrogen

efficiency of fertilizer and inhibits nitrification of ammonium-yielding fertilizer in soil which, in turn, reduces nitrate contamination of surface and ground water [13,14].

Management of resistant pest populations

Neem extracts have been effective in controlling populations of cabbage moth, which had developed high resistance against various synthetic insecticides. In laboratory experiments, Vollinger [10] found that feeding neem for 42 generations did not induce any resistance in the pest, while it developed 22- to 35-fold resistance against two synthetic insecticides.

Bioactivity of botanical pesticides

Plant extracts have a wide spectrum of microbial, fungicidal, weedicidal, nematicidal, molluscicidal, acaricidal, insecticidal and rodenticidal activities, which vary with the compounds present. Antiviral activity of oils of *Pongamia pimata, Madhuka longifolia* and *Callophylum inophyllum* has been demonstrated against rice viruses, while extracts of a few other plants controlled chilli mosaic virus. Neem formulations have moderate effects on viruses [16]. Bactericidal action of neem is species-specific. Neem cake coated with urea reduces incidence of rice blast and is synergistic to the insecticidal action of *Bacillus*. Many more plant extracts reportedly possess fungicidal activity. Neem cake had adverse effect on fungal diseases of cotton seedlings, soyabean and on human pathogenic fungi [11, 12]. Many plant extracts and products have adverse effects on fungal diseases of plants. Neem products have high activity against aflatoxins and diseases of cotton, tobacco, rice, citrus, chilli and cardimon [13,14]. Oil cakes of neem, mustard, castor and *Eruca sativa* have adverse effects against most fungi but promoted the growth of others [4, 13-15].

Weedicidal activity was reported in only 14 of over 2,100 plant extracts screened. However, neem cake and oil, and extracts of *Cassia sericea* and oil of *Cymbopogon martini* had reduced the germination of two of the most dreaded weeds in India, *Phalaris minor* and *Parthenium martini* [6]. In fact, parthenin from *Parthenium* is a potent growth inhibitor of certain weeds.

Compounds with nematicidal and antihelminthic activities are fairly widespread in plants. Neem extracts and formulations are highly successful against many plant nematodes [14-15]. Nigerian folklore has identified 106 plants with molluscicidal activity, of which only 22 have been confirmed [8]. Promising molluscicidal activity has also been reported from 21 of 51 Brazilian plant extracts [18].

Acaricidal activity was demonstrated in 50 of nearly 60 plants investigated. Extensive research on the bioassay-driven chemical characterization of some plants has revealed that the cattle ticks, *Boophilus microplus* and *Amblyomma cajanensee,* are moderately susceptible to most plant extracts but highly susceptible to tobacco, *Hibiscus* and a few other plant extracts. Besides inflicting mortality, several plant extracts severely inhibit oogenesis, embryogenesis and survival of larvae, which may hatch from eggs laid by treated ticks [4, 19-23].

Literature is replete with multiple insecticidal action of over 50% of extracts of about 2,500 plants examined, ranging from acute toxicity, and antifeedant, growth regulatory, semiochemical and photoensitiser effects [5, 9, 11-15, 24]. Stimulation of deterrent-sensitive neurons in gustatory sensilla of insects leads to antifeedant or repellent actions of neem and many other plant extracts. The bioactive compounds from various plants with antifeedant properties have been isolated and chemically characterized and identified. Disruption of neuoendocrine system causes morphogenetic abnormalities in insects. In fact, the isolation of plant compounds could lead to the synthesis of various insect growth regulators, which are sold commercially.

Semiochemicals, such as pheromones, kairomones and synamones, which modify the behaviour of insects, have been synthesized by taking the lead from natural products. Indian scientists have synthesized a synamone and about a dozen pheromones for commercial production [6]. The most significant aspect of the Indian strategy is to prepare kits with chemicals with dispensers and train farmers on their field use.Many plant extracts are repellent at higher concentrations and antifeedant at lower concentrations [4]. This is an area, which needs to be explored more as the application of such dual-purpose extracts would be easier, cheaper and more effective than commercially available synthetic repellents or attractants.

Photosensitisers, such as polyacetylenes, furanocoumarins, beta-carbolines and extended quinines which induce phototoxicity in insects, are present in many plants. Polyacetelynes, which have over 500 diverse compounds present mainly in *Asteraceae* family are powerful compounds [24]. At least one S-derivative, alpha-terthienyl is more toxic to mosquito larvae than DDT. The toxicity is mediated by the production of singlet oxygen and probably some free radicals. Some of them interact with DNA and cause chromosomal abnormalities. Much more focus is needed on such compounds, which could synergize the toxic action of Az and similar compounds in the tropics.

Animal products

Fermention of cow, goat and pig urine and whey, often integrated with inter-cropping and spraying with plant extracts, has been the indigenous pest management technique among the poor African farmers, which is presently popularized in certain regions for certain crops [2]. The urine-technology needs modern technological input.

Companion plants

Certain plants, inter-cropped with main crops, repel insect pests and deter the growth of weeds. Inter-cropping of cabbage with dill, garlic, sunflower, oat, barley, onion and pepper reduced infestation by the diamond-back moth in cabbage. When integrated with a couple of sprays with botanical extracts or synthetic insecticides, intercropping provides almost complete control of the pest in Mauritius and other parts of the world [5, 14]. On the contrary, cultivating mustard hedge around cabbage plots attracts the pests away from the main crop.

Wheat intercropped with marigold completely suppressed the propagation of *Parthenium* weed. Other plants such as *Abuitlion indicum, Croton sparsiflora, Tephrosia purpurea, Leucas aspera* and *Prosopis juliflora* also competed successfully with *Parthenium* [6]. It would be interesting to isolate the active ingredients from these plants and develop stable formulations for repelling or attracting the pests and inhibiting weed growth.

Biotechnology products

Biotechnology may range from simple grafting, through modification of processed foods and selection and breeding of resistant varieties of plants to genetic engineering. Grafting of *Coffea arabica* on drought and nematode-resistant roots of *C. robusta* is promoted in India. Processed foods may be protected from insect pests by creating dietary imbalance for insects [25]. Such imbalances would not affect human beings, as they can balance with other food intakes.

Transgenic or genetically transformed plants, which are resistant to specific pathogens or pests are developed by r-DNA technology in which genes responsible for producing delta-endotoxins from *Bacillus thuringiensis* (or any other source) are inserted into the plant genome. The transgenic plant produces toxic protein, which kills the pests or pathogen. Various pest-resistant cultivars have been developed for sorghum, wheat, corn and other major crops. Introgression of natural resistance and/or tolerant genes into papaya has provided almost complete protection against Papaya Ringspot Virus (PRSV), which was first described in Jamaica in 1989 [26].

Sterile Insect Technique (SIT) involves releasing a large population of arthropod pest/ ecto-parasites, which were sterilized by feeding chemosterilants or by exposure to radiation, in the field at an estimated ratio of 1:10 sterile individuals to one normal individual. By regular release of the sterile population over a period of a few years, complete eradication of the New World Screwworm (*Cochliomyi hominivorax*), a dreaded ectoparasite of cattle, wildlife and dogs and the Mediterranean fruitfly, have been achieved in several countries [27].

Parasitoids and predators

Introduction, inundation and conservation of parasitoids and predators, arthropods or entomorphagous nematodes, have been practised fairly successfully for a long time in the biological control of the pests. Insect parasitods and predators are a major component of integrated management of crucifer pests in Asia [28]. India has over 70 projects for mass production of *Trichogramma* and *Chrysoa* at commercial insectories in the country [6]. In the Caribbean, parasitoids of cabbage moth and coffee berry borer have been

introduced but their effects have not been so successful. However, the pink mealybug, which had been devastating a number of big and small trees in Eastern Caribbean in 1996 was successfully controlled by a lady bird beetle from India.

Entomorphagous nematodes offer great promise as biocontrol agents. Eighty species of nematodes and some of their strains have been tested under field and laboratory conditions on about 230 species of insects (86 Coleoptera, 80 Lepidoptera, 28 Diptera, 13 Heteroptera, 9 Hymenoptera, 6 Orthoptera, 5 Homoptera and 5 other orders). Generally, the infectivity of nematodes has been good against most hosts [29]. It has also shown great promise against citrus root weevil and sweet potato weevil in Jamaica [30].

Field performance of botanical pesticides

India launched the search for alternatives to synthetic pesticides in the 1950s, at a time when the motto of the entomologists around the world was 'pest eradication' and the multinationals were successfully inculcating a 'pesticide subculture' within agriculture. Since Pradhan's group at the I.A.R.I. initiated the scientific validation of indigenous usage of neem leaves, kernel and cake in pest management, it has been the main focus of attention internationally, though dozens of other plants have shown promise as pesticides [4].

However, the objectives of 'East' and 'West' were quite different. Scientists in India aimed at achieving the Gandhian self-reliance in pest management at village level. The developing countries of Africa, Asia and Central America and the Caribbean adopted the Indian policy, and started to scientifically explore their own folk practices, while concentrating on neem cultivation and utilization. Today, the Indian scientists are integrating products of high technology and indigenous practices in pest management with remarkable success.

Scientists in the West became interested in neem, more for understanding its chemical structure and synthesizing the active ingredient for commercial manufacturing of synthetic compound. Medicinal use of neem in India has already provided safety-certificate to its products. The activity-directed extraction, purification and isolation of compounds, mainly by German and Indian scientists, have generated extremely valuable data on mode of action of Az. Similar

properties of many other plants have been demonstrated but not on a wide spectrum of pests. Though neem has antiviral, bactericidal and antifungal action, its most important action is on morphogenesis (moulting, oogenesis, embryogenesis) of arthropods, followed by its antifeedant, repellent and toxic actions, respectively. Its mode of entry into the plant could be through epidermis or roots (systemic) [4].

Field trials have confirmed that neem formulations alone, or in combination with BT, synthetic insecticide, parasitoids or companion plants are capable of managing pests of vegetables, rice, chillies, potato, cotton, corn, pulses, stored products, forest and ornamentals. Neem products are also very effective in controlling mosquitoes. Neem extracts have also been effective in controlling forest pests in Nigeria and aphids on ornamentals by soil drenching. Neem kernel powder, mixed with pulses, wheat, corn, rice, tobacco and spices, or treating the bags with dust provide protection to the commodities against all insect pests [4, 10-15]. Some scientists have reported satisfactory control of various rice pests by neem products in the fields of Asia [13, 14]. Neem formulations were found to perform better than delta-methrin against the rice leafhopper, *Nephotettix virescene*. However, a five-year field study reported only moderate effect of a variety of neem formulations against rice pests, particularly during the summer [4].

Economic threshold-directed application of neem products, in combination with synthetic pyrethroids or quinalphos, was found to be most cost-effective in controlling the pink ballworm of cotton and increasing the yields [13]. Methanol and ethanol extracts of neem kernel were effective in controlling the corn borer, *Ostria nubilalis* and leaf miner in Israel [12]. Oil and other compounds of *Acorus calamus* were effective in reducing field infestation by the African corn borer, *Prosptephamus truncates.* Also, infestation by the stalk borer of maize was reduced by neem extracts. Neem oil sprays on cassava and corn grown in the Republic of Benin repelled the grasshopper, *Zonocerus variegates*, without antifeedant, morphogenetic and toxic effects [13].

The best cost-effective ratio for controlling *H. armigera* on pulses was obtained with two sprays of neem seed extract and one of

cypermethrin; soil treatment with phorate plus nuclear polyhedrosis virus plus neem extract also gave good results. Neem extract with karanjia oil is recommended for tobacco pest management [13, 14]. Neem extract and its commercial formulations provided the most convincing results in managing pests of a variety of vegetables, particularly when integrated with the use of synthetic insecticides and companion crops. Crushed neem suspensions were effective in field trials against the insect pests of sorrel, cucumber, tomato, cabbage and sweet potato, but not the nematodes [10]. Neem formulations provided good control of whitefly, jassids, aphids, tuberworm and black cutworm of potato crops in Sudan, where it has been incorporated in an IPM package for the crop. Neem formulations with or alternated with nicotine sulphate gave as good control of *Heliothis* on okra, as carbaryl [10-14].

Commercial formulations of neem can be used in large-scale cultivation of peppers, melons, lettuce, pears, citrus and celery in U.S.A. [14]. Neem extracts have been used successfully against vegetable pests in the Dominican Republic, Nicaragua, Egypt, West Africa, Mauritius, Sri Lanka, Thailand and Myanmar [4, 15]. Cuba has been utilizing tobacco debris in exemplary management of vegetable pests.

Challenges to scientists

With such an impressive performance of neem extracts in the integrated management of crop pests, one wonders why the total reliance on the 'technique' developed is so slow. Is it a matter of confidence in existing data, gaps in scientific knowledge, logistics, inadequate application of technology to neem and other botanical formulations? Critical analysis would reveal that all these issues pose challenges to entomologists, chemists, agricultural engineers and farmers, before botanicals can fully replace synthetic pesticides in IPM.

The scientists must appreciate the following facts which have constrained the usage of botanical pesticides:

1. The quick lethal action of synthetic pesticides is not as dependent on agro-ecosystems (which have characteristic differences

between tropical and temperate regions), as natural pesticides and biocontrol rational.

2. Variations in different ecosystems in the tropics determine the distribution of pests and their natural enemies, which is the major challenge to IPM strategy.
3. Environmental conditions in the tropics effectively favour multiplication of pests more than the growth of crops; hence emphasis on crop protection should be more than on crop production.
4. When pests multiply rapidly and their population build up rapidly, they must be controlled immediately. The botanical formulations and natural enemies require a 'lag period' before manifesting their effects, but the fast-acting synthetic insecticide does not.
5. The influence of companion plants is significantly less when the conditions favour relatively rapid growth and multiplication of pests.
6. Botanical formulations have been most effective when combined with low doses of synthetic pesticides.
7. Relatively slow action of natural pesticides and other biocontrol agents provide little margin for error in their implementation, compared with toxic and persistent synthetic pesticides.
8. Data on the basic IPM principles of the following issues have yet to be generated: (a) why any action is required, (b) what options are available, (c) what options are to be integrated, (d) when is action to be taken, which requires knowing the action-threshold for each option, (e) which application technology to apply when action is taken, and (f) how often.
9. Pest management with botanical formulations is labour-intensive and has been successful mainly in countries with cheap labour, such as Africa and Cuba.
10. Very little analytical data have been generated on the persistence of residues of plant compounds on field crops, which are correlated with pest population. Successes reported may have been more due to ecological factors than pesticidal action.
11. The major constraints in the usage of botanicals are their slow action and low persistence.

It is obvious that concerted inter-disciplinary research and development effort by entomologists and chemists are required for widespread acceptance of natural pesticides. These efforts should focus on:

1. generation of basic entomological data on IPM principles, as outlined above in point # 7.
2. intensive research on fast-acting natural products, such as nicotine, pyrethrins and developing formulations with slow-acting antifeedant and growth-regulating plant compounds.
3. development of photostable botanical formulations.
4. generation of analytical data on persistence and residue levels of active ingredients of botanicals on different crops under field conditions.
5. exploration for photosensitizing chemicals in plants, understanding their chemistry and developing formulations for their usage with other plant extracts. Such a formulation would be most effective under tropical sunny conditions.
6. development of microbial insecticides. This should be accelerated by focussing on the propagation of host insect-cell lines for pathogens, which grow easily on artificial medium and have wider spectrum of activity.
7. development of appropriate application technology by designing nozzles, which can uniformly spray dust, granules and liquid on both sides of the leaves,
8. development of technology for manufacturing botanicals at cottage industry levels, which would reduce the production and distribution costs.

CONCLUSION

Natural pesticides and other biocontrol rational are indeed the future of IPM, particularly as the consumer demand in developed countries for organically cultivated produce is increasing. Plants would be the major source of future pesticides and medicines. Globalization, intellectual property rights and international patenting have accelerated bioprospecting in the tropical countries by multinational corporations and scientists from Western countries. So far, the research and

development of botanical formulations such as neem has been more 'amateurish', compared with the professionalism in the development of synthetic pesticides.

Tropical countries must maximize their resources by initiating holistic approach within each country and share it with others by establishing South-South collaboration. It would accelerate national bioprospecting activities, leading to commercial development of natural products at a lower cost. India and Singapore have the expertise, infrastructure for science and technology and natural and financial resources to launch an international joint venture programme on developing botanical formulations and other natural products.

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Bioactivity of Hexane Extract of *Blighia sapida* Konig. on the Cattle Tick, *Boophilus microplus* and the Sweet Potato Weevil, *Cylas formicarius elegantulus*

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INTRODUCTION

The ackee (*Blighia sapida* Konig) fruit is consumed as an ethnic Jamaican cuisine and provides significant export earnings to the country. The aril of the fruit is quite perishable and about 40 % of the fruits are discarded as waste. The economic use of the waste could increase the earnings of the sector. Jamaican folklore claims the ackee fruits and leaves as good termiticides; scientific investigations have confirmed its insecticidal and acaricidal properties [1-2]. This has prompted the exploration of different parts of the fruit for their pesticidal potential against the cattle tick (*Boophilus microplus* Canestrinii) and the sweet potato weevil (*Cylas formicarius elegantulus* Summer).

MATERIALS AND METHODS

Preparation of extracts

Fruits of ackee were collected fresh from the trees and the aril and seeds removed. The plant parts were dried to constant weight in an oven at 40°C and then blended with hexane. The extract was filtered and concentrated with a rotary evaporator under vacuum at 45°C. Different concentrations of the extracts were made up and applied topically by Eppendorf pipettes to test for effects on ticks and weevils.

Treatment of Cylas formicarius elegantulus

Two-week-old adult *C. formicarius* were reared in the laboratory from population collected from a sweet potato field. The insects were kept in glass aquaria at room temperature (27-30°C) and 55-75% relative humidity. They were allowed to feed and complete their life cycle on sweet potato. Emerging adults were transferred daily to new aquaria and provided with fresh food; two-week-old adults were collected for bioassays. Oils were applied topically to the dorsal surface of the insect. Control insects were treated with solvent. Mortality was recorded after 24 hours. Ten adult *C. formicarius* were used in replicates of three for each treatment. The insects were noted as dead if they showed no signs of movement when aroused by external stimuli.

Treatment of *Boophilus microplus*

Cattle ticks, *B. microplus*, were collected from the local abattoir and used within 4 hours. Fully engorged females of *B. microplus* weighing 150-200 mg were used for the bioassays within four hours of collection. Oils were applied topically to the dorsal surface of the ticks. Ten ticks were used in replicates of three. Controls were treated with solvent. Mortality was recorded every 24 hours and the dead ticks removed. The surviving ticks were allowed to oviposit for up to 12 days after treatment. For every two days after treatment, the ticks were observed for commencement of oviposition and the data on the number of ticks laying and the weight of the eggs were collected. The eggs oviposited by *B. microplus* were incubated in test tubes under laboratory conditions at room temperature (27-30°C) and 55-75% relative humidity for two months, when larvae aggregated to the top were removed. The unhatched eggs and eggshells of each replicate were mixed thoroughly and three samples (each containing about 200 unhatched eggs or empty shells) were examined under a microscope. The percentage of hatched eggs to total number of eggs oviposited was recorded.

Statistical analysis

Mortality, ovipositional and embryogenesis data were analysed by Analysis of Variance (ANOVA). Means comparison was based on the least square method.

RESULTS

C. formicarius elegantulus was highly susceptible to 0.6 µl of the aril oil as 95% adults died after 2 hr of treatment (Table 1).

Table 1. Toxic Effects of Aril Oil of *B. sapida* on *C. formicarius* after 2 hr of treatment.

Dose (µl)	% Mortality
0.2	30 ±10
0.4	62 ± 8
0.6	95 ±5
Control (0)	0

*$p < 0.05$ (compared to control)

At concentrations ranging from 2 to 16 µl, the seed oil of *B. sapida* did not inflict mortality on *B. microplus* after 24 hr. Even after 72, 144 and 168 hr, mortality was not significantly different from that observed for the control animals. The aril oil of *B. sapida*, however, inflicted high mortality on the ticks (Table 2). Oviposition by *B. microplus* was significantly affected by the aril oil ($p < 0.05$). The mean weight of eggs laid was significantly lower in the aril oil-treated ticks compared to that obtained with the seed oil and control ($p < 0.05$) (Table 3). Highest inhibition of oviposition was obtained at the lowest concentration of 2 µl of aril oil.

Table 2. Toxic effects of seed and aril oils of the fruit of *B. sapida* on mortality of *B. microplus* after 24, 72, 144 and 168 hr.

Treatment	Mortality (Mean % ± S.E) At Different Times			
	24 hr	72 hr	144 hr	168 hr
Aril Oil	73.33 ± 2.8 (a)	73.33 ± 7.7 (a)	96.66 ± 9.3 (a)	100 ± 8.3 (a)
Seed Oil	0 ± 2.8 (b)	23.33 ± 8.3 (b)	30.00 ± 9.3	36.67 ± 7.7 (b)
Control	5 ± 2.0 (b)	11.67 ± 5.5 (b)	20.00 ± 5.9 (b)	21.67 ± 6.6 (b)

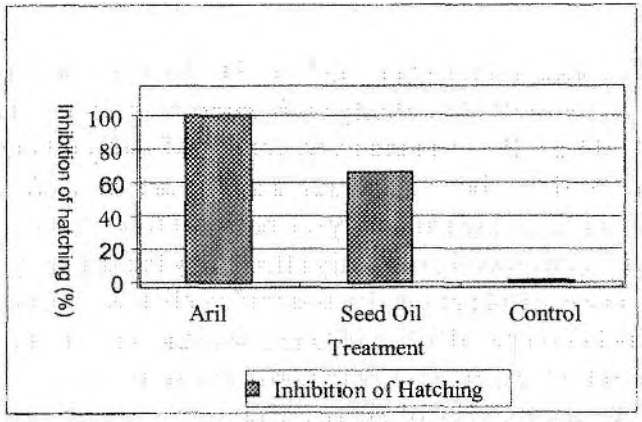
*Means are pooled for different dosages.

Table 3. Effects of aril and seed oils of *B. sapida*. on weight of eggs laid and inhibition of oviposition after 168 hr of treatment.

Treatment	Total Weight of Eggs Laid (mg)	Inhibition of Oviposition (%)
Ackee Oil	0.237 (a)	99.9
Ackee Seed Oil	**84.1 (b)**	**60**
Control	**210.9 (c)**	**0**

S.E.M = 0.054; $P = 0.028$
Numbers represent pooled means for data collected for 7 days on 30 ticks.

The aril oil of *B. sapida* inhibited embryogenesis by 100% compared to the 66% inhibition observed with the seed oil ($p < 0.0001$) [Figure 1].



S.E.M. = 1.963; $P<0.0001$

Figure 1. Effects of aril and seed oils of *B.sapida* on hatching in *B. microplus*

DISCUSSION

B. sapida contains oleic, palmitic and stearic acids as the major fatty acids [3], the amino acid, hypoglycin A (L-α-amino-2-methylenecyclopropane-propionic acid) and the dipeptide, hypoglycin B (γ-L-glutamyl-α-amino-β-(2-methylene-cyclopropyl) propionic acid) [4]. In mammalian systems, hypoglycin is metabolised to its toxic form, methylene-cyclopropyl acetic acid, which strongly inhibits transport of long chain fatty acids into mitochondria, thereby suppressing their oxidation (5).

The inhibitory action of oviposition and hatching of plant extracts on *B. microplus* may be attributed to the interference with the synthesis of sex-specific proteins, methyl esters and fatty acids. Increased sequestration of protein and the inhibition of sequestration of all types of fatty acids and methyl esters by the developing oocytes in *B. microplus* treated with extract of *A. altilis* have been reported (1). This is worth further investigation, especially *B. sapida* which already contains several fatty acids. The chain of events may also have been triggered by the inhibition of neuromuscular activity (6, 7). *B. sapida* which is stable for at least 4 weeks (2) shows strong potential as a pesticide and further detailed research involving the possible modes of action is to be undertaken.

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Antibacterial Screening of *Tragia involucrata L.* (Euphorbiaceae) — A Tribal Used Medicinal Plant

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INTRODUCTION

Since time immemorial, folk medicinal plants have been used to cure human ailments. Up till now, traditional healers (present in tribes) are using plants for the preparation of various herbal drugs based on their own traditional methods without any scientific basis. There were 6000 species of medicinal plants used by folk practitioners during 1985–1990 in India. It is estimated that nearly 50,000 herbal formulations were developed from 4600 tribal communities. Many of these formulations have been used to treat cold, inflammation, snake bite, mental illness and skin diseases, as well as for birth control and delivery of babies. A leaf paste obtained from *Clistanthus colinuswas* is used to commit suicide by tribes in many native settlements. The leaf paste obtained from *Phyllanthus amarus* is used as herbal therapy for jaundice [1]. Medicinal plants have been used to treat a wide range of human diseases because of their therapeutic values [2]. A large proportion of the population in Uganda still rely on the use of herbal remedies against measles, a killer disease of children.

Knowledge derived from the use of traditional medicines has indeed played a crucial role in the research and development of plant derived-drugs in the commercial market. Recently, researchers have

reported the antimicrobial activity of traditional medicinal plants worldwide. *Osmitopsis asteriscoides,* a medicinal plant used in traditional drug preparations in South Africa, has been evaluated for antimicrobial activity against *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa* [3]. The antimicrobial potential of *Carpobrotus* species (Aizoaceae) extracts was tested against *S. aureus, P. aeruginosa, C. albicans* and *Mycobacterium smegmatis* [4]. The efficacy of *Warburgia ugandensis* and *Zanthoxylum chalybeum* against common bacteria and fungi have also been investigated [5]. The oil obtained from the bark of *Santiria trimera* widely used by the traditional healers for wound healing [6] has also been investigated. Most people in South Africa with sexually transmitted diseases (STDs) first seek help from traditional healers [7]. Moreover, ethnobotanical investigations on the medicinal uses of *Combretum* (Combretaceae) and *Terminalia* species have shown the potential of medical applications against various bacterial infections such as gonorrhoea, syphilis, diarrhoea, hypertension and cancer. Antimicrobial screening of crude extracts of selected *Combretum* and *Terminalia* species were performed by the agar-diffusion method [8].

Tragia involucrata L. is a commonly available medicinal plant belonging to the family Euphorbiaceae. The Malaiali tribes use different parts of this plant for treating wounds and skin infections in Western Ghats of India [9]. The leaves are used to cure headache and the roots to treat fever. A paste prepared from the root inactivates and kills worms. The paste is mixed with leaf juice of *Ocimum sanctum* and employed for the treatment of skin and venereal diseases. The fruit extracts are rubbed on the head to treat baldness. The efficacy of this plant is well-known in Indian traditional medicine systems [10]. However, the stinging hairs of the plant have sharp siliceous points that break off when touched and can penetrate the skin. Baily and van Puyvelde [11] reported the antimicrobial activity of methanol extracts of *Tragia brevipes* against *Staphylococcus aureus*. Thus far, only taxonomic studies have been carried out on this plant [12]. Hence, the present investigations have focused on the *in vitro* screening of various extracts of *Tragia involucrata* tested against bacteria.

Figure 1. *Tragia involucrata* as a tribal "Malaiali"-used traditional therapy for bacterial infections.
(a) Whole plant in its natural habitat, (b) Roots, (c) Fruits with stinking needles, (d) Seeds

MATERIALS AND METHODS

Plant collection and extraction

Tragia involucrata (Figure 1) was collected from local healers of the Malaiali tribe in 1997, based on information on their medicinal use in the Kalrayan hills in the Western Ghats of Tamil Nadu, India. The plant was taxonomically identified and authenticated with the help

of "Flora of Tamil Nadu Carnatic" [13]. Different parts of the plant — fresh leaf, root and seed — were dried and ground to powder. 100 g of powder from each of these plant parts was extracted successively with 600 ml of water and the following solvents — hexane, diethyl ether, dichloromethane, ethyl acetate and methanol. The extraction was carried out at room temperature (31°C) and with mechanical shaking for 24 hrs. The extracts were filtered and evaporated using a vacuum rotary evaporator at 40°C. 100 g portions of dry powdered extract were then suspended overnight in 600 ml of distilled water before being filtered and evaporated in a water bath at 60°C [14].

Culture of microorganisms

Pure cultures of *Escherichia coli* and *Proteus vulgaris* (both Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria) were obtained from the Department of Microbiology, Dr. A.L.M PGIBMS, Taramani, Chennai, Tamil Nadu, India. The bacteria were maintained by frequent sub-culturing on Mueller Hinton (MH) agar plates (pH 7.4) and stored at 4°C. The following antibiotics were included in the test as references: chloramphenicol (30 µg/ml) and streptomycin (30 µg/ml). All the products were supplied by HiMedia Chemicals, Mumbai, India.

Antibacterial activity

Antibacterial activity was determined by the "well diffusion" method. Bacteria were grown on MH liquid broth to approximately a mid-logarithmic phase ($A_{600} = 0.1 \times 10^5$ cells/ml, CFU). 20 ml of sterile MH agar medium was mixed with 20 µl of bacterial inoculums poured into the petri-dishes and left to solidify. Wells (each 6 mm diameter) were prepared with the help of borer and sealed with a drop of sterile MH media. 25 µg/ml of the different extracts (at different dilutions ranging from 0.05 µg/ml–50 mg/ml) were dissolved in 70% acetone (w/v). Five replicates were used for each concentration of the extract. The standard drugs, chloramphenicol) and streptomycin, were dissolved in 0.9% saline (pH 7.4) and introduced into wells as drug controls while the solvent alone served as normal control. The plates were then incubated at 37°C for 18 and 48 hrs. Zones of inhibition were measured with vernier calipers [15].

Table 1. Evaluation of antibacterial activities of different extracts of the leaves of *Tragia involucrata*

Leaf extracts (25 µg/ml)	Incubation with bacteria at 37°C for 18 hours							Incubation with bacteria at 37°C for 24 hours						
	Hexane	Ethyl acetate	Diethyl ether	DCM	MeOH	Water	Hexane	Ethyl acetate	Diethyl ether	DCM	MeOH	Water	Chlor	Strep
E. coli	-	+	+	-	++	++	-	+	+	-	++	++	++	++
P. vulgaris	-	++	+	-	+++	+++	-	++	++	-	++++	++++	+++	+++
S. aureus	+	++	++	-	+++	+++	++	+++	++	-	++++	++++	++++	+++
Root extracts														
E. coli	-	-	-	-	+	++	-	++	+	-	++	++	-	-
P. vulgaris	+	++	+	-	++	+++	+	++	++	-	+++	+++	-	-
S. aureus	+	+++	++	-	+++	+++	+	+++	++	-	+++	+++	-	-
Seed extracts														
E. coli	-	+	+	-	+	++	-	+	+	+	++	++	-	-
P. vulgaris	+	+	+	+	+	++	+	+	+++		++	++		-
S. aureus	+	+	+	-	++	++	+	++	++	-	++	++	-	-

Zones of inhibition (mean ± SD, n=5); + 9-11 mm; ++ 12-14 mm; +++ 15-18 mm; ++++ 19 mm and above; No activity (-); Chlor = Chloramphenicol (30 µg/ml); Strep = Streptomycin (30 µg/ml); DCM = Dichloromethane; MeOH = Methanol.

Minimum Inhibitory Concentrations (MIC)

MIC was determined using the tube dilution method of Hufford *et al.* [16]. To sterile test tubes containing 3 ml of liquid medium, 0.5 ml of bacterial suspension and 0.1 ml of each of the plant extracts were added. Chloramphenicol and streptomycin were used as positive controls while the tubes containing growth medium with inoculums and solvent served as normal controls. After incubation at 37°C for 24 hrs, the turbidity caused by the bacterial growth was estimated using a spectrophotometer at 560 nm. Data were analysed as means ± standard deviation of five replicates (n=5). Two-way analysis of variance (ANOVA) was used for statistical analysis, with p values <0.05 considered statistically significant.

Phytochemical screening

Phytochemical screening of different extracts of *T. involucrata* was performed by chemical tests for alkaloids, flavonoids, terpenoids, phenols, saponins, tannins and steroids [17].

RESULTS AND DISCUSSION

The results of the *in vitro* screening of different extracts of *T. involucrata* tested against pathogenic bacteria are shown in Table 1. After 18 hr incubation, the ethyl acetate, diethyl ether, methanol and water fractions of the leaf extract showed a broad spectrum of antibacterial activity. The water extract was most effective against *S.*

Table 2. MIC values of antibacterial activities of the water and methanol extracts of *Tragia involucrata* leaves

Extract concentrations (mg/ml)	Water extracts			Methanol extracts		
	E. coli	*P. vulgaris*	*S. aureus*	*E. coli*	*P. vulgaris*	*S. aureus*
Bacterial control	0.607	0.705	0.613	1.060	1.010	0.921
50 mg/ml	0.323	0.421	0.422	0.323	0.421	0.422
0.5 mg/ ml	0.226	0.350	0.332	0.226	0.350	0.332
0.05 mg/ ml	0.236	0.231	0.221	0.336	0.362	0.221
5 µg/ ml	0.239	0.262	0.345	0.394	0.311	0.345
0.5 µg/ ml	0.256	0.245	0.217	0.401	0.325	0.367
Chloramphenicol 30 µg/ml ml	0.10	0.030	0.027	0.120	0.037	0.042
Streptomycin 30 µg/ml	0.020	0.025	0.042	0.056	0.028	0.039

aureus. A similar result was reported by Peres *et al.* [18] on the aqueous and ethyl alcohol extracts of *Croton urucurana* (Euphorbiaceae) against *S. aureus*. In our study, hexane and diethyl ether exhibited antibacterial activity against one or more bacteria at 25 mg/ml. However, the dichloromethane leaf extract of *T. involucrata* was the only extract to have bacterial activity against *P. vulgaris*.

Hexane, ethyl acetate, diethyl ether, methanol and water extracts of the roots of *T. involucrata* inhibited the bacteria more significantly than the dichloromethane extract. This corresponds with the finding

Table 3. Phytochemical content in various extracts of *Tragia involucrata*

Compounds	Hexane	Ethyl acetate	Diethyl ether	Dichloromethane	Methanol	Aqueous
Alkaloids	-	-	-	-	+-	-
Flavonoids	+	+	+	-	+	+
Phenols	+	+	+	-	+	+
Saponins	-	-	-	-	-	+
Sterioids	+	+	+	-	+	-
Tannins	-	+	-	+	-	+
Terpenoids	-	-	-	+	-	-

(+) Indicates the presence of compounds; (-) no compounds detected

that the methanol, ethanol, acetone and hot water root extracts of *Terminalia sericea* had good antimicrobial activity but contrasts with the finding that the methanol leaf extract of *Terminalia kaiserana* was the only extract to have a bactericidal effect on *E. coli* [18]. Methanol extracts of the root of *T. sambesiaca* and leaf of *T. sericea* were reported to be the most effective against *Candida albicans* [18]. In the present study, the diethyl ether and methanol extracts of the seed *T. involucrata* showed more effective killing of bacteria than the other extracts. Takaisi-Kikuni *et al.* [19] reported that the essential oil of *Cymbopogon densiflorus* showed a wide spectrum of activity against Gram-positive and Gram-negative bacteria. All the leaf extracts were incubated at 37°C for 24 hrs, among them ethyl acetate, methanol and water extracts. These inhibited the growth of bacteria more than the other extracts even after 48 hr incubation, though the activity was reduced at the later time. A similar trend was recorded in the root extract in our study while the seed extracts did not show any antimicrobial effects after 48 hr incubation. The results

when compared to the water extract of leaf were less potent than that of the standard, chloramphenicol.

The effective water extract was further investigated for its MIC values (see Table 2). The acetone used as a control did not show any toxic effect at 7% (v/v). The higher sensitivity of both Gram-positive and Gram-negative bacteria was confirmed by this dilution method. The MIC values of the water extract ranged from 0.05 µg/ml–500 µg/ml. These results correspond to the report of Irobi and Banso [20] where ethanol, methanol and aqueous extracts of the leaf of *Acalypha tora* showed inhibitory activity at low concentrations. Similar results with ethanolic extracts of *Ixora coccinea* were reported by Latha *et al.* [21]. After 24 and 48 hrs incubation, the inhibitory level was gradually reduced at all the dilutions, with no activity recorded at 0.5 µg/ml. However, Ogundipe *et al.* [22] reported that the acidic fraction (HAF) of the hexane extract of *Mallotus oppositifolium* had significant antimicrobial activity, with MIC values of 32.5 µg/ml and 65 µg/ml against *P. aeruginosa* and *S. aureus,* respectively. In our study, the hexane extract did not show any promising results.

The results of phytochemical screening of six different extracts of *T. involucrata* are reported in Table 3. All the extracts produced positive reactions as follows: hexane extract (flavonoids, steroids and phenols), ethyl acetate extract (flavonoids, steroids, phenols and tannins), diethyl ether extract (flavonoids, steroids and phenols), dichloromethane (terpenoids and tannins), methanol extract (alkaloids, flavonoids, phenols, steroids and tannins) and water extract (flavonoids, phenols, saponins and tannins). The present study suggested that there was a wide range (20%) of positive reactions for phenol/triterpenoids in this plant. However, the majority of the extracts gave positive results for flavonoids, phenols, steroids and tannins. In line with this, Okoli *et al.* [23] detected glycosides, tannins, saponins, flavonoids and alkaloids in the aqueous extracts of the leaf of *Harungana madagascariensis.* These were evaluated for antimicrobial activity against *B. subtilis, E. coli, S. typhi* and *S. aureus.* These bacteria showed susceptibility only to the hot water extract. The results provide a rationale for the traditional use of *H. madagascariensis* leaf extracts for the treatment of gastrointestinal disorders. Our study was supported by Chhabra *et al.* [24] who also

evaluated traditional medicines, an important part of the health-care system in Tanzania. Phytochemical screening of the 52 plant samples indicated that 94% of them contained steroids/triterpenoids, 50% saponins, 40% carotenoids and 17% alkaloids.

The phytochemical screening of *T. involucrata* identified components from the leaves that were active against bacteria. Most of the extracts showed the presence of phenolic compounds; further purification and chemical characterisation are in progress. These plant components are currently available as traditional drugs so there is an urgent need to evaluate their toxicities.

In conclusion, this study shows a high degree of bacterial activity in the aqueous extract of leaves of *T. involucrate* against *S. aureus*. Furthermore, the study describes, for the first time, strong antimicrobial activity at the lowest dilution of the aqueous and methanol extracts. This gives scientific validation to the extensive use of this plant as a traditional "antibiotic" in India.

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Effect of Crude Papaya Latex on Rats' Pregnancy

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INTRODUCTION

Indigenous beliefs and anecdotal evidences indicate that parts of *Carica papaya* L. (Caricaceae) may possess activities with potential reproductive importance. In different parts of the world, preparations from the seeds, leaves, roots and unripe fruits of papaya have been used in folk medicine as galactagogues, emmenagogues, oxytocics and abortifacients [1-5]. In India, papaya is widely classified as harmful in pregnancy. Women are forbidden from eating papaya fruits for the fear of its teratogenic and abortifacient effects [6]. For example, a survey of 1,200 women from all districts of Tamil Nadu in India showed that 82% of the women among avoided papaya fruits during pregnancy [7].

Crude papaya latex (CPL), a milky liquid is present in unripe papaya fruits in high concentrations but absent in fully ripened fruits [8]. The latex contains a mixture of cysteine proteinases such as papain, chymopapain, glycyl endopeptidase, caricain and other enzymes [9]. These enzymes are thought to be natural defense system for wound healing and protection of the plant against predators [10]. Intra-vaginal application of CPL to induce abortion and labour has been reported [1,11]. This could be due to the potent uterine stimulating activity of CPL and its proteinases [12,13].

We have previously reported that fully ripened papaya fruit lacks significant oxytocic activity and did not disrupt rats' pregnancy [12]. In the present study, we examined the potential effects of CPL on pregnancy following oral administration.

MATERIALS AND METHODS

Animal housing and mating protocols

Virgin Sprague-Dawley female (180-220 g) and male (200-250 g) rats were housed in the Animal Holding Unit of the Faculty of Medicine, National University of Singapore (NUS). The animals were housed under a constant 12 hours light and dark cycle and an environmental temperature of 21-23^{o}C. Food and water were made available to the rats *ad libitum*. This study was conducted in accordance with NUS guidelines on animal experimentation. After acclimatization, female rats were paired with males (1:1) until mating was confirmed by the presence of dislodged vaginal plugs. The day when vaginal plugs were found was designated as day 0 of gestation.

Effect of CPL on pregnant rats

CPL was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). According to Sigma-Aldrich, the dried latex was neither cleaned nor purified; it was packed in a crude and powdered form as received from Africa. The pregnant rats were assigned to 3 treatment groups of 5 rats per group. The pregnant rats were dosed daily by gavage with CPL at a dose of 200, 400, or 800 mg/kg bodyweight on days 1-5 of gestation (pre-implantation period) or days 7-13 of gestation (post-implantation period). Control rats were given an equivalent volume of the vehicle (saline).

Observations and parameters measured

Bodyweight changes of the experimental rats were monitored. Pregnant females were observed for vaginal bleeding and signs of clinical toxicity like reduced activity, ruffled fur, perinasal staining and hunched posture. Rats treated before and after the foetal implantation period were euthanized by CO_2 asphyxiation on days 16 and 20 of gestation, respectively. Following necropsy, the number of viable implantations, viable foetuses and fetal resorptions/deaths were recorded. The weights of viable foetuses were also noted. Foetuses were observed for external malformations.

Data analyses

Data analyses were performed using the SPSS (SPSS Inc., Chicago, IL USA) and GraphPad InStat software (GraphPad Software Inc., San Diego, CA, USA). Maternal and foetal body weights were analyzed by one-way analysis of variance and Dunnett's multiple-comparison tests. Data on the number of viable foetuses, resorptions and implantations were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney test. Data were expressed as mean ± SEM. p-values < 0.05 were considered statistically significant.

RESULTS

Exposure of pregnant rats to CPL prior to fetal implantation

No signs of clinical toxicity, vaginal bleeding or maternal death were observed in rats exposed to CPL orally. Maternal weight changes did not reveal any important findings. In contrast to saline (control)-treated group, few foetal resorptions were recorded in the rats treated with CPL but the numbers were not significantly different from those in control rats. The numbers of viable implantations in CPL-treated rats were lesser than the control but the differences were also statistically insignificant (Table 1).

Table 1. Pregnancy outcomes of rats treated orally with CPL before foetal implantation

Group of Pregnant rats (each n=5)	Dose (mg/kg)	Treatment day (of gestation)	Number of foetal resorptions	Number of viable implantations (mean ± s.e.m.)
1	(control)	1-5	0	13.0 ± 0.9
2	200	1-5	3	12.0 ± 1.6
3	400	1-5	2	12.4 ± 0.4
4	800	1-5	2	9.4 ± 1.9

Data are expressed as mean ± SEM

Exposure of pregnant rats to CPL after fetal implantation

Table 2 summarizes maternal findings in rats treated with CPL after foetal implantation. No symptoms of clinical toxicity, vaginal bleeding or maternal death were observed in rats exposed to CPL orally. Maternal weight changes also did not reveal any vital findings.

Table 2. Pregnancy outcomes of rats treated orally with CPL after fetal implantation

Group	Pregnant rats (*n*)	Dose (mg/kg)	Treatment day (of gestation)	Fetal resorption (*n*)	Viable fetuses (*n*)	Weight of viable fetuses (g)
1	5	Control	7-13	0	14.2 ± 0.6	3.7 ± 0.4
2	5	200	7-13	0	13.8 ± 0.4	3.7 ± 0.2
3	5	400	7-13	0	14.4 ± 0.4	3.7 ± 0.6
4	5	800	7-13	1	*10.4 ± 1.6	3.6 ± 0.4

*$p<0.05$; data are expressed as mean ± SEM

Only 1 resorbed fetus was detected. This was in the group that received the highest dose of CPL (800 mg/kg). However, a statistically significant ($p< 0.05$) reduction in the number of viable foetuses was recorded in the group that received 800 mg/kg of CPL. Foetal weights in all the groups did not show any significant differences. Examination of all the foetuses revealed no external abnormalities.

DISCUSSION

Crude papaya latex (CPL) caused an insignificant amount of foetal resorptions as well as reduction in implantation sites. However, there was a significant reduction in the number of viable foetuses in the rats that received 800 mg/kg of the latex after foetal implantation, but the lack of dose-response effect and the insignificant increase in the number of fetal resorptions or abortions suggest that CPL may have minimal negative impact on pregnancy. Nevertheless, it can not be presumed that exposure to higher concentrations of CPL will not disrupt pregnancy. Our previous studies indicated that papaya latex and its proteinases, papain and chymopapain (both phytochemicals of unripe papaya fruits), are potent uterine stimulants [12, 13] (see sample tracings (Figure 1) of the effect of CPL on the contractions of uterine strips isolated from pregnant Sprague-Dawley rats). The lack of significant abortifacient effect of CPL via the oral route could be due to poor absorption of CPL as it has been reported that only 6% of orally administered papain is capable of being absorbed without degradation in rats [14]. It is hence possible that maternal exposure to very high concentrations of CPL via the oral route may cause pregnancy disruption.

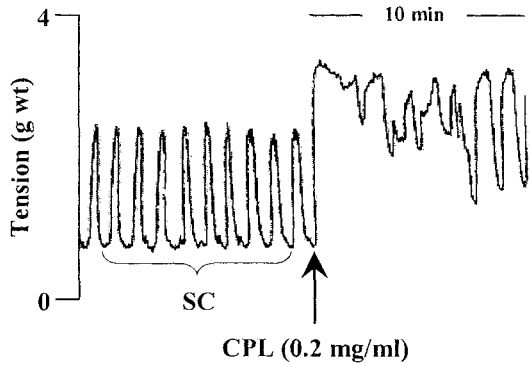

Figure 1. Sample tracing showing the oxytocic activity of CPL on uterine strips isolated from pregnant rats. SC: spontaneous contractions. CPL provoked strong and tonic contractions of the pregnant rat uterus.

The standard oxytocics used clinically (e.g. oxytocin and PGF_{2a}) are less active orally, but are largely administered via the systemic or intra-vaginal route. Gelfand et al. [15] also noted that the majority of herbal oxytocics used in folk medicine are applied intra-vaginally. Indeed, CPL is applied intra-vaginally as folk medicine to induce labour and abortion [1, 11]. Intra-vaginal application of papain to women (as a mucolytic agent) has also been reported to contract the uterus and cause uterine evacuation [16-18] suggesting that, like standard oxytocic agents, CPL and its phytochemicals are more effective as uterine contractants when applied via the systemic or intra-vaginal route.

In conclusion, oral exposure of pregnant rats to 200-800 mg/kg of CPL did not cause significant maternal toxicity or abortion. However, at 800 mg/kg, CPL did cause a significant reduction in the number of viable foetuses in pregnant rats. It is possible that exposure to high levels of unripe papaya fruits (that contain high concentrations of CPL) may lead to adverse effects of obstetric importance.

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1-Cyano-2-Hydroxy-3-Butene: A Plant Nitrile that Induces Apoptosis in Pancreatic Acinar Cells and Reduces the Severity of Acute Pancreatitis

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INTRODUCTION

Acute pancreatitis is a common clinical condition, whose incidence has been increasing over recent years [1, 2]. In the majority of patients the condition is mild but about 25% of patients suffer a severe attack and between 30 to 50% of these will die [3-5]. Most cases are secondary to biliary disease or excess alcohol consumption. The events that regulate the severity of acute pancreatitis are, for the most part, unknown. The exact mechanisms by which diverse etiological factors induce an attack are still unclear, but once the disease process is initiated common inflammatory and repair pathways are invoked. There is a local inflammatory reaction at the site of injury, if marked this leads to a systemic inflammatory response syndrome (SIRS), and it is this systemic response that is believed to be ultimately responsible for the majority of the morbidity and mortality [3-5]. Several recent studies have, however, suggested that acinar cell response to injury may, itself, be an important determinant of disease severity.

Apoptosis and necrosis in acute pancreatitis

Human as well as experimental acute pancreatitis is characterized by progressive cell death, the mechanisms of which remain poorly understood. Necrosis has classically been considered the major form of cell death in acute pancreatitis [6, 7], whereas apoptosis was

suggested to mediate atrophy in the organ [8, 9]. However, careful biochemical and morphological examination of experimental models of acute pancreatitis has shown that severe acute pancreatitis is associated primarily with necrosis but little apoptosis (e.g. that induced by pancreatic duct ligation in the opossum, by choline-deficient and ethionine supplemented diet in the mouse, and by caerulein-hyperstimulation in the mouse), whereas mild acute pancreatitis (e.g. that induced by pancreatic duct ligation and by caerulein-hyperstimulation in the rat) is associated primarily with apoptotic cell death and little necrosis [10,11]. In other words, the severity of acute pancreatitis is inversely related to the extent of acinar cell apoptosis (Figure 1).

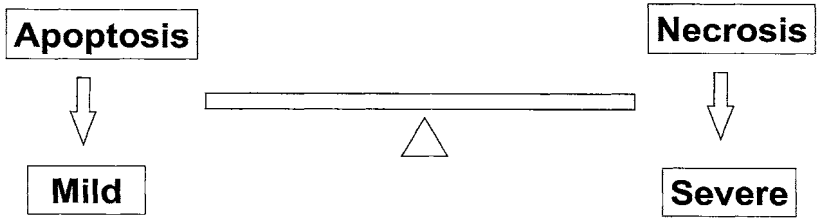

Figure 1. The severity of acute pancreatitis is inversely related to the extent of pancreatic acinar cell apoptosis.

A recent report [12] has shown that cholecystokinin (CCK) stimulates death signaling pathways in rat pancreatic acinar cells, including caspase activation, cytochrome c release, and mitochondrial depolarization, leading to apoptosis. The mitochondrial dysfunction is mediated by upstream caspase(s). CCK causes mitochondrial alterations through both PTP (permeability transition pore)-dependent (cytochrome c release) and PTP-independent (mitochondrial depolarization) mechanisms. In addition to apoptosis, caspases also regulate other processes in the pancreatic acinar cell that play key roles in pancreatitis; in particular, caspases negatively regulate necrosis and intra-acinar cell activation of trypsin [12]. Caspase-mediated protection against necrosis and trypsin activation can explain the inverse correlation between the extent of apoptosis on the one hand and necrosis and the severity of the disease on the other hand observed

in experimental models of pancreatitis. Indeed these signaling mechanisms may play an important role in acinar cell injury and death in pancreatitis. High doses of the CCK analog caerulein, however, also cause acute pancreatitis. Therefore, an ideal agent to study pancreatic acinar cell apoptosis and its effect on acute pancreatitis would be one that does not by itself cause acute pancreatitis.

1-Cyano-2-Hydroxy-3-Butene (CHB – Crambene): An inducer of pancreatic acinar cell apoptosis

1-Cyano-2-Hydroxy-3-Butene (CHB – Crambene) [13] (Figure 2) is a stable plant nitrile found in many cruciferous vegetables. Crambene is a potent inducer of "phase II" detoxification enzymes, including certain glutathione S-transferases and quinone reductase, which are important enzymes associated with conjugation and elimination of reactive chemical intermediates and carcinogens [14,15].

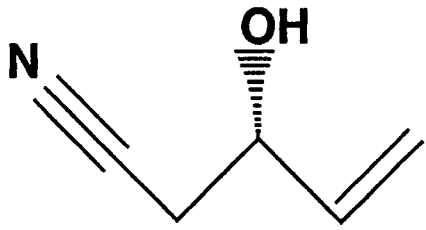

Figure 2. Structure of crambene.

Also, it has been shown to cause cell cycle arrest of the Hep G2 cells or colonic adenocarcinoma cells in G2/M phase [16]. Furthermore, mixtures of individual cruciferous breakdown products that include crambene have shown synergistic activity with regard to induction of these two protective detoxification enzymes [17]. In these mixtures, crambene was shown to be a major contributor to the effect. Crambene as a chemoprotectant is relatively unique in that it is a nitrile rather than an isothiocyanate, the type of compound typically associated with anticarcinogenesis. It is derived from (2S)-hydroxy-3-butenyl glucosinolate, also known as *Epi*-progoitrin. *Epi*-progoitrin is the counterpart of (2R)-hydroxy-3-butenyl glucosinolate, or progoitrin. *Epi*-progoitrin is the predominant glucosinolate in *Crambe abyssinica*, the Mediterranean kale, whereas progoitrin is the major

glucosinolate in rapeseed (*Brassica napa*). Upon hydrolysis of *Crambe abyssinica* meal, *epi*-progoitrin will break down to several nitrile compounds, including highly reactive epithionitiriles as well as crambene, whereas in commercially processed meal, only crambene is found. Breakdown of *epi*-progoitrin yields the S form of crambene, whereas breakdown of progoitrin yields the R-form. Both forms have equivalent biological effects [15].

Upon intravenous administration, a single dose crambene induces extensive and time dependent apoptosis of the pancreatic acinar cells which is maximal 12-24 hours after injection (Figure 3). Apoptosis is an energy consuming, genetically regulated form of cell death which is characterized by cell shrinkage, blebbing of the nuclear membrane, condensation of chromatin, internucleosomal cleavage of genomic DNA, formation of apoptotic bodies, and cell deletion with little or no inflammatory reaction. Although incompletely understood, apoptosis is generally believed to involve a series of events and to occur over differing time spans in different cell types. In a general sense, apoptosis can be considered to progress through three phases: an *initiation phase* which depends on the type of apoptosis-inducing stimulus, an *effector phase* which involves intracellular regulatory events, and a *degradation phase*. While the initiation phase and the effector phase (which is still subject to regulation) may result in reversible changes, those in the degradation phase involve irreversible changes such as DNA degradation, chromatin condensation, and formation of apoptotic bodies. Irreversible changes in apoptosis (the degradation phase) in pancreatic acinar cells appear to occur 12 hours after crambene administration (Figure 3).

Effect of crambene administration on acute pancreatitis

Mice given 12 hourly intraperitoneal injections of a supramaximally stimulating dose of caerulein (50 mg/kg/h) develop severe pancreatitis (Figure 4) which is characterized by hyperamylasemia, interstitial pancreatic edema, and extensive pancreatic acinar cell necrosis. The severity of this model of acute pancreatitis can be evaluated by quantitating these changes. As apoptosis is a teleologically beneficial form of cell death in acute

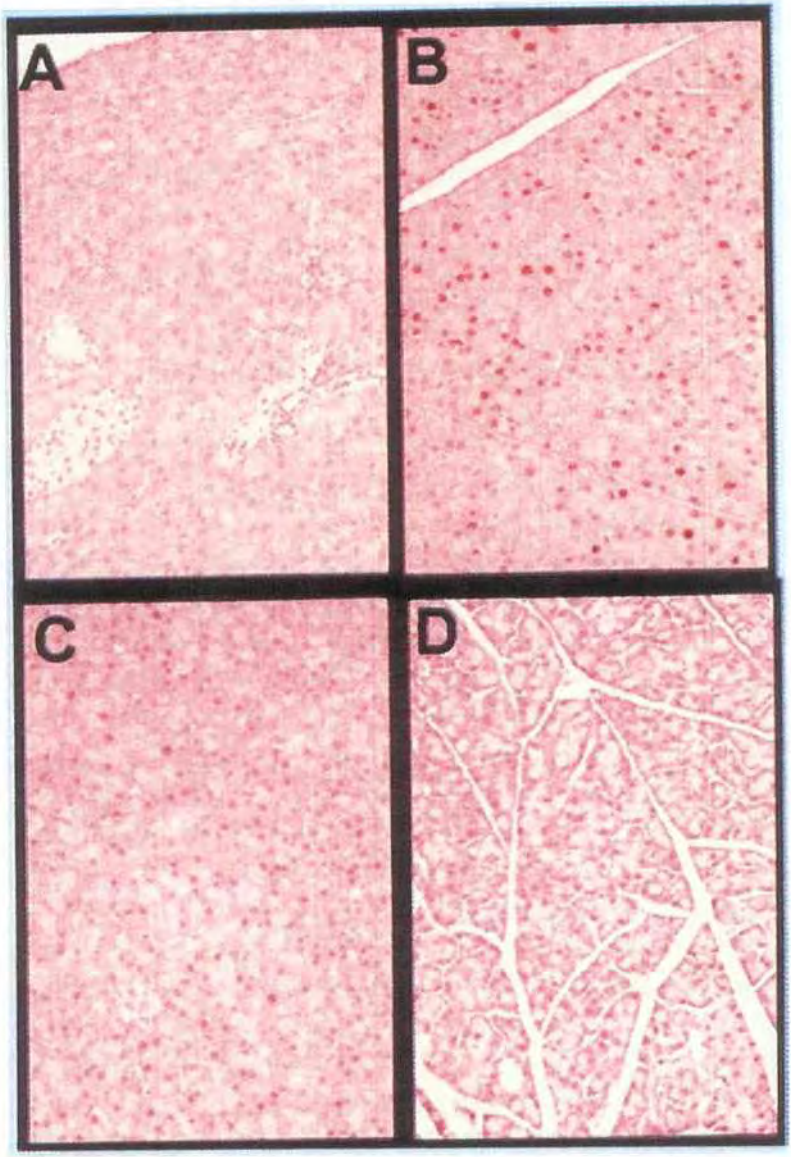

Figure 3. Intravenous administration of crambene causes apoptosis of pancreatic acinar cells. Mice were given a single intravenous injection of crambene (70 mg/kg) and sacrificed at 4 hrs (Panel A), 12 hrs (Panel B), 24 hrs (Panel C) or 48 hrs (Panel D) after crambene administration. *In situ* detection of apoptosis was determined by terminal transferase mediated nick end labeling. Nuclei from apoptotic cells show red color compared to the background nuclei which appear bluish (counterstaining). (reproduced from ref. 18 with permission).

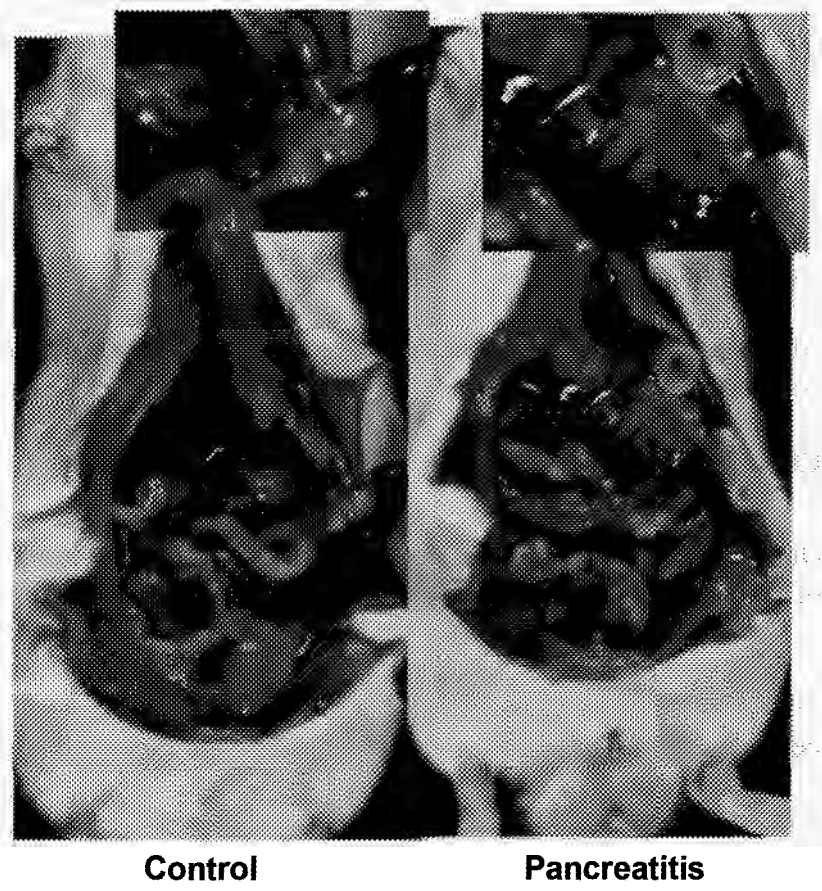

Figure 4. Caerulein-induced acute pancreatitis.

pancreatitis, it is reasonable to hypothesize that induction of apoptosis would have a protective effect against acute pancreatitis.

To that end, we chose to begin the 12 hours of caerulein administration at different times after crambene was administered. Indeed, induction of pancreatic acinar cell apoptosis with crambene protects mice against acute pancreatitis induced by caerulein (Table 1). This is despite the fact that crambene does not alter the interaction of CCK to its receptor on pancreatic acinar cells [18]. The maximal protective effect of crambene against acute pancreatitis is observed caerulein treatment is started 12-16 hours after crambene

Table 1. Effect of crambene administration on the severity of acute pancreatitis

Hyperamylasemia:	**Reduced**
Pancreatic edema:	**Reduced**
Acinar Cell Necrosis:	**Reduced**

administration, that is, when the pancreatic acinar cells have become committed to apoptosis.

CONCLUSION

Both apoptotic and necrotic forms of cell death are seen in clinical, as well as experimental acute pancreatitis. The mechanisms of pancreatic acinar cell death — apoptosis and necrosis — in acute pancreatitis are just beginning to be identified. The extent of pancreatic acinar cell apoptosis has been shown to be inversely related to the severity of the disease, suggesting that apoptosis is a teleologically beneficial form of cell death in acute pancreatitis. Indeed, in an experimental setting, induction of apoptosis with the plant nitrile, crambene, protects against acute pancreatitis. Active research in this direction will facilitate a transition of our knowledge of necrosis versus apoptosis of pancreatic acinar cells from the bench to the bedside.

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The Liver Protective Effect of Sesamin

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INTRODUCTION

Sesamin is a lignan obtained from sesame seeds, and has evidence to support its role in exerting several biological functions. It is a potent and specific inhibitor or δ-desaturase, an enzyme which catalyzes the desaturation of dihomo-γ-linolenic acid (20:3 n-6, DGLA) to yield arachidonic acid [1]. Preventing formation of this inflammatory mediator, suggests that it has anti-inflammatory action, a property that has been observed in other studies [2].

Sesamin has also demonstrated anti-hypercholesterolemic activity via its inhibition of cholesterol absorption and synthesis, thus resulting in lower serum and liver levels [3]. This is supported by a human study of hypercholesterolic male patients who experienced a significant reduction in total cholesterol and LDL-cholesterol levels when taking sesamin at 32.4-64.8 mg/day and vitamin E (162-324 mg/day), an effect not observed when the equivalent dose of vitamin E was taken alone [4].

Protective effects of sesamin against liver damage in ethanol-exposed mice

In a study investigating the effect of sesamin on ethanol-induced liver damage, 14 CDF male mice (9 weeks old) were raised in a chamber containing 12 ppm of ethanol to mimic the effects of chronic alcohol consumption. The mice were fed powdered commercial non-purified diet, and one group (n=7) was fed the same powdered feed supplemented with 1% sesamin. The control mice (n=7) were not exposed to ethanol. This procedure was continued for 1 week,

following which mice were fasted and anesthetized for blood collection and liver excision.

Alcohol exposure resulted in a large and significant increase in the serum triglyceride, bilirubin, GOT and GPT activity levels in exposed animals [5]. The daily intake of sesamin, as calculated from feed consumption with 1% sesamin, was approximately 24.3mg per mouse (800mg/kg). Sesamin feeding significantly reduced the triglyceride and total bilirubin increases resulting from ethanol inhalation. GOT and GPT activities, which are taken as a measure of liver cell damage, were also significantly reduced in sesamin-fed animals (Table 1 and Figure 1).

Table 1. The effect of sesamin feeding on serum triglyceride, total bilirubin, GOT and GPT activity levels and the prevalence of fat droplets in hepatocytes of ethanol-exposed mice

Group	Triglyceride mg/dl	total Bilirubin mg/dl	GOT activity IU/l	GPT activity IU/l	Fat droplets in hepatocytes [a]
Control	58.6±12.1	0.47±0.15	149.7±76.3	26.1±7.8	1
Ethanol	237.3±124.2††	1.61±1.32†	312.4±203.8†	39.6±31.9	3.5
Ethanol+Sesamin ~800mg/kg)	83.0±19.0†*	0.41±0.04*	81.6±15.4*	18.3±1.6*	1.8

[a] The liver with no lipid particles in lobules is scored as one, and livers with a number or lipid particles scored as 4. † $p < 0.05$ and †† $p < 0.01$ vs control group; *$p < 0.05$ vs ethanol group

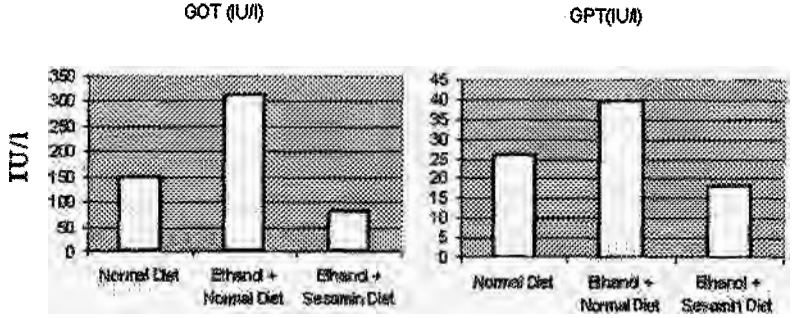


Figure 1. Sesamin incorporated into the diet at 1%, reduced the ethanol inhalation-induced GOT and GPT activity in exposed mice. Values obtained from Akimoto et al [5].

Histological staining of liver sections with oil red-O revealed ethanol-induced accumulation of lipid particles (fat droplets), particularly in the periportal regions of the liver [5]. Sesamin intake decreased lipid accumulation in the hepatic lobules (Figure 2).

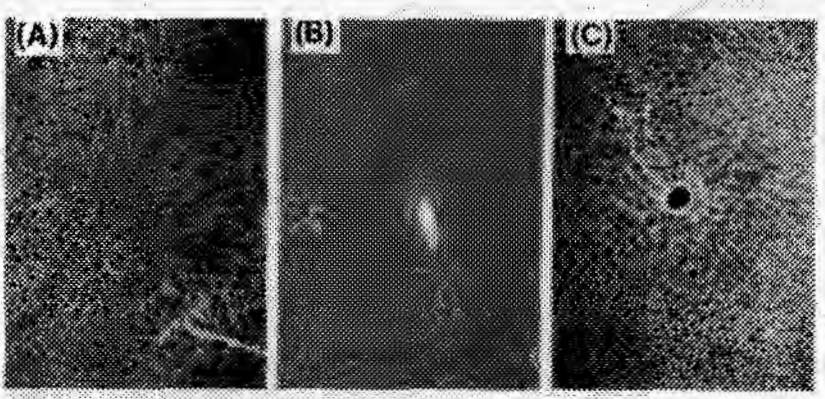


Figure 2. Light-microscope examination of the liver with oil red-O staining, indicating the degree of lipid accumulation. (A) Control rat, (B) rat subjected to ethanol inhalation and (C) ethanol inhalation + Sesamin. scale = 100mm [5].

Protective effect of sesamin against ethanol intoxication in human

The potential use for sesamin in reducing the damaging effects of ethanol in the liver has also been investigated in humans. The chosen parameters reflected the ability of sesamin to increase alcohol metabolism. Alcohol metabolism is frequently impaired in Asian populations and 50% of Japanese people lack the aldehyde dehydrogenase gene, $ALDH_2$, which catalyses the conversion of the alcohol metabolite, acetoaldehyde, to acetic acid. Acetoaldehyde is the toxic intermediate alcohol metabolite responsible for the red flushing observed in people deficient in $ALDH_2$. As shown in Figure 3, sesamin is proposed to induce the activity of catalase and the microsomal ethanol oxidising system (MEOS) enzymes, which can compensate for $ALDH_2$ deficiency by providing an alternative path for metabolic removal of acetoaldehyde.

The effect of sesamin was observed in a group of 9 male adults deficient in $ALDH_2$, identified by ethanol patch test, in a cross-over study. The subjects were given 100mg/day of sesamin in the form of

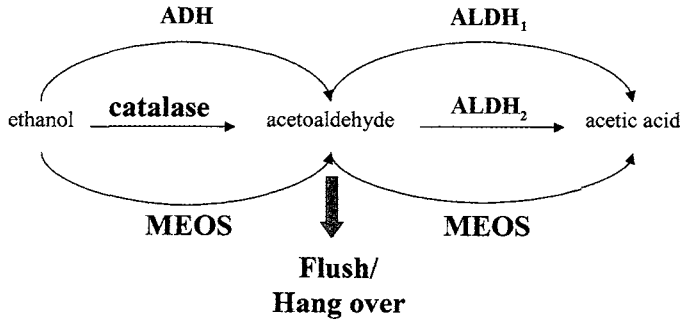


Figure 3. Metabolism of ethanol by ADH ($ALDH_1$ and $ALDH_2$) enzymes and the alternative pathway involving catalase and MEOS.

chocolate or placebo for 7 days, following which they consumed 60 ml of whisky in a single drink. The skin temperature of all subjects was recorded using infra-red camera and observed to reach a rapid peak 30 minutes following whisky consumption. As shown in Figures 4 and 5, there was a gradual reduction in skin temperature, which was significantly and clearly in the group receiving sesamin [6-8].

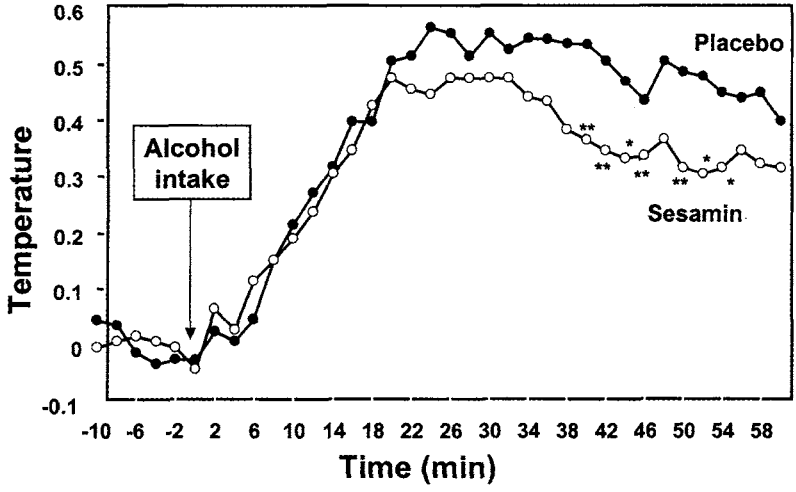


Figure 4. The effect of sesamin on the skin temperature at the chin and neck area of the human subjects before and after drinking 60 ml of alcohol, compared to placebo. Values are presented as the mean temperature as measured by infra-red camera. * $p < 0.05$;
** $p < 0.01$ vs placebo. Graph was obtained from Sugano and Akimoto [7] and Nakamura et al [8].

Figure 5. Thermogram of face temperature in male subjects who consumed 60 ml alcohol following 7 days of 100mg/day sesamin administration or placebo [6]. The more intense red colour indicates higher face temperature than the yellowish-blue colour mixture. – means without sesamin (placebo); + means sesamin intake.

Additional unpublished research has reinforced the role of sesamin in increasing alcohol metabolism with evidence that sesamin administration reduces the serum levels of ethanol, and improves the urinary excretion of acetoaldehyde (Figure 6).

These findings support the role of sesamin in reducing the symptoms observed following alcohol ingestion in $ALDH_2$-deficient

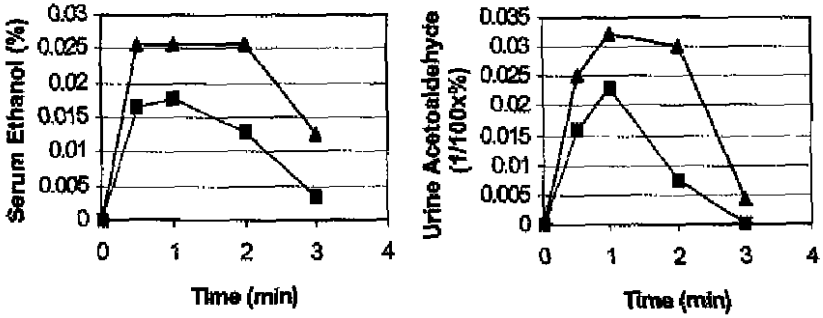


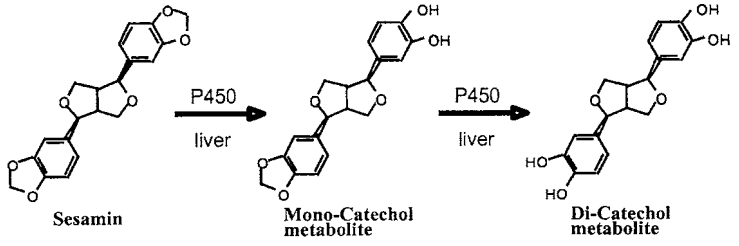
Figure 6. Serum ethanol levels (%) and Urine Acetoaldehyde in 9 male subjects after consumption of 60 ml alcohol, having taken 100mg/day sesamin (■) or placebo (▲).

individuals as well as the level of ethanol and its metabolite within the body system. The effect of sesamin can be explained by the induction of an alternative metabolism pathway, involving catalase and MEOS in the oxidation of ethanol.

Anti-oxidative role of sesamin

In vitro and *in vivo* studies have found that sesamin itself is not active in anti-oxidative processes, but instead is a 'pro-drug'-type molecule which can be metabolized in the liver to an active species which is capable of antioxidant and free radical scavenging activity [9, 10]. Orally administered sesamin has been shown to undergo oxidative metabolism by cytochrome P450 enzymes in the liver to catechol forms which are responsible for the antioxidant potential of sesamin. This activity has been demonstrated both *in vivo* and *in vitro* when sesamin is combined with liver homogenate [10].

The di-catechol form showed greater free radical scavenging activity against •OH, O_2^-, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and lipid peroxidation *in vitro* than the mono-catechol form when enzymatically demethylated and activated by rat liver homogenate (Figure 7). *In vivo*, this same metabolite demonstrated superior •OH



	O_2^-	· OH	Lipid Peroxidation
Sesamin	3.0 %	2.3 %	0 %
Mono-Catechol metabolite	61.3 %	5.4 %	38.2 %
Di-Catechol metabolite	74.6 %	59.2 %	81.0 %

Figure 7. The comparative anti-oxidative activities of sesamin and its mono and di-catechol metabolites.

and O_2^- scavenging activity in rats, following oral administration at 500 mg/kg [10].

Sesamin feeding (0.2% diet) has been shown to reduce plasma, liver and tumor lipoperoxides in female rats subjected to 7, 12-dimethylbenz[a]-anthracene (DMBA)-induced mammary carcinogenesis. In addition to the reduction of lipoperoxides, there was a concurrent reduction in the incidence of tumour formation and number, thus reinforcing the link between antioxidants and prevention of chemical carcinogenesis [11].

The effects of sesamin on liver carcinogenesis in rats

The effects of 0.01% and 0.1% sesamin (w/w) diet were similarly investigated in a rat liver model of carcinogenesis. The initiation stage was induced by Diethylnitrosamine (DENA) injection (50 mg/kg) in 3-week-old F344 mice, and repeated after a 1-week interval. The rats were fed the sesamin diet for either the initiation period (first 3 weeks), the promotion period (final 14 weeks) or the entire study duration (17 weeks) [12].

The number of DENA-induced pre-neoplastic foci, present in the liver of the rats was taken as a measure of carcinogenic progression (Figure 8). Sesamin feeding at 10 and 100 mg/kg resulted in a significant reduction in the number of pre-neoplastic foci, when compared to the control normal diet rats. The protective effect was most evident when sesamin was administered in the initiation phase or was maintained throughout the initiation and promotion phase, where maximal effect was observed.

In line with the previous study that observed the concomitant reduction in tumour incidence with reduced lipoperoxide levels [11], this study found that sesamin feeding at a relatively low concentration could reduce the DENA-induced pre-neoplastic foci in the liver of rats. This protective effect of sesamin suggests that it may act as an inhibitor of liver carcinogenesis, possibly through its inhibition of the chemically-induced processes of peroxidation and free radical damage which can lead to DNA damage and cancer development.

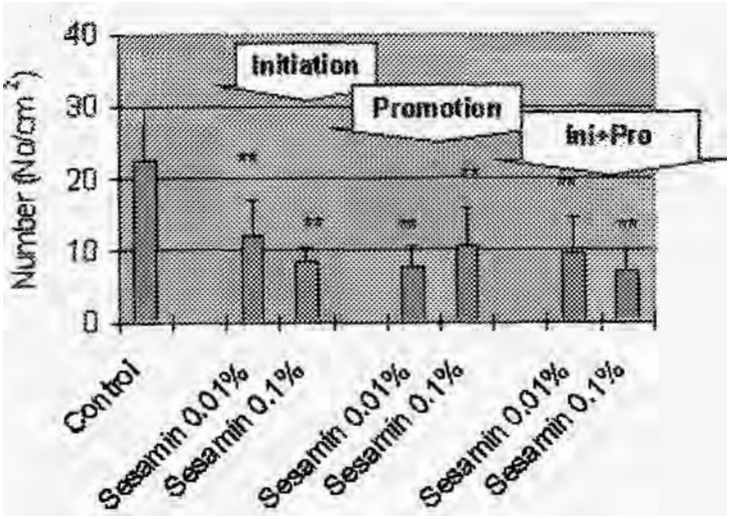


Figure 8. The number of preneoplastic foci present in the liver of DENA treated rats fed with and without sesamin (control) [12]. ** $p < 0.005$ compared to control group.

CONCLUSION

Sesamin has shown several functions in protecting the liver from toxicity due to alcohol or chemical exposure. The protective effect is attributed to its anti-oxidative and free radical scavenging abilities as well as its modulation of metabolizing enzymes. Following consumption, sesamin is metabolized to an 'active' catechol form in the liver, which exerts activity against lipoperoxides and free radicals such as •OH and O_2^- both *in vitro* and *in vivo* [10]. The protective effect is inferred further via its reduction of pre-neoplastic foci in a rat model of liver carcinogenesis, proposing its potential as a preventive measure against liver cancer development. In addition, several roles in preventing ethanol-associated toxicity have been demonstrated [5-8]. Animal studies have shown evidence for protection from ethanol-induced liver toxicity in the form of lipid accumulation and liver enzyme leakage as well as a human study which observes that sesamin can improve the metabolism of the acetoaldehyde intermediate, responsible for the alcohol-induced symptoms of flushing, which is evident in subjects deficient in $ALDH_2$.

These findings suggest that sesamin has potential for reducing liver pathologies resulting from chemical and alcohol exposure.

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Endothelium-Dependent Vascular Relaxation Induced by Extracts of Some Chinese Medicinal Herbs Traditionally Used to Remove Circulatory Stagnation: An Update

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INTRODUCTION

Botanical herbs are consumed globally not only as essential diet, but also as medicines, or as functional/recreational food supplements. Food therapy, as one form of the complementary medicine, whether for therapeutic or preventive purpose, is based on the principle of achieving the physiological homeostasis by non-pharmacological means. Indeed, plants such as vegetables and fruits are of significant dietary importance for the maintenance of good health not only for their carbohydrates, minerals, vitamins and fibres, but also for the many protective antioxidant substances, keeping the cardiovascular system in check.

Many traditional Chinese medicinal herbs, which are conventionally used to treat hypertension (that precipitates cardiac problems and stroke) or to remove blood stagnation (that causes inflammation and pain) thereby improving the circulation, usually elicit vascular relaxant properties [1-3]. Many of them act directly on the vascular smooth muscle cells via a variety of signaling mechanisms. These herbs include *Tetrandra stephania*, *Panax ginseng* and *Ligustrium wallichi*. Some active chemical substances from these herbs were identified and their antihypertensive effects as well as vasodilatory actions demonstrated [3-5]. For example, the bis-benzylisoquinoline alkaloid, tetrandrine, isolated from the root of

Tetrandra stephania, has been shown to directly dilate vascular smooth muscle by virtue of its Ca^{2+}-antagonistic action [4,5]. The alkaloid of *Ligusticum wallichi*, tetramethylpyrazine, has been shown to dilate the blood vessels by blocking the α-adrenoceptors [4] and activating the K^+-channels [6]. The glycosides from *Panax ginseng* has been reported to dilate blood vessels in an endothelium-dependent, nitric oxide (NO)-mediated manner, probably via activation of K^+-channels [7-10]. Some herbal drugs that contain a large amount of polyphenols also elicit endothelium-dependent vasorelaxation which is mediated via NO [11].

All the above medicinal herbs are examples of major ingredients in Chinese herbal prescriptions for the treatment of ailments due to blood stagnation, which reduces blood flow, aggravates inflammation, precipitates pain, increases blood pressure and enhances atherosclerosis [12]. In this communication, we have updated some recent findings to include the vascular effects of extracts from the bark of *Encommia ulmoides*, widely known in China as *Tu-Chung* or *Du-Zhong*, the root of *Eleutherococcus senticosus*, globally known as Siberian ginseng and also known as *Ci-Wu-Jia* in China, and the root of *Panax notoginseng*, known in China by the name *Tien-Chi* or *San-Chi*.

EUCOMMIA ULMOIDES

The bark of *Eucommia ulmoides* Oliv. has been widely used as a tonic medicinal herb in the Orient, but is less well-known to the West [12, 13]. It is used for the treatment of hypertension either as a single herb or in combination with one or two of the above-mentioned herbs in the traditional herbal prescription. According to the ancient writing of Chinese medicinal herbs [12], *Eucommia ulmoides,* prepared from the leaf or bark, is commonly used as a tonic for the liver and kidney, thus improving detoxification (by liver) and circulation (via kidney), respectively. The antioxidant effect of some of the chemical constituents of *Eucommia* leaf and bark may also contribute to its anti-inflammatory action [14]. Many studies have focused on the blood pressure-lowering effect of *Eucommia* leaf and bark [13, 15-19] and their chemical constituents [14]. Surprisingly, little is known

about its pharmacological profiles on the cardiovascular tissues. Recently, we have reported for the first time that the aqueous extracts of *Eucommia* leaf and bark exerted *in vitro* relaxation in rat aorta and dog carotid artery [20, 21], which is entirely endothelium-dependent, and mediated by nitric oxide (NO). However, these extracts do not act at the NO-releasing receptor sites, such as endothelial muscarinic receptors and appear to involve K^+-channels. While this relaxant effect of *Eucommia* extracts may be a plausible explanation, at least in part, for its antihypertensive action, we also investigated whether such *Eucommia*-induced endothelium-dependent relaxation is generally applicable to other vasculature, especially the smaller muscular arteries (such as mesenteric artery), which are more important for blood pressure regulation than the large conduit elastic arteries, such as aorta and carotid artery used in our previous study [21]. Also, as blood vessels become smaller, endothelium-derived hyperpolarizing factor (EDHF) becomes functionally more active aside from NO in the endothelium-dependent relaxant events [22, 23]. Therefore, we also examined whether the *Eucommia* extract can cause the release of EDHF in addition to NO in smaller muscular vessels, such as rat mesenteric arteries, as compared to a large artery such as rat aorta.

We found that all three types of vessel preparations elicited endothelium-derived vascular relaxation (EDVR) in response to *Eucommia* bark extract concentration-dependently [22] in a similar manner as the relaxant responses to carbachol (CCh). Although the NO synthase inhibitor L-NAME totally abolished the EDVR in aorta, it only partial abolished EDVR in mesenteric arteries isolated from each end, the distal end being more resistant to L-NAME. However, the residual L-NAME-resistant relaxation of the rat mesenteric arteries could be further inhibited by preincubation of the vessels with the combination of L-NAME and 15-20 mM KCl (KCl itself at this low concentration caused little or no contraction). Therefore, the EDVR induced by the *Eucommia* extract and CCh in aorta is mediated entirely by NO, and that in mesenteric arteries by NO as well as EDHF, with the EDHF component (inhibited by KCl) larger in the smaller distal end of the rat mesenteric artery. Results in our study offer a plausible mechanistic basis for the vasorelaxing action of

Eucommia ulmoides Oliv., which may account for its well documented antihypertensive action.

ELEUTHEROCOCCUS SENTICOSUS

The medicinal herb, Siberian ginseng (*Eleutherococcus senticosus* Maxim or *Acanthopanax senticosus* Harms), is botanically different from the Korean ginseng (*Panax ginseng*) and North American ginseng (*Panax quinquefolia)*, but the healing power of its roots is very similar to the root of the other two types of ginseng and has therefore been traditionally used in China as a ginseng-substitute for centuries because of its relatively lower cost. In fact, its medicinal value lies in its anti-fatigue effect following strenuous exercise and was claimed to be more superior to that of the ginseng [24]. Siberian ginseng (SG) was named so, because it was naturally grown in Siberia of Russia and the earliest studies (1955-1965) of its health effects were made extensively by Russian scientists [25]. *Acanthopanax senticosus* is also widely grown in northern China and Hokkaido, Japan. In China it is commonly referred to as "*Ci-wujia"* because of its thorny stem (*Ci* means thorny and *wujia* means five-petal leaf, hence *Acanthopanax*), whereas in Japan, it is referred to as "*Ezoukogi*" [14, 26].

According to ancient Chinese medical writings [12], *Ciwujia* root is traditionally used in China to fight against ailments due to stagnation of blood circulation and other body fluids and help clear excessive fluid cumulated in the body (as in edema). These ailments range from indigestion, urinary stagnation, general fatigue, arthritis, mild hyperglycemia to hypertension. Russian scientists reported that Siberian ginseng (SG) improves red blood cell production and perfusion of tissues, and thus efficient oxygen delivery and consumption [25]. These effects of SG on circulation noted by people in different cultures conceivably would contribute collectively to its well-known anti-fatigue effect, which has also been confirmed in animal studies of physical endurance by scientists in Hokkaido [14, 26]. Accordingly, SG has been widely used by athletes and people involved in stressful activities. Despite its wide and huge global consumption of SG as a health food, reports on its pharmacologic profile on vascular system

are scanty. PubMed search indicated that the general study of SG represents a very small portion (<1%) of the large body of studies on ginseng.

Although the health effect of SG, like that of the *Panax ginseng* or *Panax notoginseng,* may be attributed to a general improvement of body circulation [12], probably via vasodilatory effect elicited by its active ingredients [27-29], direct *in vitro* studies of SG or its extracts on vascular function are so far not available. On the other hand, in both *Panax ginseng* and *Panax notoginseng*, the total glycosidic saponins (also referred to as ginsenosides) are the major active substances known to cause vascular relaxation by endothelium-dependent [7-8, 28] and endothelium–independent [27, 29] mechanisms, respectively. SG, however, does not contain these vasodilatory ginsenosides [14] and a direct vasodilatory effect of SG has not been experimentally demonstrated despite historical claims suggesting vasodilatation as exerting its health effect. We therefore investigated the *in vitro* vasorelaxant effect of the aqueous extract of the roots of SG (*Eleutherococcus senticosus* Maxim) using several vascular rings prepared from dog carotid artery, rat aorta and rat mesenteric artery. SG extract (0.04-0.8 mg/ml) caused concentration-dependent relaxation in dog carotid arterial rings pre-contracted with 100 μM phenylephrine (PE), and the relaxation was primarily endothelium-dependent. Treatment with 100 μM L-NOARG (a nitric oxide synthase inhibitor) either prevented or reverted SG-induced relaxation suggesting that the endothelium-dependent relaxation was mediated by NO. Similar endothelium-dependent vascular relaxant responses were also obtained with rat aortic and mesenteric arterial rings, except that it occurred over a relatively higher concentration range of SG (0.5-2.0 mg/ml). When tested in the presence of 300 μM L-NAME, the vasorelaxant effect of SG was inhibited totally in rat aorta but only partially in rat mesenteric artery. The relaxation to SG that was insensitive to L-NAME in rat mesenteric arterial rings was eliminated when the rings (both proximal and distal ends) were pre-treated with a combination of 300 μM L-NAME and 15 mM KCl indicating the involvement of endothelium-derived hyperpolarizing factor (EDHF). This vasorelaxant response of the SG extract was inhibited partially by atropine (1 μM), completely by TEA (5 mM),

but not by indomethacin (1 μM) or propranolol (10 μM). SG, up to 2 mg/ml, had no effect on KCl-induced contraction in any of the vascular rings studied. When compared with CCh-induced relaxation, SG resembles CCh in that the sensitivity to L-NAME inhibition is dependent on vascular size, i.e. aorta > proximal end of mesenteric artery > distal end of mesenteric artery. However, SG exhibited different potencies for relaxation while CCh showed similar potency (EC_{50} of about 0.2 μM) in all three vascular segments. These studies have demonstrated that the vascular effect of SG is endothelium-dependent and mediated by NO and/or EDHF depending on the vessel size. Other vasorelaxation pathways, such as inhibition of K^+-channels and activation of muscarinic receptors, may also be involved.

PANAX NOTOGINSENG

As mentioned earlier, the ginsenosides isolated from *Panax notoginseng* appears to dilate vessels via blockade of a putative receptor-operated Ca^{2+}-channels [27, 30]. A vasoactive lipid component, trilinolein which has linoleic acid as the fatty acid residue at all 3 esterified positions of glycerol, isolated from the traditional Chinese herb, *Panax notoginseng or Sanchiÿor Tienchi)* [31], was also shown to elicit endotheliuim-dependent vasorelaxation.

In studies of isolated rat aorta, trilinolein, at strikingly low concentrations ranging (0.1 nM to 1 μM), relaxed phenylephrine (PE)-induced contraction. The concentration-response curves for the interaction between trilinolein and PE showed that trilinolein was unlikely to be a competitive antagonist of α-adrenoceptors. The vasorelaxant effect of trilinolein was dependent on the presence of intact endothelium [32]. Both L-NAME and methylene blue antagonized this vasorelaxant effect. L-arginine partially reversed the effect of L-NAME on trilinolein. Linoleic acid itself had no vasorelaxant effect. This study suggests that trilinolein is an endothelium-dependent vasorelaxant, and the underlying mechanisms may be through stimulation of NO production and cyclic GMP pathways.

Trilinolein has been shown to have other various beneficial effects on the cardiovascular system, including improvement of erythrocyte

deformability, inhibition of platelet aggregation induced by epinephrine but not by collagen, thrombin, ADP or arachidonic acid [33, 34]. It also possesses an effective antioxidant action in various experimental models [35-39]. Furthermore, *in vivo* studies have also demonstrated that trilinolein has antiarrhythmic effects [39-42]. All of these findings suggest that trilinolein is a potential medication for the treatment of cardiovascular diseases.

The myocardial protective effects of trilinolein were investigated in the coronary artery ligation model [39]. Pre-treatment with trilinolein at a dose of 0.1 μg/kg given 15 min prior to coronary ligation produced a significant reduction in infarct size. It was concluded that trilinolein may protect the myocardium against ischemic injury during ischemia and reperfusion. Moreover, the mechanism of the myocardial protective effect of trilinolein was further investigated in isolated cardiomyocytes to determine if inhibition of calcium influx and alteration of the activity of superoxide dismutase were involved [43]. In isolated cardiomyocytes, Ca^{2+} influx stimulated by hypoxia/normoxia was effectively reduced by 34% after pre-treatment with trilinolein at a low concentration of 1 nM. Furthermore, in isolated perfused rat hearts subjected to 60 min of global hypoxemia without reperfusion, pre-treatment with 0.1 μM trilinolein for 15 min reduced infarct size by 37%.

Trilinolein also elicited antiarrhythmic effects in ventricular arrhythmia induced by digitalis intoxication and coronary artery ligation for 15 min and 30 min [39-42]. Ventricular arrhythmia is a frequent complication in the use of digitalis. The mechanism underlying the generation of delayed after-depolarization by cardiac glycosides is complex. It involves the over-loading of intracellular Ca^{2+} stores, caused by the inhibitory effect of digitalis on the Na^+/K^+ pump which subsequently activates the reverse mode of the Na^+/Ca^{2+} exchanger [44]. In support of this hypothesis, after pre-treatment with 0.1 μg / kg trilinolein, strophanthidine-induced ventricular extrasystoles decreased significantly and this was accompanied by reduced Ca^{2+} influx at low concentrations in isolated rat cardiomyocytes.

Another anti-ischemic mechanism of trilinolein is through the improvement of the erythrocyte deformability *in vitro* [31, 40]. It

was suggested that it may act by modifying membrane fluidity rather than by competing with Ca^{2+} at the channel sites.

RED WINE AND TEA

Red wine and tea are two very common and popular recreational drinks derived from plant. Long-term consumption of wine (from grapes), especially red wine, may lower blood pressure [45] and protect against cardiovascular diseases due to its high content of polyphenolic compounds [46]. These polyphenols originate from the skins, seeds and vine stems of the grapes while some are formed during the process of vinification.

The effects of short-term oral administration of red wine polyphenolic compounds on haemodynamic parameters and on vascular reactivity have been investigated in rats [46, 47]. Daily intragastric administration for 7 days of red wine polyphenolic compounds (20 mg/kg) in rats (5% glucose for control rats) produced a progressive decrease in systolic blood pressure. Aortas from rats treated with red wine polyphenolic compounds displayed increased endothelium-dependent relaxation to acetylcholine that was related to increase endothelial NO activity and involved a mechanism sensitive to superoxide anion scavengers [47]. The endothelium-dependent NO-mediated relaxant effect of red wine was also observed in human coronary arteries [47]. Also, in the aorta, red wine polyphenolic compounds increased the expression of cyclooxygenase-2 and increased the release of endothelial thromboxane A_2, which compensated for the extra-endothelial NO-induced hyporeactivity in response to norepinephrine, resulting from enhanced inducible NO synthase expression. Therefore, the NO-promoting effects of red wine polyphenolic compounds may be a potential mechanism for preventing cardiovascular diseases and account for the "French paradox".

Tea, especially the non-fermented green tea, also contains a high content of polyphenols, termed tea catechins, which have been shown to have cardiotonic and antifatique effects [48]. Catechins may also prevent atherosclerotic lesion due to oxidized LDL [49] and cancer progression through mitotic signal transduction blockade

in experimental animals [50]. The purified tea catechin, (-)Epicatechin (EC), caused both endothelium-dependent and -independent relaxation in rat mesenteric artery [51]. N^G-Nitro-L-arginine methyl ester (L-NAME, 100 μM) and methylene blue (10 μM) significantly attenuated EC-induced relaxation in endothelium-intact tissues [51]. L-Arginine (1 mM) partially antagonized the effect of L-NAME. EC (100 μM) significantly increased the tissue content of cGMP and N^G-nitro-L-arginine (100 μM) or removal of the endothelium abolished this increase. Iberiotoxin at 100 nM attenuated (-)-epicatechin-induced relaxation in endothelium-intact arteries and this effect was absent in the presence of L-NAME.

CONCLUSION

In summary, in addition to our recent review [11], more experimental evidence has been provided here to suggest that many botanical medicinal herbs, including fruits and vegetables, may provide cardiovascular protective effects via NO-mediated, endothelium-dependent vasodilatation [52]. Such an effect may result in improved blood flow and lowering of blood pressure, thus being responsible for their traditional therapeutic effect via the removal of blood stagnation. These herbal extracts may interact either directly with the endothelial cells or act indirectly via its antioxidant effects [53]. However, one should exercise caution in extrapolating *in vitro* experimental findings to *in vivo* clinical situations. First, many of the above studies were not performed on human vascular tissue, but on vascular tissues from experimental animals. Second, as is usually the case in the study of therapeutically effective herbal extracts, the high concentration required to produce a maximal acute *in vitro* relaxant effect may dampen its physiological significance. On the other hand, one should also realize that unlike the use of potent synthetic drugs for their acute therapeutic effects, many herbal medicinal drugs are said to take effect over a longer period of time; indeed, they are taken within their original cultural context for preventive measure rather than for therapeutic purpose. Therefore, one should be open to the possibility that many herbal extracts may require a relatively longer administration period for conditioning to elicit its *in vivo* health effect,

compared to the acute *in vitro* cellular effects over a very short exposure. Third, it is also unknown whether the herbal extract administered (usually orally in the form of food or as powders in capsules) would reach the concentration needed for the *in vitro* study. Furthermore, it is possible that *in vivo* biotransformation could result in more potent vasoactive metabolites. However, resolution of the above limitations in terms of relevance of clinical application would require a totally different methodological approach and warrant future investigations.

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Effect of Chronic Administration of Aqueous *Azadirachta indica* (Neem) Leaf Extract on the Cardiovascular System in the Rat

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INTRODUCTION

Azadirachta indica (Neem) *A. Juss.* [synonyms *Melia indica* (*A. Juss*), *Melia azadirachta L.*] belongs to the Meliaceae (mahogany) family and is commonly called Indian Lilac, neem tree or the margosa tree in English or *nim* in India. It grows best in the tropics and is very common in the Indo-Pakistan sub-continent and in subtropical areas of Africa, the Americas and Australia [1].

The neem tree is used extensively in agriculture and medicine because of the biological properties of several limonoids and flavonoids found in this plant. Different parts of the neem tree have been used in *Ayurveda* (the traditional Indian system of Medicine) in the treatment of various aliments. For instance, the bark exerts strong anti-microbial effects. The sap is used for leprous ulcers and various skin diseases; the gum for sore-throats; the leaves for skin diseases and hemorrhoids or in gynaecological practice for induction of labour; young twigs for oral hygiene; and flowers as an antihelminthic or a cough remedy [2].

Neem has also been proven scientifically to have significant pharmacological activity. It reduces plaque formation [3], and have hypoglycaemic [4], contraceptive [5] and anticancer [6, 7] effects, useful dermatological properties [8], and is also a central nervous system (CNS) depressant [9]. Research reports of the chronic effects of neem on the cardiovascular system are very few, even though neem has been cited in the Indian system of medicine (*Ayurveda*) as having

antihypertensive properties when taken regularly as neem tea. Acute administration of both aqueous and alcoholic extracts of neem have been reported to reduce blood pressure in guinea pigs and rabbits and to act as a spasmogen in guinea pig ileum [10, 11], but even though neem is consumed daily as tea in the normal population of people who use it, there are no known reports on the effect of chronic neem ingestion on the cardiovascular system.

Thus, the aim of this study was to determine the effect of chronic administration of aqueous neem leaf extract on the cardiovascular system in normal, conscious rats with a view to having scientific evidence regarding the long term blood pressure lowering effect of neem.

MATERIALS AND METHODS

Preparation of aqueous neem leaf extract

The aqueous neem leaf extract was prepared using a modified method of Parshad *et al* [12]. Briefly, neem leaves including small stems were collected from the botanical gardens in the University of the West Indies. They were oven-dried at 40°C and 200g of crushed leaves were boiled under reflux in 2L of distilled water for 4 hours, sieved through a muslin cloth, then filtered through a Whatman No. 1 filter paper. The cationic concentrations in the filtrate were determined and the filtrate was freeze-dried. The dried extract (68 g) was stored refrigerated in a desiccator and dissolved in distilled, deionised water when required.

Animals

The studies reported in this work have been carried out in accordance with regulations for protection of animals as stipulated in the University of the West Indies. Male Wistar rats, 5-6 weeks old, were obtained from the Animal House of the University of the West Indies. They were divided into three groups of eight. The first group (control) received daily oral boluses of 0.5 ml distilled water; the second (Low-neem) received 20 mg of neem extract /kg body weight; and the third (High-neem) received 40 mg of neem extract /kg body

weight, both in 0.5 ml of water. The various regimens were administered daily for 8 weeks by oral intubation. All groups had water and food *ad libitum*.

Measurement of blood pressure in conscious animals

Blood pressure was measured in conscious rats using a tail cuff pressure meter (Letica, LE 5002 Storage Pressure Meter) placed in the middle part of the animal's tail. Recordings were made when a regularly-paced, stable pulse was established. Data were transmitted to a computer for storage and further processing.

Preparation of the aortic rings

The rats were anaesthetized with pentobarbitone sodium at 50 mg/kg body weight, and killed by cervical dislocation. The thoracic aorta was carefully dissected free of adhering connective tissue, removed and placed in a trough of Physiological Salt Solution (PSS) of the following composition (mM): sodium chloride (NaCl), 119.0; potassium chloride (KCl), 4.7; potassium biphosphate (K_2HPO_4), 1.2; magnesium sulphate ($MgSO_4$), 1.2; sodium bicarbonate ($NaHCO_3$), 24.9; calcium chloride ($CaCl_2$), 1.6; glucose, 11.5. The aorta was cut into 2 mm ring segments from the thoracic end of the tissue. The rings were mounted vertically in 10 ml organ baths containing PSS, bubbled with 95% O_2 / 5% CO_2 gas mixture (pH 7.35 – 7.40) maintained at 37°C. The rings were allowed to equilibrate for at least 90 min and rinsed every 15 min with fresh, pre-warmed PSS (13). Changes in isometric force were recorded under an initial tension of 2 g, using a Grass FT03 isometric transducer. During the equilibration period, each aortic ring was exposed to successive doses of 10^{-7}M noradrenaline (NA) for about three minutes each. Uniformity of consecutive contractions was used as an index of adequate stabilization of the tissue.

Following equilibration, cumulative dose-response curves for noradrenaline and potassium chloride were determined on aortic rings from control and 20 mg/kg neem-fed rats in order to compare the sensitivity of the tissues to the two agonists.

Statistical analysis

Results are expressed as Mean ± SEM. Statistical analysis was by Student's t-test or one way analysis of variance (ANOVA) as required. Fisher's least square difference (LSD) procedure was used for *post hoc* comparisons. P values < 0.05 were considered to be statistically significant and *n* represents the number of animals studied.

RESULTS

With 34 g solid extract per litre of solution, the concentration of cations (mM/L) was as follows: K^+ (40) > Mg^{++} (9.16) > Ca^{++} (3) > Na^+ (0.57). At these concentrations, the quantities of the various ions (say K^+) administered to the animals per day, would cause no more than a 5-6 % change in ECF K^+ concentration, even if given as an *i.v.* bolus. This would be unlikely to contribute to the effect of the neem extract on blood pressure, heart rate or body weight.

Figure 1 shows the effect of daily administration of the neem extract on body weight. There were no significant differences in rat weights at the beginning of the experiment. The rats all gained weight between weeks 1 and 8, the high-neem rats gaining more weight than the other groups. The difference in weight gain between the groups, however, was not significant.

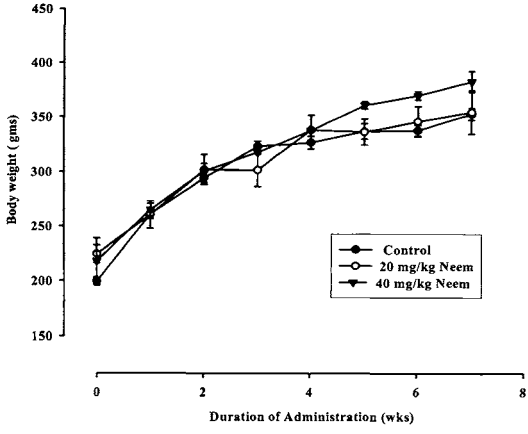

Figure 1. The effect administration of different concentrations (high – 40 mg/kg; low – 20 mg/kg and control – 0 mg/kg) of neem on body weight in normal rats over an eight week period. Each point represents the mean value ± SEM (indicated by vertical bars) from 6 – 8 rats.

Figure 2 shows the effect of chronic administration of neem extract on MAP. The blood pressures were not significantly different in the 1st week of study (control: 79 ± 9.7; low-neem: 75 ± 3.9 and high-neem: 73 ± 3.5 mmHg). From weeks 3 to 8, the mean arterial pressures were significantly lower in Groups 2 and 3 than in the controls ($p<0.05$), the terminal values being: control, 102.5 ± 1.3; low-neem, 87.7 ± 1.5; high-neem, 80.4 ± 1.6 mmHg. Neem-extract did not have a statistically significant effect on heart rate (Figure 3).

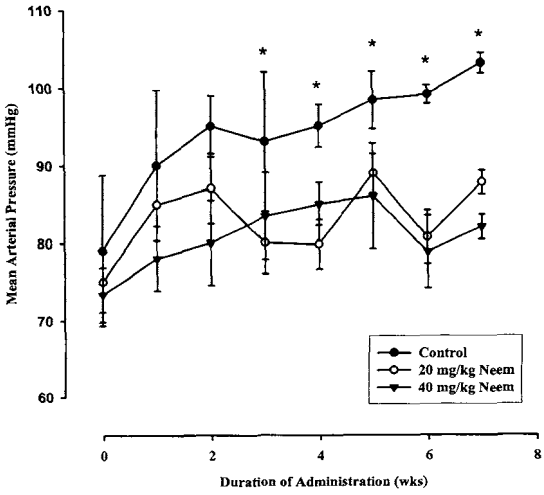

Figure 2. The effect administration of different concentrations (high – 40 mg/kg; low – 20 mg/kg and control – 0 mg/kg) of neem extract on Mean Arterial Pressure (MAP) in normal rats over an eight week period. Each point represents the mean value ± SEM (indicated by vertical bars) from 6 – 8 rats. (* = statistically significant difference at $p<0.05$).

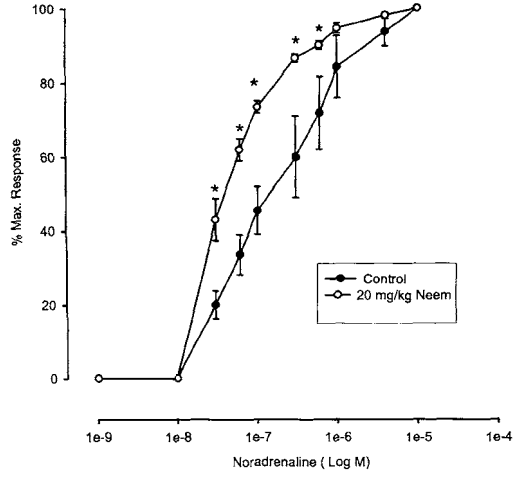

Figure 3. Dose-response curves for noradrenaline in thoracic aortic rings from control and neem-fed rats. Each point represents the mean value ± SEM (indicated by vertical bars) from 6 – 8 experiments. (* = Statistically significant at $p<0.05$).

Exposure of the aortic rings to cumulative concentrations of noradrenaline (Figure 4) showed that the tissues from neem-treated animals (EC_{50} = 39.9 ± 5.62 x 10^{-9}M) were more sensitive ($p<0.05$; $t = 3.4$; $n = 8$) than the control rings (EC_{50} = 266 ± 58.5 x 10^{-9}M). There was no statistically significant difference in response to cumulative concentrations of KCl between aortic rings from the neem-treated animals (EC_{50} = 21.2 ± 0.75 mM) and from the controls (EC_{50} = 19.6 ± 1.55 mM) as shown in Figure 5 ($p>0.05$; $t = 0.81$; $n = 8$). Experiments with the 40 mg were no longer necessary as there was no difference in the effects between 40 and 20 mg/kg.

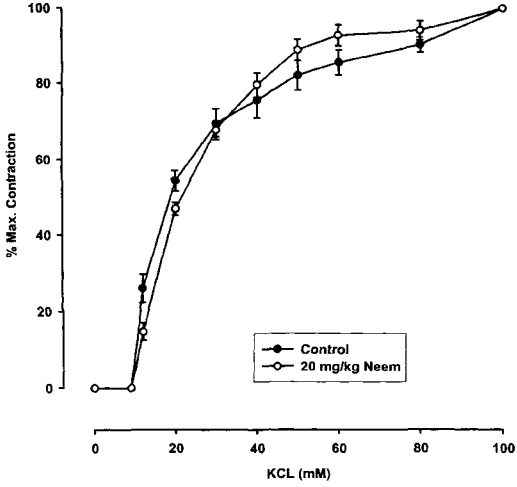

Figure 4. Dose response curves for potassium chloride in thoracic aortic rings from control and neem-fed rats. Each point represents the mean value ± SEM (indicated by vertical bars) from 6 – 8 experiments.

DISCUSSION

The concentrations of cations in the neem extracts were such that at the intra-gastric dosages given, they would have had negligible effects on the body fluids of the rats, and so would be unlikely to contribute to the effects observed. The fact that animals in all three groups had a steady, similar weight gain, being highest in the high-neem group, suggests that the aqueous neem extract did not adversely affect growth of the animals, was probably not toxic and may in fact even promote growth.

We do not know how the fall in blood pressure in the neem-treated animals was produced and more experiments are needed to

investigate this. We report the present findings however, since the long term pressure- lowering effect of chronic administration of neem has not been documented before, and there is some evidence to suggest a possible mode of action.

The depression of mean arterial pressure in the neem-treated rats is consistent with reports that neem affects conduction in the heart and has vasodilatory effects [11]. Pillai and Santhakumari [14] reported that nimbidin, one of the active ingredients of neem, exerts a mild suppressive effect on CNS function in mice and rats after intraperitoneal administration of doses over 250 mg/kg. They also reported anti-histaminergic (H_2-receptor blocking) effects which may interfere with neurotransmission within the CNS. Neem leaves have been reported to reduce stress when ingested in small quantities [15]. Thus, neem could contribute to the lower blood pressure observed in the neem-fed rats by lowering stress responses.

Neem treatment increased sensitivity of the aortic rings to NA whilst not altering sensitivity to KCl. Chronic drug treatments which alter NA blood levels can cause marked changes in the number of NA post-synaptic receptors [16]. Sporn *et al.* [17] showed an up-regulation of α-adrenergic receptors after 7-9 days of NA depletion with 6-hydroxy-dopamine (6-OHDA). Menkes *et al.* [18] demonstrated up-regulation of α_1-adrenoceptors in the thalamus 2-7 days after 6-OHDA treatment. These changes were accompanied by increases in receptor density, affinity and physiological responsiveness.

The lack of a differential response to KCl suggests that the change in the aortic tissue occurs upstream of the depolarisation of the cell membranes and is not associated with changes in muscle contractility. It reinforces the idea that the increased responsiveness to NA is due to up-regulation of the receptor sensitivity of the blood vessels to NA in compensation for chronically diminished blood NA and adrenaline levels in the neem-treated animals compared with the controls. The chronicity of the changes would allow adaptation of the baroreceptor responses, thus decoupling compensatory heart rate increases from the fall in blood pressure. This, along with the proposed fall in circulating catecholamines, could account for the lack of differences in heart rate in the different groups of rats.

In conclusion, chronic administration of neem reduces blood pressure in normal conscious rats and increases sensitivity of receptors in the aortic rings to noradrenaline, a receptor mediated agonist, but had no effect on responses to potassium chloride, a depolarization mediated agonist, perhaps as a result of chronic lowering of circulating NA and adrenaline levels associated with reduced stress responses in the treated animals.

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The Anti-Hypertensive Effect of Sesamin

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INTRODUCTION

Sesamin is a lignan exclusively and abundantly found in sesame seeds. Scientific research has demonstrated its potential efficacy in several forms of preventive healthcare. Animal studies have revealed an anti-hypercholesterolemic role for sesamin via its inhibition of cholesterol absorption and synthesis, thus resulting in lower serum and liver cholesterol levels [1]. These effects have been reflected in a human study involving hypercholesterolaemic male patients where sesamin combined with Vitamin E resulted in a significant reduction in total cholesterol and LDL-cholesterol levels compared to the placebo group receiving vitamin E alone [2].

In addition, it exerts anti-oxidative [3] and anti-inflammatory activities, which are believed to contribute to its prevention of conditions exacerbated by oxidative or inflammatory damage [4-6].

Early unpublished studies that we conducted revealed that sesamin exerts a vasorelaxant effect on the rat aortic ring. These findings instigated our investigation into the potential anti-hypertensive properties of sesamin. Several studies were performed and the results will be presented here in an amalgamated form.

MATERIALS AND METHODS

Materials

Sesamin was prepared from refined sesame oil and purified as described previously [7]. Sesamin was admixed into the standard diet (Oriental Yeast Co. Ltd) at 0.1% or 1% (w/w).

Animal experiments

Deoxycorticosterone acetate (DOCA)-salt induced hypertensive rats

Male Sprague-Dawley rats (6 weeks old) were anaesthetized with ip 40mg/kg sodium pentobarbital and the right kidneys were removed via right flank incision. 1 week following the post-surgical recovery period, the animals were separated into a sham-operated group (sham) and a DOCA-salt group. The DOCA-salt group was further separated into a normal diet (control) and a group fed with 0.1% or 1% (w/w) Sesamin admixed diet (sesamin group). DOCA-salt group was treated twice weekly with 15mg/kg DOCA (subcutaneous) suspended in corn oil and 1% NaCl added to the drinking water. Systolic blood pressure was monitored weekly with a tail cuff and pneumatic pulse transducer (BA-98A, Softron, Tokyo, Japan).

After 5 weeks of feeding, the animals were sacrificed by exsanguination under anaesthesia (40mg/kg sodium pentobarbital by the intraperitoneal route) [8].

Stroke-Prone Spontaneously Hypertensive Rats (SPSHR)

6-week-old male SHRSP rats (Kinki University School of Medicine, Osaka Japan) were used. Rats were separated into unloaded and salt-loaded groups (provided 1% NaCl tap water ad libitum). Each of the groups were further divided into two groups, normal diet and sesamin diet (1%w/w) group. Systolic blood pressure was monitored weekly with a tail cuff and pneumatic pulse transducer (BA-98A, Softron).

After 19 weeks (salt-unloaded) and 24 weeks (salt-loaded) the animals were killed by exsanguination under anesthesia and the heart and left kidney were removed [9].

The whole heart and the left ventricle including septum were removed and weighed accordingly in both DOCA and SPSHR rats. The thoracic aorta and superior mesenteric artery were also removed in some rats from each group for morphometric analysis.

Morphometric analysis

5 mm cross-sections of the thoracic aorta and mesenteric arteries were made and stained with Elastica-van Geison and vessel wall area

and thickness was determined using a digital computer system (IBAS II, Carl Zeiss, Germany) [8,9]. In the case of the SPSHR rats, the renal tissues were paraffin embedded and cut into thin sections according to conventional techniques. The sections were stained with haematoxylin and periodic acid-Schiff. The severity of the morphological damage was determined under a light microscope and scored using a blind protocol as follows: 0 for none and 1 for mild [9].

Aortic O_2^- production

O_2^- production was determined using a lucigenin-enhanced chemiluminescence assay [10]. Thoracic aortas from DOCA-salt-induced hypertensive rats fed 0.1% and 1% sesamin were examined (see above). The thoracic aorta was isolated and cut into strips whilst preserving the endothelium. In a few strips, the endothelium was deliberately removed with gentle rubbing. Three aortic strips were incubated in Krebs-HEPES buffer and equilibrated in the dark at 37°C for 15 minutes following which lucigenin (5mM) was added to the tube. Luminescence was measured using a luminometer (Sirius-2, Funakoshi, Tokyo, Japan) and the relative light unit (RLU) integrated every 3 seconds for 15 minutes and averaged. Background counts of identically treated vessel-free readings were obtained and subtracted from vessel readings [11].

Isometric tension study

Thoracic aortas from DOCA-salt induced hypertensive rats fed 0.1% and 1% sesamin, were examined (see above). The thoracic aorta was isolated and cut into strips whilst preserving the endothelium. In a few strips, the endothelium was deliberately removed with gentle rubbing. The specimens were suspended in organ chambers containing 10 ml Krebs-Ringer bicarbonate solution, under resting tension of 1.5g at 37° and gassed with 95%O_2-5% CO_2. Contractions and relaxations were measured as changes in isometric tension by a force displacement transducer (TB-612T, Nihon Kohden, Osaka, Japan) coupled to a polygraph (RM 6000, Nihon Kohden). Approximately 1.5 h equilibration period was allowed before the start of experiments. Vasodilator response was assessed after strips were precontracted

with L-phenylephrine (Phe, 10^{-6} M). The aortic strips were then exposed to acetylcholine (ACh, 10^{-9} to 10^{-5} M) to induce vasorelaxation in the absence or presence of 30-min pretreatment with 10^{-4}M N^{G}-nitro-L-arginine, a nitric oxide synthase inhibitor. Aorta vasodilator response to Ach was expressed as a percentage of the response to Phe. Contractile responsiveness was examined in strips with and without endothelium by exposing to Phe at 10^{-9} to 10^{-5} M and expressed as a percentage of the response to 60 mM KCl in each tissue [12].

Statistical analysis

All values are expressed as mean ± S.E.M. Several forms of statistical analysis were conducted and are specified in the 'Results' section.

RESULTS AND DISCUSSION

Effects of sesamin on the systolic blood pressure of DOCA-salt induced hypertensive rats

In DOCA-salt induced hypertensive animals, salt loading significantly increased systolic blood pressure levels compared to the sham-operated animals. Sesamin feeding (1%, w/w) significantly attenuated the DOCA-salt-induced increase in systolic blood pressure (Figure 1).

There was a decrease in the body weights of the DOCA-salt fed groups, though this was only significant for the control group. The left ventricle weight of the control group was significantly increased compared to the sham, whilst the sesamin group showed a significantly lower ventricle weight to the control DOCA-salt group. This was also observed for the combined left ventricle and septum weight, where sesamin treatment significantly attenuated the weight increase observed in the control DOCA-salt group. These comparisons indicate the preventive action of sesamin on cardiac hypertrophy, which is associated with hypertension, resulting from the increased workload on the heart (data not shown) [8]. In addition, there was a reduction in the thickening of the thoracic aorta and superior mesenteric artery walls, wall area and wall-to-lumen ratio, all representative of vascular

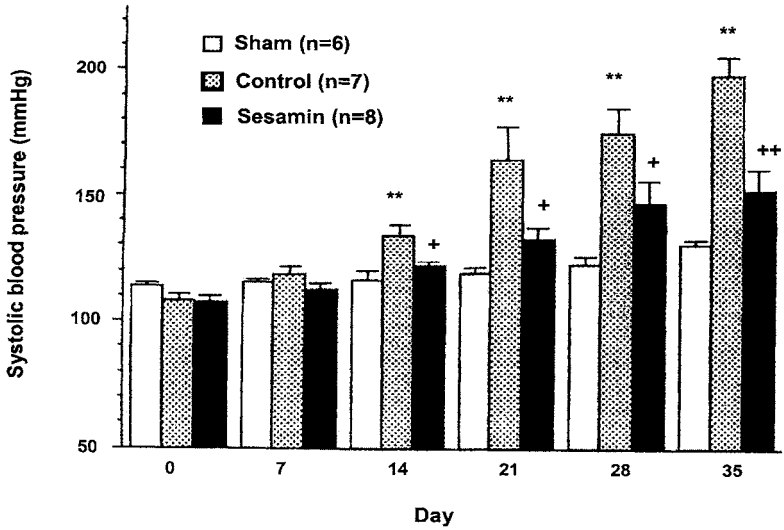


Figure 1. Changes in systolic blood pressure of sham-operated, control and sesamin-fed rats. Columns and bars are the mean ± S.E.M. $p < 0.01$, vs sham group. †, $p < 0.05$; ††, $p < 0.01$ vs control group (one-way ANOVA, Duncan's multiple comparison). Figure taken from Matsumura et al [8].

hypertrophy (Table 1). Histological cross-sections of the arteries from the salt-loaded DOCA hypertensive animals (Figure 2) reveal the anti-hypertrophic effect of sesamin on arterial thickening [8].

Inhibition of vascular superoxide production by sesamin

Increased production of aortic superoxide anion (O_2^-) has been observed in several animal models of hypertension. Whilst anti-hypertensive therapy has not always been effective in preventing

Table 1. Morphological analysis of aortas and mesenteric arteries in sham-operated, control and sesamin-fed rats after 5 weeks of treatment.

	Aorta			Mesenteric artery		
Group	Wall thickness (mm)	Wall area (mm^2)	Wall to lumen ratio	Wall thickness (mm)	Wall area (mm^2)	Wall to lumen ratio
Sham	95±1	0.41±0.01	0.27±0.02	56±1	0.12±0.01	0.33±0.02
DOCA-salt (Control)	136±5**	0.63±0.04**	0.35±0.02**	89±2**	0.24±0.01**	0.49±0.02**
DOCA-salt (Sesamin)	107±4††	0.48±0.03††	0.28±0.01††	73±3††	0.16±0.01††	0.42±0.02†

**$p < 0.01$ vs sham group; †$p < 0.05$, ††$p < 0.01$ vs control group (one-way ANOVA, Duncan's multiple comparison). Table reproduced from Matsumura *et al.* [8].

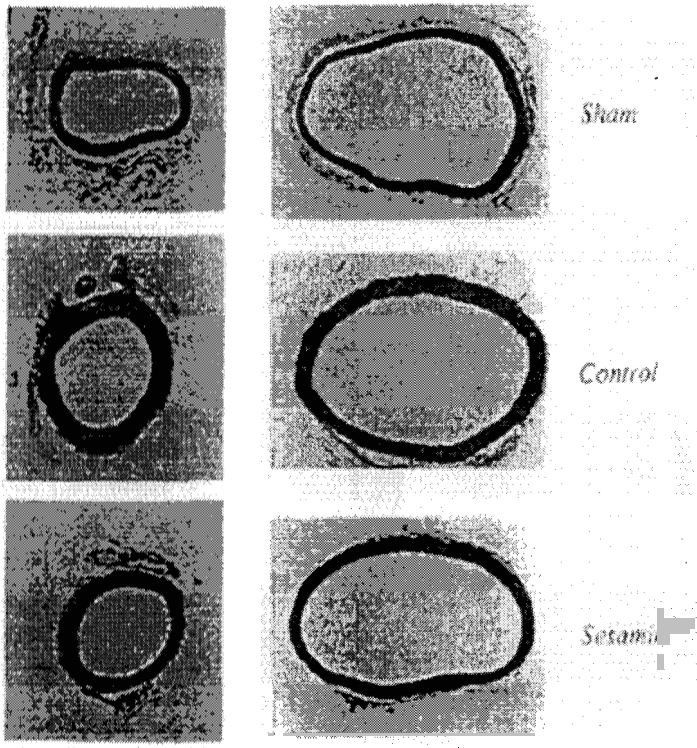


Figure 2. Representative light micrographs showing cross sections of the superior mesenteric arteries (left) and thoracic aorta (right) from sham-operated, control and sesamin-fed rats. Taken from Matsumura et al [8].

oxidative stress, there has been observation that antioxidants such as Vitamins C and E, can attenuate both the elevation of blood pressure and progression of hypertension whilst reducing oxidative stress in hypertensive animals [13, 14].

In our study sesamin at 0.1% or 1% did not alter aortic O_2^- production in sham groups. Salt feeding in 5-week DOCA-salt hypertensive rats caused a significant increase in the aortic O_2^- production in the normal diet group. It was observed that feeding of 0.1% and 1% sesamin reduced the O_2^- production in endothelium-intact aortic rings, and this effect was significant with 1% sesamin (Figure 3). Sesamin also produced a dose-dependant suppression on the increased systolic blood pressure, observed to occur in the DOCA rats. The level of aortic O_2^- production was also positively correlated to the systolic blood pressure of the animals (Figure 4),

suggesting a close association between the sesamin-induced decrease in vascular O_2- production and its hypertensive activity [11].

Effects of sesamin on vasoconstrictor responses in aortic rings of DOCA-salt hypertensive rats

In sham-operated rats, there is a dose-dependent contractile response, to L-phenylephrine (Phe) in aortic strips (with and without

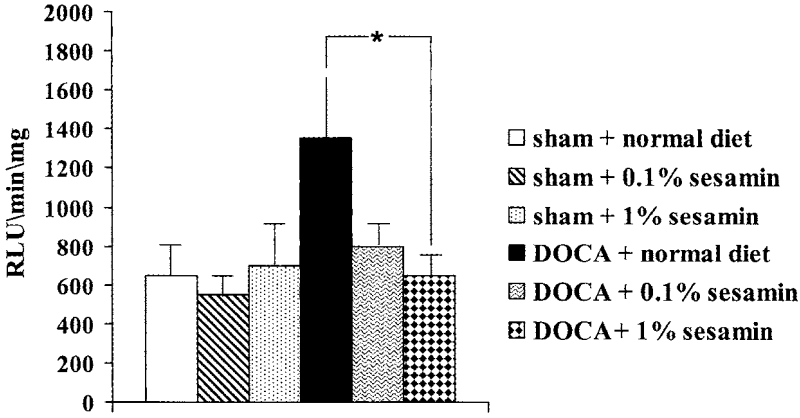


Figure 3. Effects of dietary sesamin on enhanced O_2^- production in endothelium-intact aortic rings. *$p<0.05$ vs DOCA+ normal diet (one way ANOVA, Tukey-Kramer multiple comparison). Graph obtained from Nakano et al [11].

endothelium) obtained from 5-week DOCA-Salt hypertensive rats. In the DOCA-salt rats, there was a significant increase in the sensitivity and contractile response to Phe, whereas in the normal diet-fed group, this effect was significantly attenuated by sesamin feeding (Figure 5). Therefore the normalization of vasocontractile responses by sesamin suggests a potential contributing mechanism for its anti-hypertensive activity [12].

Effects of sesamin on the development and maintenance of hypertension in SPSHR

In spontaneously hypertensive rats, salt loading initiated a progressive increase in systolic blood pressure. There was an increase from 130±4 and 132±5 mmHg in normal and sesamin-diet groups respectively, to 199±3 and 172±2 mmHg respectively at 12 weeks.

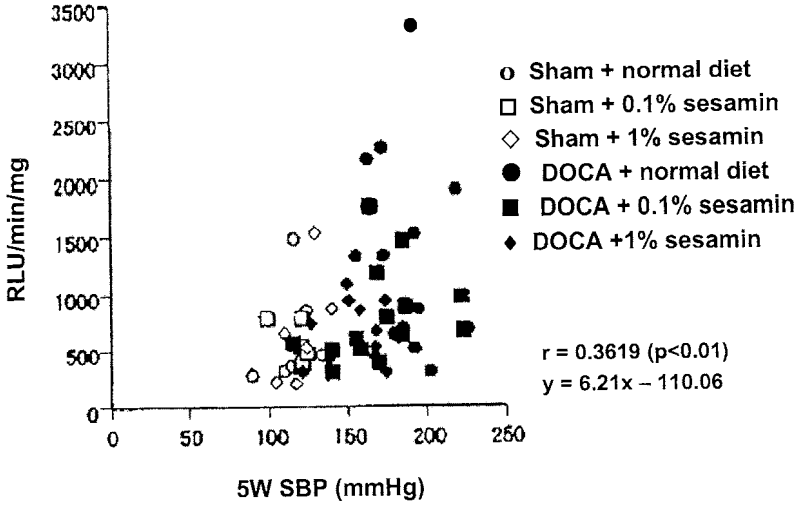
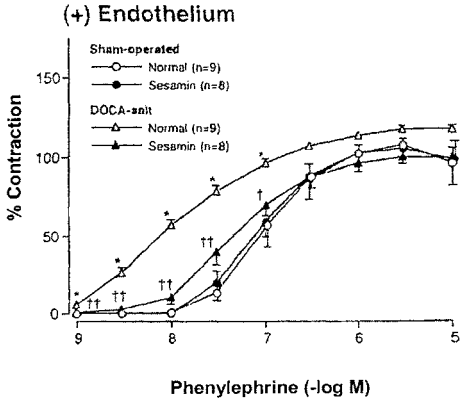


Figure 4. Correlation between systolic blood pressure and vascular O_2^- production in rats in all experimental groups (n = 56). (Pearson linear correlation analysis). Graph obtained from Nakano et al [11].

Sesamin dampened the increase in the blood pressure levels between 9-26 weeks of the 30-week study, however, by 30 weeks (trial completion) the levels between normal and sesamin-diet groups did not differ [9] (Figure 6).

In the un-salt loaded group, there was a progressive increase in systolic blood pressure from 6 to 25 weeks, which was slightly suppressed by sesamin feeding however, this effect was not statistically significant (data not shown).

Similar results were obtained for the effect of sesamin on cardiac hypertrophy as was observed for the DOCA-salt induced hypertensive rats. In both the salt-loaded and unloaded groups, sesamin significantly reduced the heart weight, however this may in part be due to the lower body weights recorded for these groups. In the salt-loaded group, there was a significantly lower left ventricle-septum to body weight ratio ($p < 0.01$, unpaired *t*-test) in the sesamin-diet fed group, compared to the normal-diet fed group (data not shown), supporting its ability to reduce cardiac hypertrophy, an action also observed in the DOCA-salt induced hypertensive rats [8]. Sesamin feeding significantly attenuated the effect of salt-loading on the wall thickening and wall area of the aorta and mesenteric arteries [9] (Table 2).



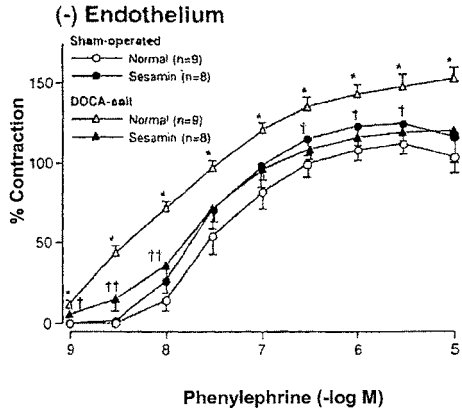


Figure 5. Dose-response curves of aortic rings (with or without endothelium) from normal- or sesamin diet-fed sham-operated and DOCA-salt hypertensive rats to L-Phenylephrine. Values are mean ± S.E.M. *p < 0.01 vs normal diet-fed sham group; †p < 0.05; ††p < 0.01 vs normal diet-fed DOCA-salt group (one-way ANOVA, Bonferroni's multiple comparison). Figure taken from Matsumura et al [12].

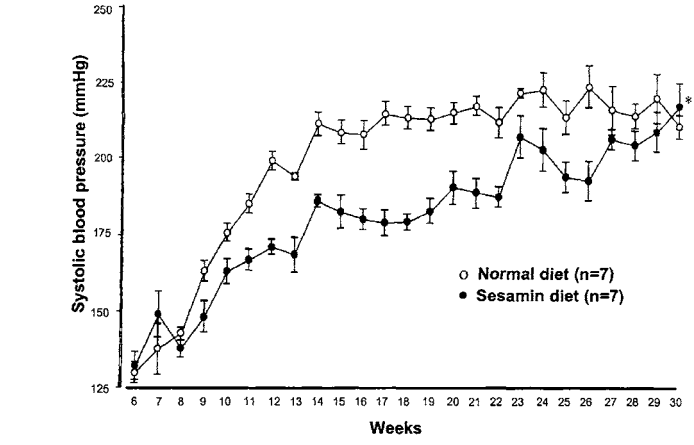


Figure 6. Changes in the systolic blood pressure of salt-loaded SPSHR rats fed normal-diet or sesamin diet. *, p<0.001, compared with normal diet (two-way repeated ANOVA). Graph obtained from Matsumura et al [9].

Morphological analysis of the renal tissues also revealed the ability of sesamin in preventing hypertension associated renal damage. Thickening of the tunica intima small artery and fibrinoid degeneration of the small arterial wall were scored using a blind protocol. In the salt-loaded group, sesamin feeding significantly reduced the incidence of fibrinoid degeneration in the arterial wall ($p<0.01$ (X^2-test)) as well as the number of arteries exhibiting thickening of the tunica intima ($p<0.01$; Mann-Whitney-test, values not shown) [Figure 7].

In this study, sesamin feeding to salt-loaded SPSHR rats markedly delayed the development of hypertension and ameliorated both vascular hypertrophy and renal damage. It appears to act by delaying the malignant status of hypertension and the salt-dependent pathology. In previous experiments on the rat aortic ring, sesamin produced Ca^{2+} antagonistic vasorelaxant activity. It is postulated that this mechanism underlies at least part of the anti-hypertensive activity of sesamin.

These studies in two animal models of hypertension confirmed our expectation that the vasorelaxant ability of sesamin contributes to its anti-hypertensive action. Sesamin attenuated increases in systolic blood pressure, cardiac and vascular hypertrophy in both the DOCA-induced and spontaneously hypertensive rats, when loaded with salt. The effect was only observed in salt-loaded animals, suggesting there

Table 2. Morphological analysis of aorta and mesenteric artery in normal- and sesamin-diet groups of SPSHR

Group	(n)	Aorta		Mesenteric artery	
		Thickness (μm)	Area (mm^2)	Thickness (μm)	Area (mm^2)
Salt-loaded					
Normal-diet	(7)	136 ± 3	0.764 ± 0.018	80.2 ± 1.5	0.219 ± 0.005
Sesamin-diet	(7)	124 ± 3**	0.670 ± 0.017**	73.6 ± 1.6*	0.201 ± 0.005*
Salt-unloaded					
Normal-diet	(5)	132 ± 3	0.678 ± 0.023	80.9 ± 0.9	0.177 ± 0.006
Sesamin-diet	(6)	121 ± 4	0.598 ± 0.025*	79.0 ± 2.0	0.169 ± 0.008

*$p<0.05$; **$p<0.01$, compared with each normal-diet group (unpaired t-test). Values taken from Matsumura et al [9].

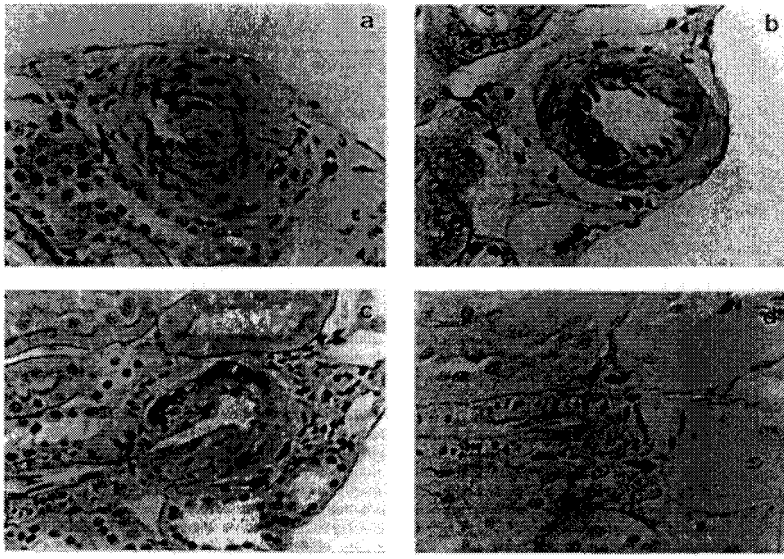


Figure 7. Representative light micrographs of renal tissues obtained from salt-loaded SPSHR fed normal-diet or sesamin-diet. a, Thickened tunica intima of small artery in normal-diet group (x 600); b, d, normal small artery in sesamin-diet group (600x); c, fibrinoid degeneration of small arterial wall in normal-diet group(x 600). Figure taken from Matsumura et al [9].

was specific interference with the pathological progression associated with salt intake.

In the DOCA-induced hypertensive animals, the systolic blood pressure increase was correlated to aortic O_2^- production. Sesamin intake reduced this oxidant production and aided the lowering of systolic blood pressure. This correlation reiterates the previously reported antioxidant activity of sesamin *in vivo* [3], and proposes a potential mechanism for retarding the manifestation of hypertension.

In sesamin-fed salt-loaded SPSHR, in addition to the reduced arterial wall thickening of the aorta and mesenteric arteries relative to the control group, sesamin also demonstrated the ability to reduce hypertension-ssociated renal pathology as measured by thickening of the tunica intima of small arteries and fibrinoid degeneration of arterial walls.

In conclusion, the increases in systolic blood pressure and development of cardiovascular hypertrophy and hypertension-

associated pathology were shown to be prevented or delayed by sesamin administration. These studies suggest the potential for sesamin as a natural prophylactic agent that may protect against the development of hypertension and its associated pathology.

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Effects of *Salvia miltiorrhiza* After Acute Myocardial Infarction in Rats

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INTRODUCTION

Acute myocardial infarction (MI) is the commonest cause of death in the developed countries, and it is on the rise in developing countries. Ramipril is a well-known Angiotensin-converting enzyme (ACE) inhibitor which inhibits conversion of inactive angiotensin I to active angiotensin II. Experimental studies have shown that ACE inhibitors administered chronically before acute MI might limit myocardial infarct size, improve cardiac function and prevent cardiac hypertrophy [1, 2]. The Chinese herb, *Salvia miltiorrhiza* (SM), has been widely and successfully used mainly for angina pectoris, MI and stroke [3]. Compared to ramipril, however, there is very limited biochemical information available to demonstrate the mechanisms of *SM's* cardio-protective effects. This study thus investigates the possible biochemical and molecular mechanisms of such effects of SM in Wistar rats in comparison with those of ramipril.

MATERIALS AND METHODS

Male Wistar rats (210-230g) were obtained from the Laboratory Animal Centre, National University of Singapore,

Singapore. Ninety rats were randomly divided into 3 treatment groups and 1 sham group as shown in Table 1. All the animals were housed under diurnal lighting conditions and allowed food and water *ad libitum*.

Table 1. Medicines, dosage and the number of rats in the different treatment groups

Medicines	Dosage	Number of Rats
Sham	--	10
SM	0.675g/kg/day	32
Saline	6.75ml/kg/day	24
Ramipril	1mg/kg/day	24

Medicines, dosage and the number of rats in the different treatment groups

SM (0.675 g/kg/day) or ramipril (1 mg/kg/day) was administered intraperitoneally once daily for 7 days. At the end of this period of treatment, the rats underwent a permanent ligation of the left anterior descending (LAD) coronary artery, which is a widely used surgical procedure to induce acute MI in animals. Treatment was continued for another 2 weeks after the surgery. At the end of the treatment period, all the rats were sacrificed by decapitation. Hearts and livers were collected for further studies including antioxidant and lipid peroxidation assays, coronary capillary density study, morphological examination of the infarcted heart and also molecular studies to evaluate the DNA-protective effect of SM.

In vitro antioxidant tests

Inhibition of Pyrogallol red bleaching by hypochlorous acid (HOCl)

Pyrogallol red (pyrogallosulphonephthalein; 100 μM) was dissolved in 100 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.4 and left at room temperature for 10min. After this time, 125 μM HOCl was added and incubated with ascorbic acid or SM. The final mixture was diluted 1:4. Absorbance values obtained at 542 nm with UV-visible spectroscopy in the presence and absence of the antioxidant and SM were recorded. The decreases in absorbance were expressed as percentage inhibition as previously described [4].

Evaluation of DNA damage using GC/MS

100 μg calf thymus DNA samples were pre-incubated with SM at various concentrations. HOCl (250 μM) was added to the DNA reaction mixture, followed by incubation at 37°C for 1 hr. DNA samples were then dialysed against water for 22 hrs. Hydrolysis was done by addition of 0.5 ml 60% cold formic acid (v/v) and heating at 150°C for 45 min in an evacuated, sealed hydrolysis tube after addition of respective internal standards. Samples were cooled and lyophilised. After acid hydrolysis of DNA, samples and calibration standards were derivatised in poly(tetrafluoroethylene)-capped glass vials after purging with nitrogen. 75 μl of a BSTFA (+1% TMCS)/ acetonitrile/ethanethiol (16:3:1 v/v) mixture was added and mixed well at 23°C for 2 hrs. Oxidized DNA was analyzed by GC/MS (Agilent 6890 gas chromatograph and interfaced with an Agilent MSD 5973) as previously described [4].

Ligation of left anterior descending (LAD) coronary artery

MI was induced by permanent ligation of LAD using a modified version of the technique described by Stauss et al. [5]. Briefly, after chloral hydrate anaesthesia, the rats were incubated and artificially ventilated. Skin and muscles were separated at the left side of the sternum, and the third and fourth ribs were cut. A rib-spreading chest retractor was inserted and the left descending coronary artery was ligated using sterile 6-0 suture material [5, 6].

Biochemical assays

Hepatic antioxidant assays

For the assays of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione perioxidase (GSH-Px) and Glutathione S-Transferase (GST) activities, frozen liver tissue (1g) was homogenized in 1mL phosphate buffer (10mmol/L, ph 7.5) by a Polytron homogenizer. After centrifugation, the pellets were discarded and the supernatants used for the assessment of enzyme activity as we previous reported [7]. SOD activity was determined by an improved method of Marklund and Marklund [8]. Catalase was assayed by the amended method of Aebi [9]. For the assay of GSH-Px activity, glutathione peroxidase was determined by the modified method of Beutler [10].

Protein assays

The protein content in the samples for the above anti-oxidant assays were determined by the method of Bradford [11], using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) [7].

Thiobarbituric Acid Reacting Substances (TBARs) assay method for measuring malonaldehyde content in the heart and liver of rats with acute MI

The TBARs are the secondary products of lipid peroxidation. These secondary products are mainly aldehydes, the major compound being malondialdehyde (MDA). The determination of MDA content in hearts and livers was performed by the thiobarbituric acid (TBA) method. When heated at a low pH, TBA reacts with these aldehydes to give a pink coloured chromogen, which absorbs at 535nm. Hence this test estimates the MDA content in the sample. This method has been widely adopted as a sensitive method for assay of lipid peroxidates in animal tissues [7, 12].

Morphological examination of rat heart with acute MI

The hearts collected from rats with acute MI were stained by Tetrazolium-Blue and kept in 4% phosphate buffered paraformaldehyde for morphological examination. The infarcted area was judged from both epicardial and endocardial sides and outlined on paper, cut and weighted. The sizes of the left ventricle and the infarct area were calculated by Scion Image software (California, USA). The ratios of the total heart weight to body weight, the right ventricular weight to body weight and the infarcted area weight to heart weight were calculated. The infarct size was expressed as a proportion of the left ventricular size [5, 6].

Coronary capillary density study of rat hearts with acute MI

H&E staining of myocardial sections was performed to differentiate coronary capillaries, after which they were covered with crystal mount (Biomeda, CA, USA). The sections were examined under a microscope (ZEISS AX10SKOP-H, USA) [13].

Statistics

Data are expressed as means ± SEM. All data were analyzed by one-way analysis of variance (ANOVA) for independent evaluation of all groups. In cases where $p < 0.05$, the differences between individual groups were analysed using the unpaired Student's t-test. These differences were considered to be statistically significant when $p < 0.05$.

RESULTS

In vitro antioxidant tests

The scavenging effect of SM on HOCl as determined by Pyrogallol red bleaching assay was significantly higher ($p < 0.05$) than that of ascorbic acid (Figure 1).

Protection against HOCl-mediated DNA damage

SM significantly prevented DNA damage caused by HOCL. Incubation of DNA with HOCl (250 μM) for 1 hour at 37°C led to increased formation of DNA base products (Table 2). However, when DNA was pre-incubated with *SM* at different concentrations (500 and 1000 μg/ml), DNA base products formation was observed to be decreased significantly. It was more significant especially with the higher concentrations as shown in Table 2.

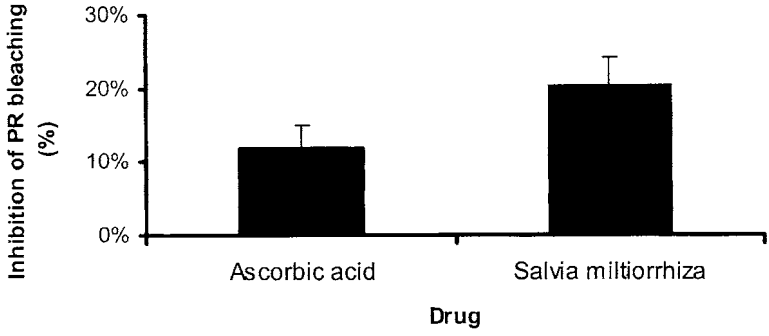

Figure 1. Effects of ascorbic acid and SM on inhibition of pyrogallol red (PR) bleaching by Hypochlorrous acid (HOCl). Both standard (ascorbic acid) and SM were tested at final concentrations of 2.5 μg/ml. Results shown are mean±S.D. of at least 3 independent experiments performed in duplicate.

Table 2. Inhibition of HOCl-induced DNA damage by SM and ramipril.

AVERAGE nmol/mg DNA			DNA+HOCl+Vit C		DNA+HOCl+SM	
DNA Base	DNA alone	DNA+HOCl	500ug	1mg	500ug	1mg
5-Cl Uracil	0.00±0	3.53±0.66	0.20±0.06	0.18±0.07	1.77±0.41	1.24±0.10
5-OH, Me Hydantoin	0.03±0	0.07±0.01	0.12±0.02	0.15±0.03	0.10±0.02	0.09±0.03
5-Formyl Uracil	0.00±0	0.15±0.01	0.01±0.0	0.00±0.00	0.12±0.05	0.06±0.01
5-OH Uracil	0.02±0.01	1.49±0.14	0.44±0.06	0.04±0.01	0.85±0.07	0.51±0.05
5-(OH, Me) Uracil	0.13±0.02	0.27±0.04	0.15±0.03	0.15±0.02	0.15±0.03	0.15±0.01
5-OH Cytosine	0.12±0.03	3.65±0.35	0.19±0.06	0.14±0.03	1.03±0.195	0.61±0.06
Thymine Glycol (cis)	0.09±0.02	7.30±1.17	0.14±0.03	0.07±0.01	3.02±0.19	0.89±0.21
FAPy Adenine	0.49±0.04	0.44±0.02	0.54±0.05	0.54±0.07	0.34±0.11	0.33±0.03
8-OH Adenine	0.02±01.0	0.86±0.06	0.08±0.01	0.07±0.02	0.04±0.01	0.04±0.01
Xanthine	0.54±0.1	5.71±0.19	2.58±0.12	1.31±0.06	0.85±0.21	0.72±0.03
2-OH Adenine	0.03±0.02	0.15±0.04	0.08±0.01	0.07±0.01	0.04±0.01	0.02±0.01
FAPy Guanine	0.04±0.01	0.51±0.11	0.11±0.02	0.12±0.05	0.07±0.02	0.06±0.02
8-OH Guanine	0.17±0.01	0.47±0.05	0.19±0.04	0.13±0.02	0.13±0.06	0.19±0.05

100μg DNA was oxidized using HOCl (250μM) for 1h at 37°C. The samples were then dialyzed against water for 22h, after being freeze dried and hydrolyzed with 60% (v/v) formic acid. Oxidized DNA base products were analyzed by GC/MS after derivatization for more than 2h. DNA alone as a negative control and DNA with HOCl as a positive control. Results are expressed as mean±SD

General survival rate after acute MI

Survival rates during the period of study are shown in Figure 2. In all treatment groups, the highest mortality occurred within 24 hrs of surgery. The mean survival rates at the end of the observation period

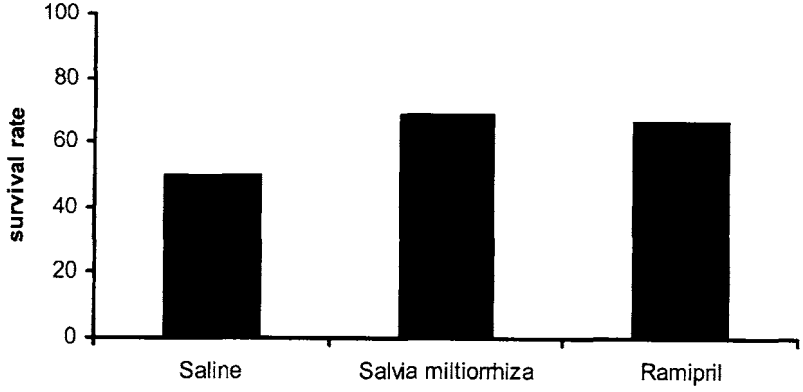

Figure 2. Mean survival rate of the different animal groups after acute MI.

for rats given SM, saline and ramipril were 68.9% (22/32), 50% (12/24) and 66.5% (16/24), respectively.

Biochemical assays

Hepatic anti-oxidant assays

As shown in Table 3, significantly higher hepatic CAT activity was observed in SM-treated rats than in saline-treated rats ($p < 0.005$). There was no significant difference between SM- and ramipril-treated rats, or between saline- and ramipril-treated rats. Hepatic SOD activity was significantly higher in SM-treated rats than in both saline- and ramipril-treated rats (both $p < 0.001$). A significantly higher SOD activity ($p < 0.05$) was also observed in saline-treated rats compared to ramipril-treated rats. Hepatic GSH-Px activity was significantly higher in SM-treated rats than in saline- and ramipril-treated rats (both $p < 0.0001$) but was significantly lower in ramipril-treated rats than in saline-treated rats ($p < 0.001$). Hepatic GST activity was significantly higher in SM-treated rats than in saline- and ramipril-treated rats ($p < 0.005$ and $p < 0.001$, respectively). A significantly higher hepatic GST activity was observed in saline-treated rats compared to ramipril-treated rats ($p < 0.001$).

Table 3. Anti-oxidant enzyme activities in the livers of SM-, saline- and ramipril-treated rats

Enzyme (U/mg protein)	SM	Saline	Ramipril
CAT	*0.674±0.016	0.583±0.031	0.630±0.019
SOD	**16.24±0.31	10.56±0.31	$8.94\pm0.67^{+}$
GSH-Px	***0.453±0.013	0.353±0.012	$0.248\pm0.012^{++}$
GST	**5.23±0.114	4.03±0.215	$2.62\pm0.105^{++}$

Values are mean±SEM (n = 6). All assays were performed in duplicate at 25°C. *$p < 0.005$; **$p < 0.001$; ***$p < 0.0001$ compared with corresponding saline- and ramipril-treated group; $^{+}p < 0.05$ $^{++}p < 0.001$ ramipril-treated compared with corresponding saline-treated group.

TBARS content in the liver and heart

The cardiac and hepatic TBARS content were significantly lower in SM-treated rats compared to both saline- and ramipril-treated rats (Table 4, both $p < 0.005$). Cardiac TBARS content was significantly higher in ramipril-treated rats than in saline-treated rats ($p < 0.05$). There was however no significant difference in hepatic TBARS values between saline- and ramipril-treated rats ($p > 0.05$).

Table 4. TBARS content in the heart and liver of SM-, saline- and ramipril-treated rats with acute MI.

Treatment	TBARS value (nmol/g wet weight)	
	Heart	Liver
SM	202.23±1.77[a]	320.23±4.22[d]
Saline	231.48±7.09[b]	385.83±3.39
Ramipril	245.35±4.33[c]	382.54±5.21[e]

Values are the mean±SEM (n=6). Assays were performed in duplicate at 25°C.

[a] $p < 0.005$ (t-test) SM-treated vs saline-treated rats

[b] $p < 0.005$ (t-test) saline-treated vs ramipril-treated rats

[c] $p < 0.005$ (t-test) ramipril-treated vs SM-treated rats

[d] $p < 0.005$ (t-test) SM-treated vs saline-treated rats

[e] $p > 0.5$ (t-test) ramipril-treated vs saline-treated rats

Morphological examination of rat hearts with acute MI

Three hearts from sham group and six hearts from each treatment group (total 21 hearts) were examined for morphological changes, using a microscope.

Infarct size

Infarct size (IS) as a proportion of left ventricular size and the absolute sizes of the infarcted areas are shown in Table 5. Infarct size and its ratio with left ventricular size were reduced significantly in SM- and ramipril-treated rats with acute MI compared to those of saline-treated rats (all $p < 0.001$); no significant difference was found in IS between SM- and ramipril-treated rats.

Cardiac and body weights

Cardiac weight parameters were expressed as the ratios (g/kg) of total heart weight (HW) to body weight (BW), left ventricular

Table 5. Absolute size (mm^2) of infarct area and the ratios of infarct size to left ventricular size.

Treatment Groups	Absolute sizes (mm^2)	Mean Ratios (IS/LVS)
SM	38.99±0.83[a]	0.42±0.01[a]
Saline	53.98±1.26	0.51±0.01
Ramipril	39.33±1.06	0.44±0.01[b]

Values are the mean ± SEM (n=6).
[a] $p < 0.001$ (t-test) SM-treated vs saline-treated rats;
[b] $p < 0.001$ (t-test) ramipril-treated vs saline-treated rats
IS: infarct size; LVS: left ventricle size.

weight (LVW) to body weight and right ventricular weight (RVW) to body weight (Table 6). The body weights were not significantly different in the three treatment groups. The ratios of heart weight to the body weight, and left and right ventricular weights to body weight were significantly lower in SM- and ramipril-treated rats with acute MI than those of saline-treated rats with acute MI (all $p < 0.001$); there was no significant difference in all the ratios between SM- and ramipril-treated rats.

Capillary density

Blood vessels of the left ventricles of rat hearts with acute MI were counted under a microscope by amplifying by 200 times. The average number of microvessels of each group was calculated in at least 6 sections of each heart and at least 6 samples for each group. Myocardial capillary density of the left ventricles of rat hearts with acute MI was significantly increased in SM- and ramipril-treated rats

Table 6. Body weights, ratios of heart, left ventricular and right ventricular weights to body weight in the different treatment groups.

Treatment groups	Body weight (g) at the end of the study	HW/BW	LVW/BW	RVW/BW
SM	302.9±11.9	***2.55±0.03	***2.03±0.04	***0.56±0.02
Saline	303.5± 5.7	3.27±0.02	2.36±0.04	0.89±0.02
Ramipril	296.7±13.9	***2.45±0.08	***1.95±0.07	***0.52±0.03

HW: heart weight. BW: body weight. LVW: left ventricular weight.
RVW: right ventricular weight. Values are mean ± SEM (n = 6).
*** $p < 0.001$ (SM- and ramipril-treated rats compared with the corresponding saline-treated group).

compared to that in saline-treated rats ($p < 0.0001$); a slight but insignificant increase in myocardial capillary density was observed in SM-treated rats compared to ramipril-treated rats ($p > 0.05$) (Table 7). Furthermore, the distribution of blood vessels in the two treatment groups appeared to be different. In the SM-treated group, the blood vessels were mainly present in non-ischemic myocardium with an increased density along the border, while in the ramipril-treated group, the number of blood vessels was slightly increased in the non-ischemic zone compared to the infarction zone, without any increase in vessels along the border zone. In the saline-treated group, blood vessels were predominantly present in the non-ischemic zone while few blood vessels were observed in the ischemic and border zones.

Table 7. Coronary capillary densities in left ventricles of different treatment groups with acute MI

Treatment groups	Saline	SM	Ramipril
Number/AMI left ventricle	259.8±2.6	292.7±4.7[a]	285.5±3.7[b]

Values are the mean±SEM (n=6).

[a] $p < 0.001$ (t-test) SM-treated vs saline-treated rats

[b] $p < 0.001$ (t-test) ramipril-treated vs saline-treated rats

DISCUSSION

This study showed that, compared to ramipril, SM produced a similar cardioprotective outcome in terms of a higher survival rate, smaller IS and reduced myocardial hypertrophy in rats with acute MI. These parameters are important "objectives" in the treatment of cardiovascular diseases, including acute MI [4, 14, 15].

SM produced potent antioxidant effects by increasing the activities of the hepatic antioxidant enzymes including SOD, CAT, GSH-Px and GST, and inhibiting hepatic and myocardial lipid peroxidation in rats with acute MI. However, there was no evidence to show that ramipril had any favourable effects on the antioxidant enzymes. Furthermore, it augmented myocardial lipid peroxidation in rats with acute MI by inhibiting the activity of GSH-Px, which is a major antioxidant enzyme that inhibits lipid peroxidation in cytomembranes.

SM increased the activities of antioxidant defense enzymes in the liver of acute MI rats in this study. These enzymes are released into the blood and circulate to the heart and other parts of the body where they scavenge free radicals. In this way, SM acts indirectly as an antioxidant. Furthermore, SM could enhance the activities of antioxidant enzymes in the heart and other parts of the body too. This could be one of the major mechanisms for protecting myocardium from free radical damage following acute MI, and improve the general condition of rats with acute MI. The increased activities of hepatic enzymes could, in part, be contributed by SM's "sparing" effects on the antioxidant enzymes. Zhao et al. [16] had shown that danshensu and tanshinone, two active ingredients of SM, could react with oxidants generated from the xanthine-xanthine oxidase system and lipid peroxidation. By such a "sparing" action, SM could contribute indirectly to the increased activities of hepatic antioxidant enzymes in the rat with acute MI.

ACE inhibitors including ramipril have been shown to be effective in preventing the development of left ventricular hypertrophy by improving myocardial tissue blood flow, stimulating myocardial capillary growth and limiting myocardial necrosis. The prevention and regression of ventricular hypertrophy are desirable beneficial effects in myocardial remodeling after acute MI [17]. In our study, a significant increase in myocardial capillary density in the non-ischemic area of the heart with acute MI was observed not only in ramipril-treated rats but also in SM-treated rats compared to saline-treated rats. These findings suggest that SM, like ramipril, can potentially prevent ventricular hypertrophy following acute MI in rats. The results of this study thus show the promising potential of using SM in human subjects with acute MI. Similar results were achieved recently from our latest study with the purified and standardized SM (know as Angino®) in the acute MI model [18].

SM (and likely its purified form as well) appears to be as good as Western drugs currently used in clinical practice in producing beneficial effects in the heart of rats with acute MI following LAD ligation. It deserves consideration for further evaluation in the animal model and in clinical situations in future.

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The Antioxidant and Free Radical Scavenging Effects of Sesamin

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INTRODUCTION

Sesamin is one of the lignans found exclusively and abundantly in sesame oil. It is known for its multiple health benefits including the reduction of cholesterol [1], anti-carcinogenic activity [2] and anti-hypertensive effect [3-5]. Sesamin has also been reported to protect against alcohol and carbon tetrachloride induced liver toxicity [6].

One of the proposed mechanisms underlying its liver protective action is via suppression of the free radical-mediated processes initiated by hepatotoxins, which potentially lead to lipid peroxidation and DNA damage. Sesamin itself, has no anti-oxidative properties, but can be metabolized to several catechol forms in the liver. Conversion of the methylenedioxyphenyl moiety of Sesamin to the dicatechol (bis (3,4-dihydroxyphenyl)) moiety, yields a metabolite, which demonstrates free radical scavenging activity against •OH, O_2^-, 1,1-Diphenyl-2-picrylhydradyl (DPPH) and lipid peroxidation in vitro when enzymatically demethylated and activated by rat liver homogenate. In vivo, the same metabolite demonstrated •OH and O_2^- scavenging activity in rats, following oral administration at 500mg/kg [7]. Similarly an alternative catechol metabolite, 2-(3,4-methylenedioxyphenyl)-6-(3,4-dihydroxyphenyl)-cis-dioxabicyclo [3.3.0]octane, has demonstrated the ability to scavenge superoxide radicals in vitro with an efficacy greater than that of vitamin E [8].

In addition to its independent anti-oxidant capacity, several studies have also shown its potential to work synergistically with vitamin E against lipid peroxidation, and increase the plasma and tissue levels of alpha-tocopherol [9, 10]. The bioavailability of the gamma-

tocopherol isoform, is increased with sesamin feeding, reiterating its role in enhancing the potential activity of known anti-oxidants [11, 12].

In this study, we examined the anti-oxidant function of sesamin, on exercise-induced lipid peroxidation. We observed the effects in both animal and human subjects, using strenuous physical exercise as a trigger for oxidative stress in the body.

MATERIALS AND METHODS

Sesamin was prepared from refined sesame oil and purified in a method described by Fukuda et al [13].

Human study

Seven healthy male university students were recruited for this triple cross-over study. Two hours on each occasion, following a 5-mins warm up, subjects were instructed to exercise at 80-95% maximal heart rate (HRmax) on a cycle ergometer for 25 mins with a 1 min cool down period towards the end of the exercise (Figure 1). This experiment was repeated after a wash-out period of 1 week, and two hours prior to exercise, the subjects were orally administered either placebo, 36 mg of sesamin, or 200 mg of vitamin E (alpha-tocopherol) on separate trial occasions. In all trials, blood samples were collected before and 5,10, 20, 30, 40, 60 and 90 mins after the onset of exercise. Plasma lipid peroxide levels were measured using the Hb-MB method.

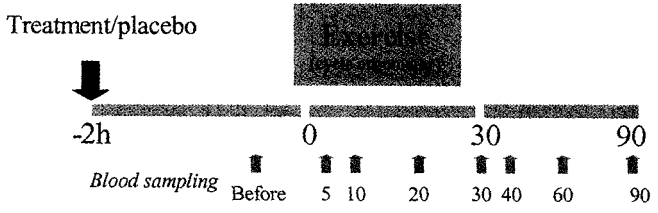

Figure 1. Exercise and blood sampling plan for 7 human male subjects pre-administered with placebo, sesamin or vitamin E.

Animal study

At rest

24 ddY mice were randomly divided into sesamin, vitamin E or control groups. 100mg/kg of sesamin (S100), and 100mg/kg of vitamin E (E100) was administered to the respective group via an orogastric tube while the control received the vehicle (1% carboxymethyl cellulose sodium salt [CMC]). Two hours following feeding, the animals were sacrificed for blood collection and liver excision.

The livers were washed in cold saline, homogenized in 40mM Tris-HCL (pH 7.4) containing 145 mM KCl, 4 mM EDTA and 5 mM dithiothreitol. Samples were then centrifuged at 8500g for 10 minutes to remove cell debris and nuclei and the supernatant retained for enzyme activity assay and protein determination.

Heparinized blood was obtained from the heart, and centrifuged to obtain plasma, which was then stored at -80°C for lipid peroxide (LPO) analysis [14].

During exercise

32 ddY mice were subjected to swimming exercise in an aquarium tank (35°C) 2 hrs after orogastric feeding of 10 mg/kg sesamin (S10), 100 mg/kg sesamin (S100), 100 mg/kg vitamin E (E100) dissolved in 1% CMC. Control group received only CMC solution. Following 30 mins of continuous swimming, the animals were sacrificed by cervical dislocation and heparinized blood samples collected from the heart.

Enzyme assay

Total GPX activity was determined by a modified Paglia and Valentine [15] coupled assay procedure, using tert-butyl hydroperoxide substrate. Se-GPX activity was measured with the same procedure, using an H_2O_2 substrate. GST activity against Chloro-2,4-dinitrobenzene (CDNB) was also determined as described by Habig and Jakoby [16]. BCA assay was used to determine protein content of the enzyme source [17].

Measurement of LPO

Lipid hydroperoxide values were measured using the Hb-MB method, which detects hydroperoxide form of lipid peroxidation [18, 19].

Statistical analysis

Statistical analysis was performed using the SPSS version 10.0 Package. Two-way analysis of variance was performed followed by Tukey's multiple comparison test. Data is presented as mean ± SEM.

RESULTS

Human Study

In the human subjects, 25 mins strenuous exercise was accompanied with progressive increase in Plasma LPO reaching statistically significant levels at 10 and 20 mins (Figure 2). The LPO levels returned to a level comparable to that before exercise, at 30 mins.

As shown in Figure 2, intake of vitamin E (200 mg) and sesamin at 36 mg, 2 hrs before exercise commencement, attenuated the increase in LPO levels in exercised subjects. Most notable was the preventative effect of sesamin, which completely prevented the increase in LPO, and there was a statistically significant difference to the LPO levels observed in the control group.

Animal Studies

Enzyme activity

As shown in Figure 3, enzyme activities of the control group values (Con) were normalized to 100% to account for the initial differences between the 3 groups. At rest, the sesamin fed group (100 mg/kg) exhibited significant increases in Total Glutathione Peroxidase (GPX) and Glutathione-S-Transferase activity compared to the untreated control group. vitamin E at 100 mg/kg (E100) showed no significant improvement of either enzyme activity.

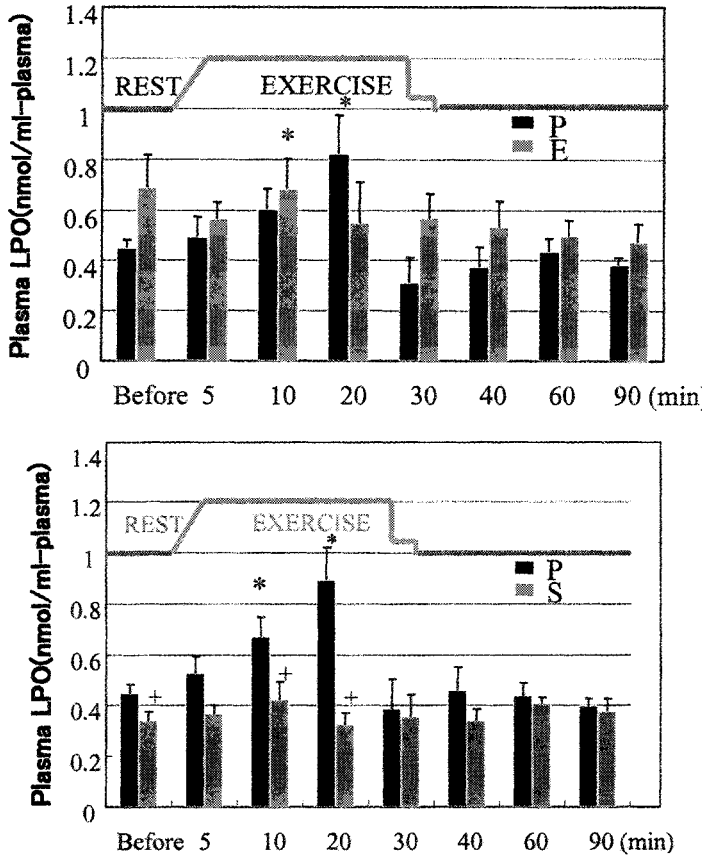

Figure 2. Plasma LPO levels in 7 human subjects who consumed 36 mg sesamin (S), 200 mg vitamin E (E) or placebo (P), 2 hrs prior to exercise at 80-90% HRmax for 25 mins. $^{*}p < 0.05$ vs Before; $^{+}$ $p < 0.05$ vs Placebo.

Plasma LPO

Plasma LPO levels were not found to be altered by sesamin at 10, 100 mg/kg nor vitamin E at 100 mg/ kg when animals were fed at rest (Figure 4). Following exercise, there was a dramatic and significant increase in the LPO levels of the control mice and also those fed vitamin E. Sesamin feeding at 10 mg/kg and 100 mg/kg prevented the increase in LPO levels; 100 mg/kg also significantly reduced the LPO levels following exercise compared to the control group (Figure 4). Vitamin E did little to attenuate this increase suggesting that sesamin at the equivalent dose could have a more potent anti-oxidant effect than that of vitamin E on exercise-induced lipid peroxidation.

DISCUSSION

In humans performing strenuous exercise, pre-treatment with sesamin at 36mg or vitamin E at 100 mg/kg, 2 hrs before exercise commencement, significantly suppressed the rise in plasma LPO levels which was observed in untreated subjects.

Animal studies supported this finding, with sesamin feeding inducing a significant increase in the liver activity of total Glutathione Peroxidase and Glutathione-S-Transferase enzymes in mice at rest. Following 30 mins of exercise, the plasma levels of LPO were significantly increased in the mice, and Sesamin significantly reduced the LPO increase when administered at 100 mg/kg, 2 hrs before exercise commencement. Both sesamin at 10 and 100 mg/kg suppressed the exercise induced increase in LPO levels, whereas vitamin E at 100 mg/kg had no suppressive effect.

The reduction in lipid peroxide levels suggests that sesamin may exert anti-oxidative or free radical scavenging activity, against exercise-induced lipid peroxidation. A prerequisite for the anti-oxidative activity of sesamin is oxidative metabolism to its catechol forms, which have previously been demonstrated to exert antioxidant and free radical scavenging activities both *in vivo* and *in vitro* [7].

In addition, the investigation of liver enzyme activity following oral administration of sesamin, revealed that sesamin can also increase the levels of the Glutathione-S-Transferase and total Glutathione Peroxidase, suggesting a role in the induction of enzymes associated with detoxification and inhibition of lipid peroxidation. In this study, sesamin demonstrated a strong protective effect against exercise-induced lipid peroxidation and the mechanisms can be explained by the anti-oxidative activity of its metabolite and also through enzyme induction of endogenous scavengers and anti-oxidants such as Glutathione-S-Transferase and Glutathione Peroxidase [20]. In our study, there was a significant increase in the activity of GST following administration of 100mg sesamin prior to exercise. Whilst exercise itself appears to induce GST, phenolic groups, which are present in the catechol metabolite of sesamin, have also been implicated in induction of phase II detoxifying enzymes including GST [20-22].

It was interesting that despite the lack of effect of vitamin E on exercised mice LPO levels, the inhibitory effect of vitamin E in Humans was observed and significant. The vitamin E administered in humans was 200 mg, which is comparatively lower when considering the greater weight of the human subjects. In addition, sesamin exerted a greater anti-LPO effect in the human subjects, despite its lower overall dose suggesting a greater potential potency that vitamin E in inhibition of *in vivo* lipid peroxidation.

In conclusion, both animal and human experiments demonstrated that sesamin could scavenge free radicals and enhance LPO metabolism resulting in strong protective effect against exercise-induced lipid peroxidation.

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Anticancer and Immunomodulatory Potential of DRF-3188, an Analogue of Andrographolide

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INTRODUCTION

Plants have been a source of medicinal substances for thousands of years, and provided many effective anticancer agents in current use, such as vinblastine, vincristine, etoposide, teniposide, and paclitaxel [1]. The contribution of plant derived compounds, as a source of active antineoplastic agents and models for the design, synthesis and semisynthesis of novel substances for the treatment of cancer is well documented. Diterpenes from many species are known for their biological activity and are amongst the most widely distributed terpenes in the plant kingdom [2]. Andrographis paniculata of acanthaceae family, a known source for diterpene lactones has been used for more than two centuries to treat a variety of disorders [3-7]. Andrographolide, a labdane diterpene, from andrographis paniculata extracts has been identified as a cytotoxic and immunostimulatory compound [8,9]. Earlier, we demonstrated the *in vitro* and *in vivo* anticancer potential of andrographolide against human tumor cells and immunostimulatory activity on human peripheral blood lymphocytes (HPBLs) [10]. Our efforts to develop a potent, novel patentable anticancer molecules based on andrographolide scaffold resulted in the synthesis and identification of DRF-3188 as a novel anticancer immunomodulatory compound. In this paper we report the anticancer and immunomodulatory activity of DRF-3188 and the possible mechanism of action.

MATERIALS AND METHODS

Cell lines and animals

Human cancer cell lines used in this study were either purchased from the American Type Culture Collection, Manassas, VA or from the National Cancer Institute, Bethesda, US. All cells were maintained in RPMI 1640 supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml) and 10% FCS at 37°C in a humidified, 5% CO_2 atmosphere. Three- to 4-week-old female athymic mice (CD-1 *nu/nu*) were used for human tumor xenografts. Mice were housed in specific pathogen-free conditions, and all studies were carried out with approved institutional experimental animal care and use protocols.

Cell growth assay

Cell proliferation was evaluated by Sulphorhodamine B (SRB) assay where the amount of dye bound to the cells after staining gives a measure of cell growth. Briefly, cells were seeded on a 96-well cell culture plates (Nunc, Denmark) at a concentration of 10,000 cells per well and incubated at 37°C in CO_2 incubator. Twenty-four hours later cells were treated with different concentrations of DRF-3188 dissolved in DMSO to a final concentration of 0.05% in the culture medium and exposed for 48 hrs. Cells were fixed by adding ice-cold 50% trichloroacetic acid (TCA) and incubating for 1 hr at 4°C. The plates were washed with distilled water, air dried and stained with SRB solution (0.4% wt/vol in 1% acetic acid) for 10 min at room temperature. Unbound SRB was removed by washing thoroughly with 1% acetic acid and the plates were air-dried. The bound SRB stain was solubilized with 10mM Tris buffer, and the optical densities were read on a spectrophotometric plate reader at a single wavelength of 515 nm. From the optical densities percentage growths were calculated (JNCI, vol 83, No.11, June 5, 1991).

Cell cycle analysis by flow cytometry and Western blot analysis

MCF-7 cells were treated with DRF-3188 (5mM) or DMSO for 24 h. Cells were harvested by trypsinization and permeabilized with ice-cold 70% ethanol for at least 1 hr. After washing with PBS,

cells were treated with 100 mg/ml RNase A (DNase free) at 37°C. After 30 min, cells were washed with PBS and stained with 50 μg/ml propidium iodide for 30 min. DNA contents were analyzed by FACScan (Becton Dickinson). The effect of DRF-3188 on cell cycle was studied in MCF-7 cells by Western blot analysis. Cells were treated with DRF-3188 (5 μM) for 24 hrs, washed with cold PBS (pH7.4) and lysed using ice cold Lysis buffer (0.15 mM NaCl/0.05 mM Tris–HCl, pH 7.3, 1% Triton X-100, 1% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 mM PMSF). The clarified cell lysate was either used immediately or stored at –70°C. Protein concentrations were estimated using Bio-Rad Protein Assay Reagent following the manufacturer's suggested procedure. Twenty micrograms of protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane, blocked overnight in TBS with 5% skim milk at 4°C, reacted with primary polyclonal antibody against human CDK4. CDK1, Cyclin B1, p27 Kip1 and actin and washed. After reaction with horseradish peroxidase-conjugated antigoat IgG or antimouse IgG, immune complexes were visualized by using the ECL detection reagents following the manufacturer's suggested procedure.

Isolation of HPBLs

Venous blood was collected asceptically from healthy donors in a preservative free heparinized tubes. The blood was diluted 50% with phosphate buffered saline (PBS), pH 7.4 and layered onto Ficoll Plus (Amersham). After centrifugation at 1500 rpm for 25 minutes the mononuclear cells were collected at the interface and washed thrice with the PBS. The cells were resuspended in RPMI 1640 medium with 10% Foetal Bovine Serum (FBS) and antibiotics.

HPBLs proliferation and cytokine secretion

1×10^5 cells per well in a 96-well plate were stimulated with 5 mg/ml of phytohaemagglutinin-A (PHA). After 24 hrs of incubation at 37°C in humidified CO_2 incubator different dilutions of test compounds were added to respective wells. Control wells received only medium containing vehicle (DMSO). The cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well), 24 hrs prior to termination. 48 hrs after

drug addition, the cells were harvested onto unifilter plates (Packard) and incorporated ^{3}H-thymidine was counted using Topcount (Packard). The proliferation of HPBLs was expressed as percentage stimulation index (SI) in DRF-3188-treated cells compared to untreated controls.

The peripheral blood lymphocytes were treated with DRF-3188 as described in proliferation assay. At the end of the assay, culture supernatants were collected and stored at –70°C until use. Cytokine levels were estimated in the stored supernatants using commercially available DUOSET ELISA kits from R & D Systems.

Immunophenotyping

The effect of DRF-3188 on specific subsets of HPBLs was studied *in vitro* by using flow cytometry analysis. HPBLs were incubated with and without DRF-3188 at 1μM concentration for 24 hrs. The cells were washed thrice with PBS and resuspended in 500 μl of PBS containing 1% FBS. 1:1000 dilution of each of the primary antibodies for CD3, CD4, CD8, CD56 and isotype were added to the compound stimulated cells and incubated on ice for 2 hrs. Cells were washed twice with PBS containing serum and 1:1000 dilution of FITC tagged secondary antibody was added to the cells and incubated on ice for 2 hrs. Cells were washed twice and samples were run on a FACS machine to determine the increase or decrease in the CD markers.

In vitro lymphocyte-mediated cytotoxicity

K562 cells (2×10^{6}/0.1 ml) in RPMI 1640 medium with 10% FCS were pre-incubated with 100 μCi of radioactive sodium chromate for 90 mins at 37°C. These cells were washed several times with complete medium to remove unincorporated radioactive sodium chromate and used as target cells. Human peripheral blood lymphocytes were incubated with 0.1 μM DRF-3188 for 24 hrs, washed twice with PBS and used as effector cells. The target and effector cells were mixed in defined ratios (100:1 and 50:1). After 4 hrs of incubation the plates were centrifuged and the 100 μl of culture supernatant was counted in a Gamma counter. The spontaneous release and the total release were determined in respective wells. The total

release was counted by lysing the cells with 1% Triton X-100. The results were expressed as percentage specific lysis determined as: % Specific lysis = [(Experimental release – Spontaneous release)/ (Total release – Spontaneous release)] × 100.

In vivo antitumoural assay

HT-29 human tumour xenografts and B16F0 mouse tumours were initiated by implantation of tumour fragments (~60 mm^3) from established tumours in athymic nude mice and C57BL/6 mice respectively. Tumour fragments were implanted subcutaneously in the right flank. The tumours were measured with calipers, and mice were weighed every alternate day. When tumours reached a volume of ~100 mm^3, mice were randomized to a control and treated group. DRF-3188 was administered orally once daily. Tumour size was measured and the volumes were calculated using the equation, $V = (D \times d^2)/2$, where *V (mg)* is tumour volume, *D* is the longest diameter in mm, and *d* is the shortest diameter in mm.

RESULTS AND DISCUSSION

Andrographolide, a diterpene lactone isolated from *Andrographis paniculata,* showed significant *in vitro* and *in vivo* anticancer activity against different types of human cancer cells and immunostimulatory activity in human peripheral blood lymphocytes [8-11]. A series of andrographolide derivatives were designed by our oncology group to develop analogues with higher potency and improved antitumour activity. One of the most potent analogues, DRF-3188, was selected on the basis of improved *in vivo* anticancer and immunostimulatory properties. DRF-3188 significantly suppressed the proliferation of cancer cells after 48 hrs of exposure (Figure 1), compared to control cells with the GI_{50} (Concentration required to inhibit the cell growth by 50%) values ranging from 4 μM to 9 μM. With DRF-3188 treatment, all cell lines studied demonstrated a dose-dependent inhibition of proliferation irrespective of their genotype and phenotypes, like EGFR, MDR over-expression and p53 status, which are indicators of poor clinical outcome.

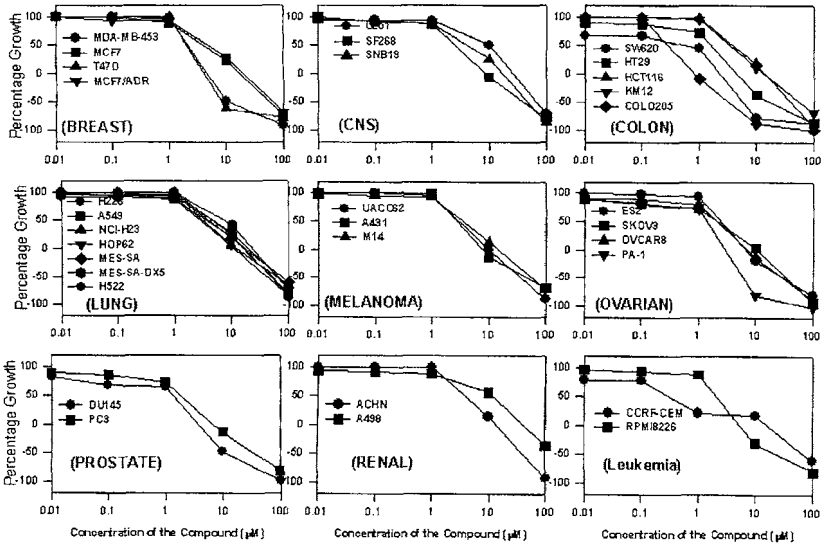

Figure 1. DRF-3188 inhibits growth of human cancer cells.
Human cancer cell lines representing different types of cancers were incubated in complete medium with and without DRF-3188 for 48 hrs and the percentage growth determined by SRB method as described under materials and methods. Percent growth of the treated cells was calculated compared to the control untreated cells. Each point is an average of two data points of an experiment.

To examine the likely mechanism(s) that might account for the effects of DRF-3188 in cancer cells, we investigated its effects on cell cycle distribution in MCF-7 (breast cancer) cells. A marked accumulation of MCF-7 cells in the G_0/G_1 phase of the cell cycle occurred with a concomitant decrease of cells in the G_2/M and S phase upon treatment with 5 mM of DRF-3188 (Figure 2A and 2B). The sub-G_1 peak which is considered as a hall mark of apoptosis increased to 14% above control after 48 hrs of incubation with DRF-3188. These findings suggest that DRF-3188 has a marked effect on MCF-7 cells proliferation due to cell cycle arrest and induction of apoptosis. Cell cycle progression is governed by cyclin-dependent kinases (CDKs) that are activated by cyclin binding and inhibited by CDK inhibitors. Our investigation for the changes in regulatory proteins in G_1 Phase of cell cycle showed the induction of cyclin-dependent kinase inhibitor $p27^{kip1}$ and inhibition of CDK4 protein expression without alteration in the levels of CDK2 and Cylin B1 protein levels

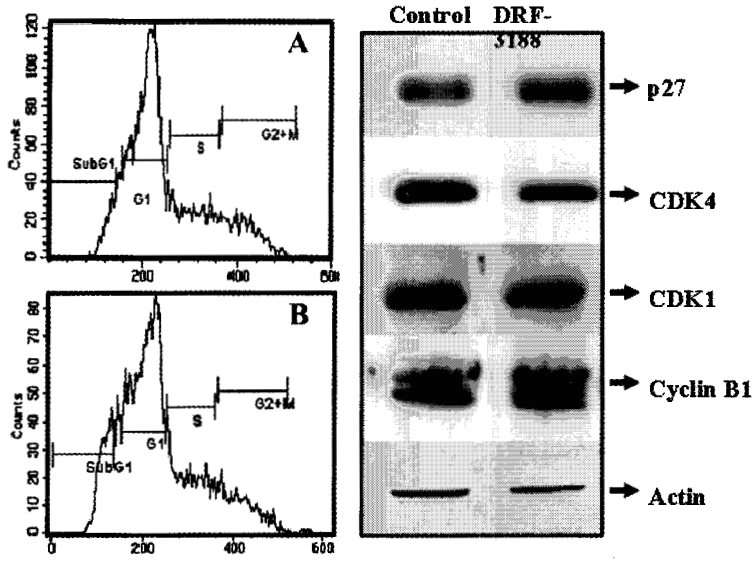

Figure 2. DRF-3188 induces a G0 – G1 cell cycle arrest.
MCF7 cells treated with DMSO or DRF-3188 at 5 mM for 24 hrs were stained with propidium iodide as described in material and methods and analysed by flow cytometry. 2A) DMSO treated control MCF7 cells. 2B) DRF-3188 treated MCF7 cells and 2C) western blot analysis of lysates from DRF-3188 treated MCF7 cells with p27, CDK4, CDK1, Cyclin B1 and actin antibodies.

(Figure 2C). CDK4 plays an important role in the G/S progression of the cell cycle by forming a complex with Cyclin D1 and this process is inhibited by p27. Many anticancer compounds that inhibit, the expression/ activity of CDK4 or formation of CDK4/Cyclin D1 complex through over-expression of CDKIs like p27 are reported [12-15].

Previously, *Andrographis paniculata* extract has been reported to stimulate both humoral and cell mediated immunity in mice [9] and increase in CDK4 lymphocyte count in HIV patients [16]. We also reported the *in vitro* immunostimulatory activity of andrographolide in human peripheral blood lymphocytes [10]. Because DRF-3188 is an analog synthesized from a scaffold of andrographolide, we investigated the *in vitro* immunomodulatory activity of DRF-3188 in human peripheral blood lymphocytes (HPBLs). DRF-3188 promoted the production of Th1 cytokines (Figure 3A and 3B) IL-2

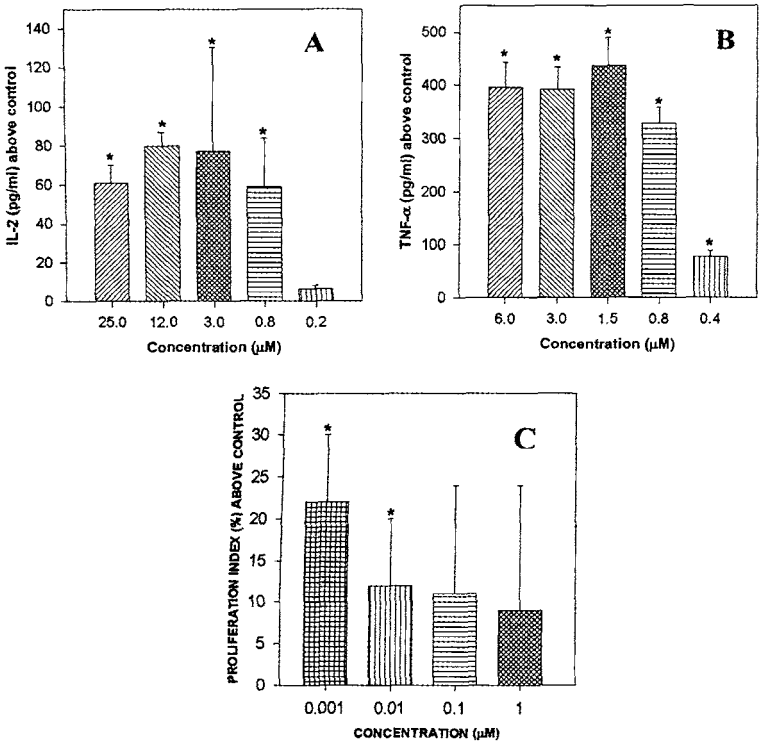

Figure 3. Proliferation and cytokine induction by DRF-3188 in human peripheral blood lymphocytes.
PHA stimulated HPBLs were treated with different concentrations of DRF-3188 for 48 h and assayed for cellular proliferation and cytokine levels as described in materials and methods. A) IL-2 levels and B) TNF-α levels and C) Proliferation by ^{3}H-thymidine incorporation. Each bar represents the mean ± SD of three experiments performed in triplicates. * $p < 0.01$ with respect to control (by Dunnett's test).

and TNF-α in PHA-stimulated HPBLs compared to untreated controls. It is known that IL-2 and TNF-α activate differentiation of naïve T-cells into effectors and enhance the cytotoxic activity of Natural Killer cells, CD8+T cells and lymphokine-activated killer cells [17, 18]. Also the pro-inflammatory cytokine, TNF-a, is known to kill the cancer cells directly [19]. Consistent with these observations, after DRF-3188 treatment, we observed the increased proliferation of HPBLs as shown in (Figure 3C), increased expression of CD3, CD4, CD8 and CD56 markers on lymphocytes (Figure 4A) and

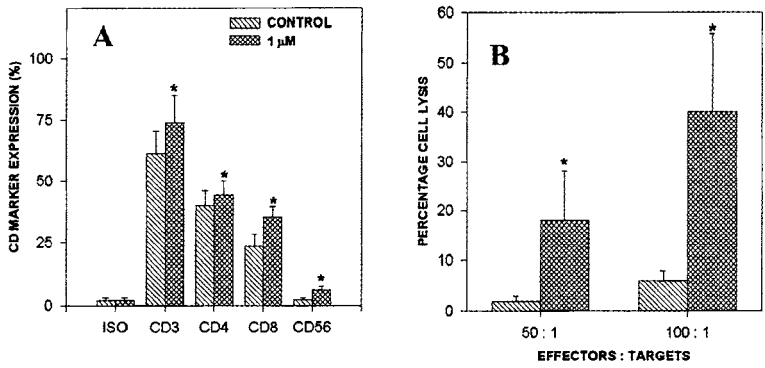

Figure 4. DRF-3188 enhances lymphocyte-mediated cytotoxicity.
Non-adherent lymphocytes isolated from human peripheral blood were incubated with DRF-3188 for 24 hrs. The lymphocytes were washed and incubated at different cell densities with K562 cells labeled with radioactive chromium^{51}. After 5 hrs, Chromium^{51} in the culture supernatants was estimated and percent cytotoxicity was determined as mentioned in materials and methods. Each bar represents the mean ± SD of three experiments performed in triplicates. * $p < 0.01$ with respect to control (by student *t*-test).

augmentation of cytolytic activity of lymphocytes against K562 cancer cells (Figure 4B). The cytokine induction and activation of immune cells by DRF-3188 is important considering the fact that patients with advanced cancer often exhibit a poorly functioning immune system resulting in poor survival. Agents that activate host immune system in diseased/immunosuppressive state can provide supportive or alternative therapy to conventional therapy [22-25]. Our results indicate that DRF-3188 can activate the immune cells and may be useful for direct and supportive chemotherapy of cancer. DRF-3188 inhibited the growth rate of B16 melanoma tumors in immunocompetent C57BL/6 mice (Figure 5A) and HT-29 tumors in nude mice (Figure 5B) without body weight loss and clinical symptoms of toxicity (data not shown). Additional *in vivo* studies are in progress in animal models to substantiate the immunomodulatory activitiy of DRF-3188 that was observed *in vitro* in HPBLs.

In conclusion, our studies demonstrate that by modulating cell cycle proteins and inducing apoptosis, DRF-3188 blocks cancer cell growth. The dual activity (anticancer and immunostimulatory) of DRF-3188 makes it an interesting molecule to develop as a novel anticancer

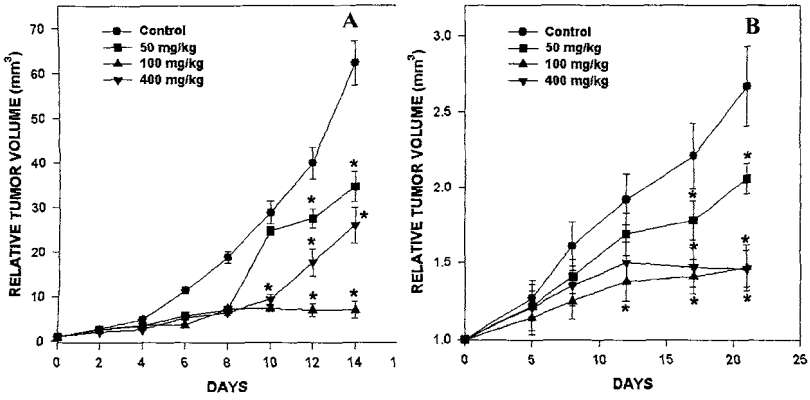

Figure 5. Dose-response effects of DRF-3188 on relative tumour volume. DRF-3188 treatment increases the expression of CD markers on HPBLs.
HPBLs from healthy donors were incubated for 24 hrs in the presence or absence of DRF-3188. Cells were fixed and stained with CD3, CD4, CD8, CD56 antibodies and analysed by flow cytometry. Each bar represents the mean ± SD of three experiments performed in triplicates. * $p < 0.05$ with respect to control (by student *t*-test).

agent. The improved *in vivo* efficacy of DRF-3188 over andrographolide substantiates the importance of natural product-derived pharmacophores as leads to develop or design potent novel anticancer agents.

ACKNOWLEDGEMENT

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Immunomodulating Property of *Astragalus membranaceus* and *Curcuma aromatica*

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INTRODUCTION

In Singapore, Traditional Chinese Medicine (TCM) is the most popular type of alternative medicine. Most of the patients seek TCM treatment because they believe it has less side effects and toxicity, and can improve their immune system. In the cases we studied, herbal TCM is used to complement conventional treatment (surgery, radio-, chemo- and hormonal therapy) in breast cancer patients. We sought a scientific evaluation of the immunomodulatory effects of some Chinese herbs used widely in breast cancer patients at 2 TCM Clinics that we surveyed. The herbs were identified through a quality-of- life (QOL) analysis [1] using the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QLQ-C30 version 3.0) and the supplementary breast cancer module (QLQ-BR23). We compared the herbal prescriptions of the patients who had better scores on the symptoms scale (i.e. less fatigue, pain, dyspnoea, insomnia and appetite loss) and selected the two most frequently used herbs, *Astragalus membranaceus* (AM) and *Curcuma aromatica* (CA). *Astragalus membranaceus* (AM) in TCM is a tonifying herb that has been used to correct the deficiency of *qi*. In Western medicine it has been reported to strengthen the natural defence mechanism by enhancing antibody production through increased T helper cell activity [2-6]. *Curcuma aromatica* (CA) in TCM is a mass-reducing herb which has been used to remove the stagnation of

qi, reduce inflammation and pain. In Western Medicine it has been reported to stimulate active immunity and to enhance fibrinolysis [2, 7].

MATERIALS AND METHODS

Preparation of extract

The dried forms of AM root and CA tuber were purchased from Teo Acupuncture & Medical Hall, where the breast cancer patients get their prescription. The herbs were crushed into small pieces with an electronic microniser. As a first step in the fractionation process, we chose the ethanolic extract of these herbs. 170 g of AM and 180 g of CA were extracted with 80% ethanol at room temperature until exhaustion. The preparation was filtered with Whatman filter paper GF/A, concentrated under reduced pressure and freeze dried.

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate buffer saline (PBS) solution, concanavalin (Con A) and lipopolysaccharide (LPS) were all purchased from Sigma, U.S.A.; dodecyl-sulphate sodium salt (SDS) was supplied by Merck, Germany.

Animals and cell culture medium

Inbred male/female BALB/c mice, 5 to 6 weeks old, were obtained from the Animal Holding Unit, National University of Singapore. RPMI 1640 medium (Sigma,U.S.A.) were supplemented with 10% heat-inactivated fetal bovine serum (FBS) [Hyclone, UK].

Lymphocyte-activation assay

Fresh splenocytes were prepared aseptically into single cell suspension [8, 9]. These were suspended at 1.6×10^6 cells/ml in medium containing RPMI 1640 supplemented with 10% heat-inactivated FBS. A total of 90 µl of the cells were added to each well of a flat-bottom 96-well plate using a multi-channel pipette. Studies were carried out to evaluate the effects of 10.0, 1.0 and 0.1 µg/ml AM and CA extracts on the proliferation of mouse lymphocytes under

various conditions, in the absence or presence of 10 l of (a) suboptimal doses of Con A (0.3 μg/ml) or LPS (1.0 μg/ml) and (b) optimal doses of Con A (3.0 μg/ml) or LPS (6.0 μg/ml). The spleen-cell suspensions were incubated for 48-72 hrs at 37°C in 5% humidified CO_2 incubator with 10 μl serially diluted extracts, starting with highest concentration of 10.0 μg/ml for each extract in quadruplicate wells. Experiments were repeated in four replicates for extracts showing stimulation of the splenocytes.

Evaluation of cell activation was carried out as previously [10, 11], using the MTT tetrazolium assay: after 48-72 hrs incubation at 37°C in a 5% humidified CO_2 incubator, 10 μl/well MTT (stock solution 5 mg/ml PBS) was added [9]. The plates were again incubated for 4 hrs after which 100 μl/well 10% SDS in 0.01N HCl was added to dissolve the formazan crystals. After an overnight incubation, the plates were read in a microplate reader (ELX 800, Bio-Tek Instruments, U.S.A.) at 570 nm. Wells with medium, MTT and SDS but without cells were used as blanks.

Statistical analysis

The significance of the differences between control and treated values was analysed using Student's t-test. Differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

	AM			CA		
μg/ml	0.1	1.0	10.0	0.1	1.0	10.0
No mitogen	+	+				+
ConA suboptimal	+		+			
ConA optimal	-	-	-	+	+	+
LPS suboptimal						
LPS optimal						

Table 1. Effect of different concentrations of 80% ethanol extract of *Astragalus membranaceus* and *Curcuma aromatica* on mice lymphocyte proliferation. (+): stimulation, (-): inhibition (both p value < 0.05); empty square: no statistically significant change.

Table 1 shows that in the absence of mitogens, both 0.1 μg/ml and 1.0 μg/ml of AM with 10.0 μg/ml of CA stimulated splenocyte proliferation. Neither extract caused significant splenocyte proliferation in the presence of B cell mitogen, LPS. In the presence of sub-optimal dose of Con A, AM at 0.1 μg/ml and 10.0 μg/ml significantly potentiated splenocyte activity while AM depressed splenocyte activity in the presence of optimal dose of Con A. CA at 0.1 μg/ml, 1.0 μg/ml and 10.0 g/ml significantly potentiated splenocyte activity in the presence of optimal dose of Con A.

CONCLUSION

The results indicate that both AM and CA have immunostimulatory effect. AM acts best in the presence of sub6-optimal dose of Con A, while CA caused significant splenocyte proliferation in the presence of optimal dose of Con A. The responder cells are probably T-cells, as there was increased splenocyte proliferation with the T-cell mitogen, Con A, but not with the B-cell mitogen, LPS. The suppression of splenocytes in the presence of optimal doses of Con A with 1.0 and 10.0 μg/ml doses of AM might be due to the toxic effect of this extract. It appears that AM has a stronger immunomodulating property than CA as it potentiated splenocyte activity at a lower dose than CA.

The results of these experiments indicate that AM and CA may have a role not only in palliative care of cancer patients, but also in enhancing the effects of conventional oncology therapy. The stimulating activity on human lymphocytes should also be investigated. We are conducting *in-vivo* studies in mice to further evaluate the immunomodulating property of AM.

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Induction of Breast Tumours in Rats for Assaying Natural Products for Anti-Tumour Properties

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INTRODUCTION

Mortality due to breast cancer is highest in countries of Northern and Western Europe and North America, lowest in countries of South-East Asia, Africa and Latin America [1]. It is of great importance to investigate synthetic or natural compounds that may have a prophylactic or therapeutic effect on breast cancer. Such studies may be carried out using an animal model or a breast cancer cell line. The use of an animal model is advantageous since toxic effects of tested compounds can be observed in a biological environment similar to humans and therefore results can be extrapolated to human subjects.

In this study, rats were used as the animal model. The strain of the rat is critical since this will influence the susceptibility to tumour induction [2,3]. The rat colony in our laboratory, the Mona strain, is a cross between the Wistar (low high susceptibility) and the Sprague-Dawley (high susceptibility). It is thus of interest to observe the Mona strain's susceptibility to tumour induction.

Breast tumours can be induced in rats by chemical carcinogens which are aromatic hydrocarbons [4]. The carcinogen, 7, 12-Dimethylbenz-(a)anthracene (DMBA), was used in this study due to its potency when administered only once, according the method of Huggins et al [5]. DMBA acts by damaging the DNA of the mammary cell, being first oxidized into an epoxy intermediate which binds to DNA [6, 7].

MATERIALS AND METHODS

Chemicals

Sesame oil and DMBA were purchased from Sigma Company, Mo, USA.

Animals

Male and female rats were obtained from the Basic Medical Sciences, Mona, Animal House and were put to mate. At 5 weeks, the litters were weaned and separated. Female rats of the litter were used.

Tumour induction

Seven virgin female Mona strain rats aged 50–55 days old were given, intragastrically, 133 mg DMBA (in 1 ml of sesame oil) per kg body weight. DMBA was dissolved in sesame oil by heating to 100°C. Another seven virgin female Mona strain rats of the same age were given only 1 ml sesame oil and served as control animals.

Data collection

Mammary tissue of female Mona strain rats were palpated three times per week. Tumour sizes were measured using a vernier caliper. Animals were sacrificed by ether euthanasia and tumour tissues were examined histologically.

RESULTS AND DISCUSSION

The effect of DMBA on the mammary glands of female Mona strain rats showed an 86% success rate for the induction of breast tumours (Figure 1). This was clearly shown in Table 1 — from a total of 7 treated animals, 6 developed breast tumours, while only 1 of 7 control animals developed breast tumour.

The mean latency period (Table 1) for tumour development in the DMBA-treated group was 203 days, compared to 356 days in the control group.

Tumour size increased by almost two-fold over one to four months (Figure 2), with no sign of regression. Histological analysis of

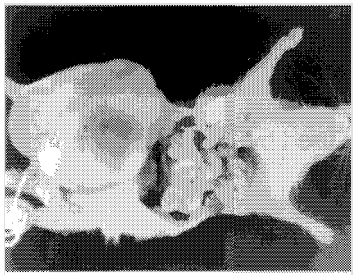
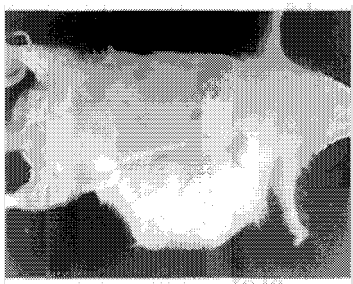
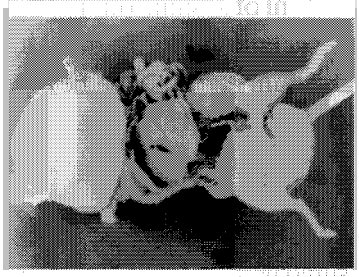


Figure 1. Gross appearance of breast tumours

(a) Abdomino-inguinal tumour



(b) Thoracic tumour



(c) Cervical, abdominal and inguinal tumours

Table 1. Effect of DMBA on Mammary Gland

Group	Number of rats	Number of rats with tumor	Mean time of onset of palpable tumor (days)
DMBA	7	6	203
Control	7	1	356

these tumors showed a proliferation in the mammary epithelial parenchyma and stromal connective tissue (Figure 3). This is indicative of fibroadenoma, a type of benign tumour [3].

CONCLUSION

This study reports the use of an animal model for breast tumour research. This is a critical finding since the use of tissue culture for breast cancer studies is costly.

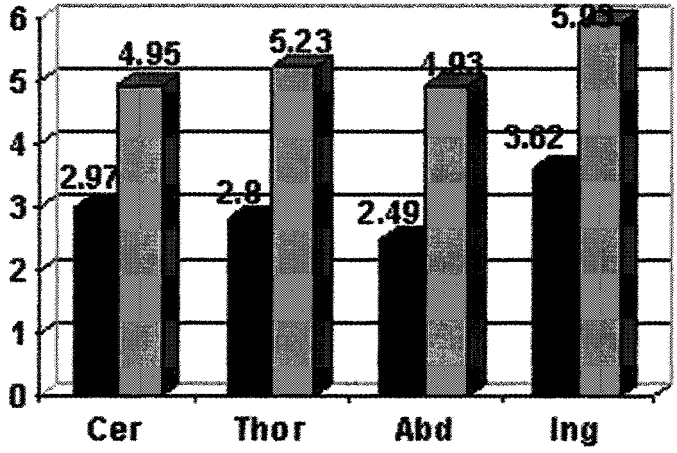


Figure 2. Size of mammary tumours of different regions at 1 month (black) and at 4 months (gray). Cer(Cervical), thor(thoracic), abd(abdominal), and ing(inguinal).

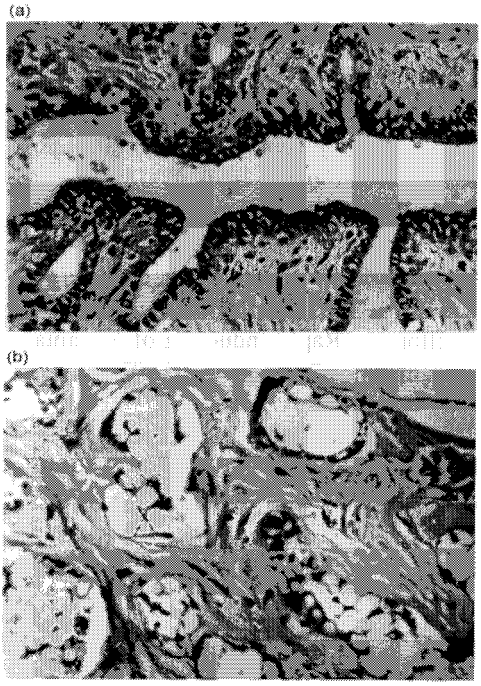


Figure 3. Histology of

(a) Normal Mammary Gland

(b) Mammary Gland Tumour

Some studies have identified garlic with its organosulphur compounds which act as prophylactic agents for breast cancer [8, 9]. Plants with such compounds are presently under investigation in our laboratory, using our rats as the animal model.

ACKNOWLEDGMENT

I gratefully acknowledge the financial support from The School of Graduate Studies and Research, U.W.I., Mona, Professor Tik The for his supervision and advice, Dr. Cyril Fletcher and Mr. Michael Gardner for their advice and support, Professor Barrie Hanchard for analyzing the tumours and Mr. Ray Lawson for taking care of the animals.

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Cancer Diagnosis Using the Novel Photosensitizer, Hypericin

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INTRODUCTION

Hypericin is a potent photosensitizer (a hydroxylated phenantroperylenequinone) derived from the plant *Hypericum perforatum*. It is a new drug which in clinical trials and studies is showing great promise as an effective treatment for depression, PMS and menopausal symptoms, wounds and gastrointestinal ailments. However, it is showing most promise as an anti-cancer agent and to date has been used in the effective treatment of many different types of cancers. Hypericin has been found to be safe for clinical diagnostics without adverse side effects and also showed no genotoxic and cytotoxic results either *in vitro* or *in vivo* [1-3].

In this study we evaluated the use of hypericin as a selective marker for bladder cancer. Bladder cancer is a prevalent disease being the 4th most common form of malignancy worldwide and accounting for 4% of all cancer cases [4, 5]. In Singapore, it is the 9th most common form of cancer [6]. Mortality rates are very high due to invasive bladder cancer. Early detection and treatment are therefore mandatory in order to reduce mortality rates [7, 8].

While the non-invasive papillary tumours are clearly visible by white light cystoscopy, flat lesions such as carcinoma in situ (CIS) are rarely detectable by this method, sonography or other radiological methods [9]. Regular white light cystoscopies with cold cup biopsies are the current methods of surveillance of patients who are at high risk of recurrent bladder cancer. However, these methods are insufficient to detect all urothelial neoplasia. Therefore methods in detecting flat urothelial neoplasia with the use of specific fluorescent

dyes are constantly being sought after. In recent years with the development of photodynamic diagnosis and therapy, many photosensitizers are being tested for use as fluorescent markers. We have previously reported on one such photosensitizer precursor, 5-Aminolevulinic Acid (ALA). We found an increased sensitivity when compared to that of conventional cystoscopy (89.1% vs 65.6%) and concluded that fluorescence guided biopsies are more sensitive than random biopsies in the detection of dysplasia and bladder cancer without additional risk of complication [10]. With current fluorescence diagnostic techniques, the sensitivity to detect urothelial neoplasia especially carcinoma in situ is much higher than with the naked eye, but valid interpretation and reliable diagnosis are difficult due to the high amount of false positive results which demonstrates the relative lack of specificity of these techniques [9, 11].

The present study explores tissue specific macroscopic and microscopic fluorescence characteristics of hypericin induced fluorescence in normal human bladder tissue and in bladder malignancy in vivo with special reference to laser confocal fluorescence microscopy and image analysis as a diagnostic aid to fluorescence cystoscopy.

MATERIALS AND METHODS

Clinical studies

30 patients suspected of primary or recurrent cancer were selected for this study. Hypericin was prepared as described in Olivo et al. [13] and instilled into the bladder of the patient. It was then allowed to incubate for 2 hours, after which fluorescence cystoscopy and routine white light cystoscopy were performed. Biopsies of suspicious and normal regions were obtained. The biopsies were frozen immediately and later sections were cut and viewed under a confocal laser scanning microscope. A sensitive color CCD video camera, connected to the endoscopy system, was used to capture the macroscopic fluorescence images of the bladder.

RESULTS AND DISCUSSION

Clinical results

No local or systemic side effects were noted in the 30 patients given hypericin in this pilot study. White light cystoscopy was used to identify non-malignant and also malignant regions of the bladder in situ. Macroscopic fluorescence imaging was also performed on these regions. Macroscopic fluorescence imaging showed that normal bladder regions showed no red fluorescence, however inflammation in the bladder showed mild red fluorescence (Figures 1 & 2). The transitional cell carcinoma (TCC) and CIS lesions showed bright red

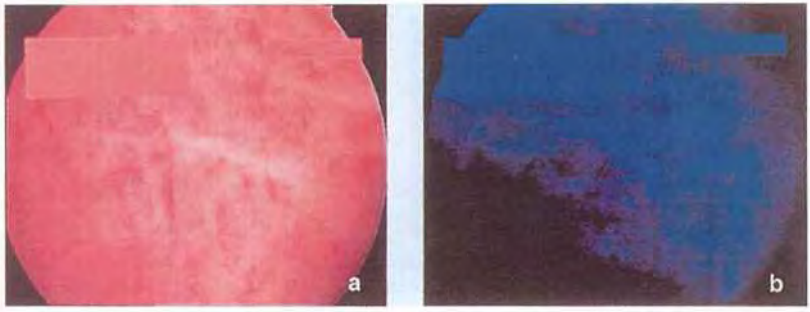

Figure 1. Macroscopic imaging of normal bladder after the instillation with hypericin.
(a) White light cystoscopy; (b) fluorescence cystoscopy.

fluorescence (Figure 3). Biopsies taken from these fluorescent regions were used in the confocal microscopic study. Results from the confocal fluorescence microscopy were correlated with standard white light cystoscopy and histopathology, which is the gold standard method of determination of malignancies in bladder tissue. Microscopic fluorescence revealed that normal bladder regions showed little or no fluorescence and inflammation in bladder had mild fluorescence. The transitional cell carcinoma (TCC) and CIS lesions showed bright fluorescence (Figure 4). The order of fluorescence was as follows: Normal < Inflammation < Grade 1 TCC < Grade 2 TCC< CIS < Grade 3 TCC. This can be seen in Figure 5 in the form of a histogram. The confocal images obtained from biopsies can be seen in Figure 4 where increase in fluorescence intensity with regards to the different

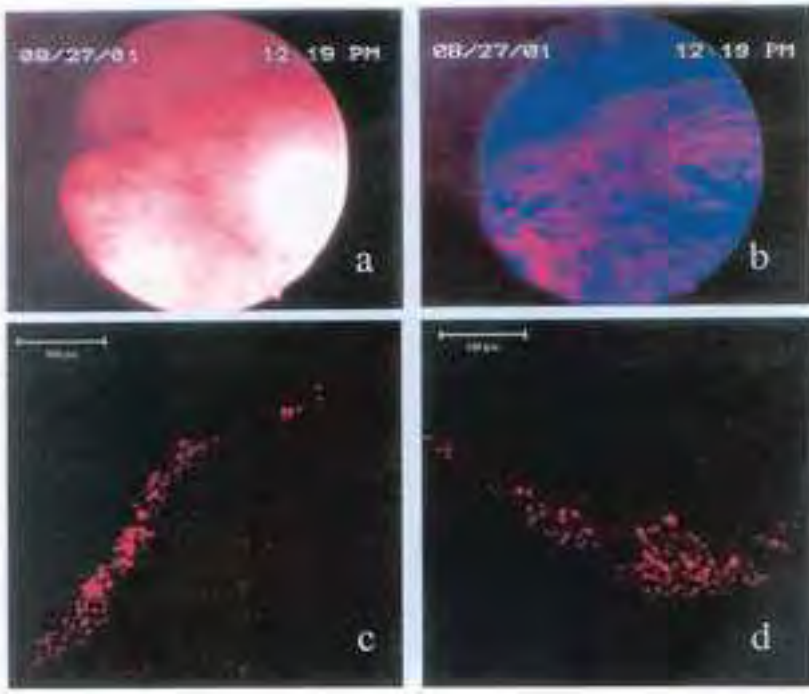

Figure 2. Macroscopic and microscopic imaging of inflammation in bladder after the instillation with hypericin. (a) White light cystoscopy; (b) fluorescence cystoscopy; (c & d) microscopic fluorescence of the same tissue.

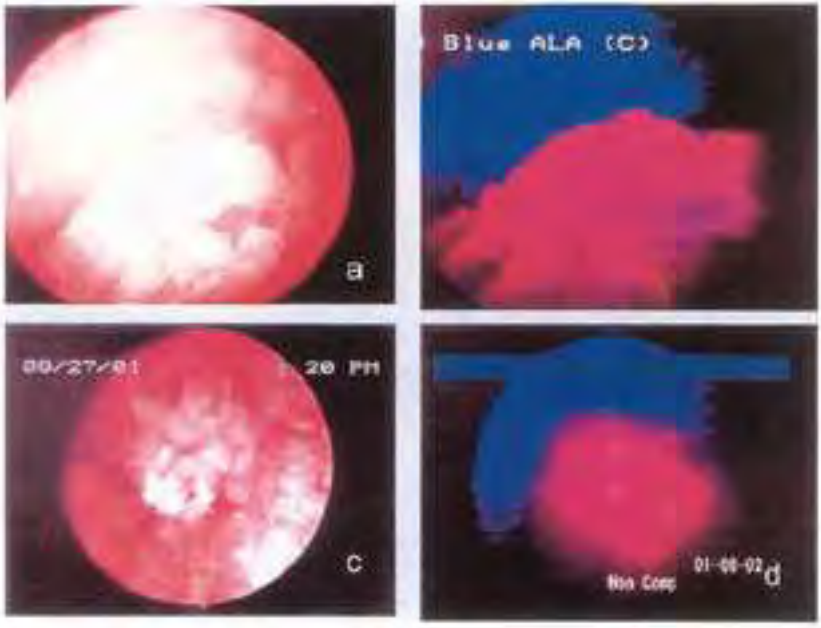

Figure 3. Macroscopic imaging of transitional cell carcinoma after the instillation with hypericin. (a) and (c) White light cystoscopy; (b) and (d) fluorescence cystoscopy.

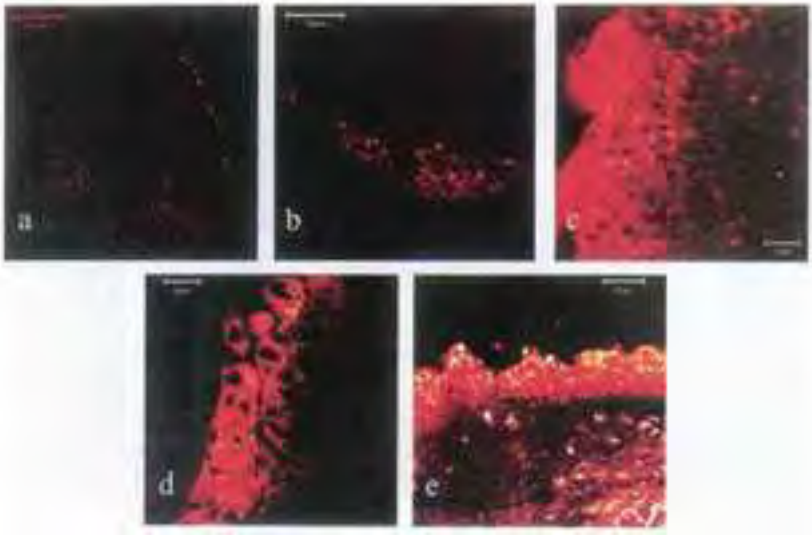

Figure 4. Fluorescence confocal microscopy of hypericin localization in different grades of bladder cancer tissue. (a) Normal bladder; (b) inflammation of bladder; (c) TCC grade 1; (d) TCC grade 2; (e) CIS.

grades of TCC can be observed clearly. The amount of fluorescence in normal and inflammation bladder tissue was far less than G1–G3 and CIS. Compared to inflammatory bladder tissue, TCC Grade1 tissue had 2.2 times more fluorescence, Grade 2 tissue had 2.9 times more fluorescence and Grade 3 tissue had 7.2 times more fluorescence. In this study we found that CIS biopsy lesions had 5.7 times more fluorescence than tissue with inflammation. Evidence derived from the macroscopic fluorescence data corroborated the microscopic fluorescence data. This shows an increased specificity of this technique over methods such as using ALA-induced PPIX fluorescence imaging of the bladder [13].

Not only were we able to differentiate between the different grades of tumour, we could also determine the degree of tumour invasion by microscopic fluorescence. In Figure 6, pT2 had 5.2 times more fluorescence intensity than pTa and 2.6 times more than pT1.

Bladder studies using hypericin as a photosensitizer by D'Hallewin et al. [14] have revealed a high level of sensitivity (93%) and specificity (98.5%) in the detection of bladder cancer with hypericin instillation with no local or systemic side effects in patients post

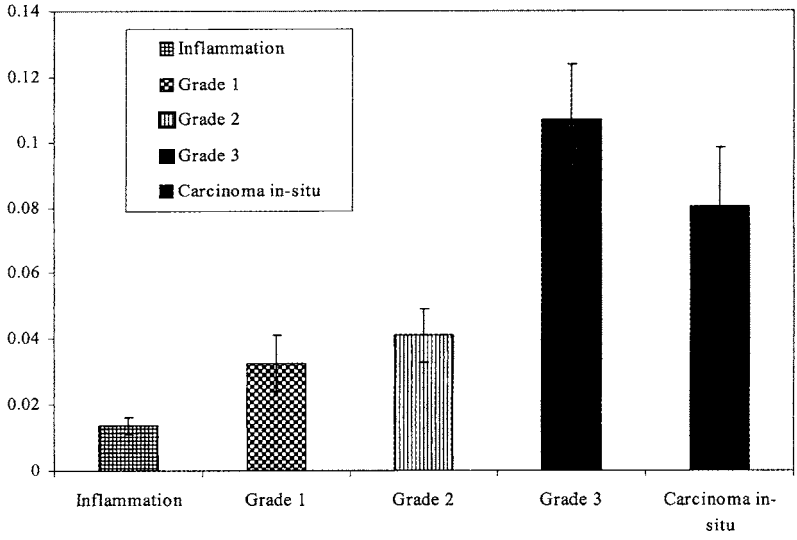

Figure 5. Average fluorescence intensity in different histological grades of bladder cancer.

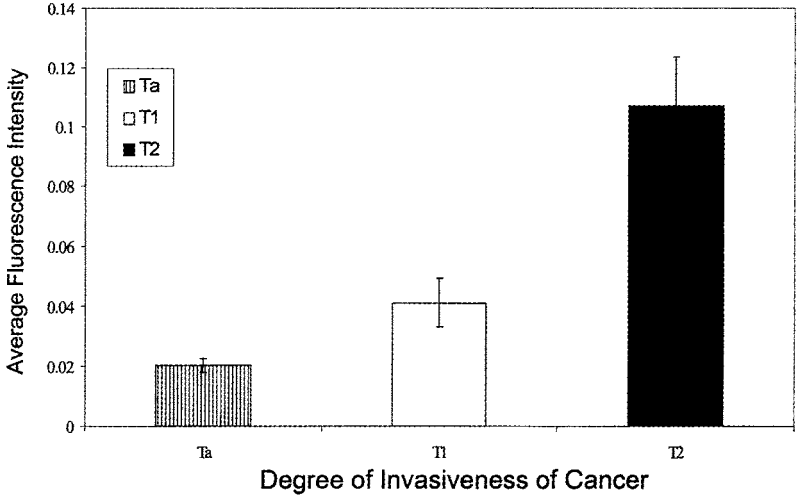

Figure 6. Average fluorescence intensity of hypericin in different stages of cancer.

instillation of hypericin for 2 to 4 hours at a maximal dosage of 160 mg. This promising study by D'Hallewin et al. [14] initiated the present study primarily to analyze the efficacy of hypericin fluorescence

cystoscopy and confocal laser microscopy and fluorescence image analysis as a diagnostic tool in identifying bladder malignancies. Incomplete tumour resection is recognised as an important factor in recurrence and progression of superficial bladder cancer [9]. Therefore the sensitivity of the tools used for diagnosis will play an important role in the prognosis of the patient.

In this study, after a low dose of hypericin (20mg/patient) was instilled for 2-4 hours, we were able to image and quantify the relative amounts of hypericin in various stages of disease in the progression of bladder cancer ranging from TCC grade 1, 2, 3 and CIS for the first time in human bladder carcinoma using image analysis. It was evident from our study that hypericin tissue microfluorescence can be used to as a prognostic marker and also as an aid to routine histopathology. These findings with hypericin microfluorescence are similar to our findings with ALA induced microfluorescence in bladder cancer [13]. In fact we find hypericin microfluorescence to be more specific than ALA-induced PPIX in differentiating TCC Grade 1, 2, 3 and CIS based on the differences in the relative fluorescence intensities between different histopathologies of the biopsies examined [13]. More interestingly, we found that the microfluorescence analysis revealed that the degree of fluorescence increased with the stage of disease (pTa, pT1, pT2). The same observation was made by us with ALA-induced PPIX [13]. However, the reasons why this is so remain unclear to us. Again we have shown in this study that the relative increase in drug-induced fluorescence intensity could potentially be used as an indicator for determining the invasive stage of the bladder malignancy. This information could appropriately be used as a prognostic marker in predicting the extent of disease as an adjunct to traditional histopathology.

Preliminary macroscopic fluorescence in 30 patients using hypericin revealed that the technique is safe for human use with no ill effects and also revealed greater degree of both sensitivity and specificity (data not shown) compared to ALA-induced PPIX [13]. However, further work is underway to ascertain both specificity and sensitivity in both transitional cell carcinoma of varying grades 1, 2 and 3 and also carcinoma *in-situ* in a larger patient sample size treated with hypericin.

In conclusion, early detection of carcinoma *in-situ* and other flat lesions are essential for the good prognosis of the patient, and hypericin proves to be very promising in this field. Its short half-life and high sensitivity coupled with high specificity for cancerous cells makes it a good candidate as a tumour marker. The method also does not pose any adverse side effects to the patient. More importantly fluorescence diagnosis at both macroscopic and especially the microscopic level reveal that this method could be used as a diagnostic aid to augment conventional histopathology. With the advent of confocal endomicroscopy [15] and the concept of optical biopsy [16-18], one could envisage the use of *in-vivo* hypericin fluorescence image analysis to obtain 3-dimensional fluorescence imaging of the bladder in order to develop diagnostic algorithms to obtain increased sensitivity and specificity in bladder cancer with a diagnostic accuracy of almost 100%.

We hope optical biopsies using either drug-induced tissue fluorescence or auto-fluorescence could increase diagnostic accuracy and also possibly replace the need to take unnecessary biopsies for routine histopathology. We conclude that the use of hypericin is indeed a very promising clinical application in the diagnosis of bladder cancer.

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Chemopreventive Efficacy of Apigenin against *N*-Nitrosodiethylamine-Induced and Phenobarbital-Promoted Experimental Hepatoma

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INTRODUCTION

Plant polyphenols have drawn attention in recent years because of their possible anticancer effects. In particular, flavonoids, which are diphenyl propanoids, possess antiproliferative and anticarcinogenic properties. Apigenin (4',5,7-trihydroxyflavone), a common dietary flavonoid, is a non-toxic and non-mutagenic flavone subclass of flavonoids. It inhibits cell proliferation and cell cycle progression in various biological systems [1,2].

N-nitrosodiethylamine (Diethylnitrosoamine, DEN), one of the most important environmental carcinogens in the *N*-nitrosoamine class, primarily induces tumors of the liver. It is normally used as a hepatocarcinogen to induce hepatocellularcarcinoma (HCC) in animal models, especially in rats [3]. The general population may possibly be exposed to unknown quantities of DEN present in foods, beverages, tobacco smoke, herbicides, pesticides, drinking water and industrial pollution. An electrophile is a highly reactive (electron loving) compound that will covalently interact with nucleophilic centers. Proteins, RNA, DNA and all biological molecules are loaded with nucleophilic centers. The electrophilic species formed during the metabolic activation of DEN cause functional disturbances by directly interfering with these biological molecules [4].

Reactive oxygen species (ROS), such as hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$) and H_2O_2, and their subsequent modification of macromolecules (such as protein, RNA and DNA) may be involved in the development of multistage

carcinogenesis, especially tumor promotion [5,6]. As a potent tumor-promoting agent, phenobarbital (PB) can cause oxidative damage to liver as one among its many effects [7]. Here the generation of ROS is related to the induction of certain cytochrome P_{450} isoenzymes by phenobarbital (PB) treatment [8]. Oxidative stress caused by free radicals has been reported to result in membrane lipid peroxidation, DNA damage and mutagenesis and has been associated with various stages of tumorigenesis.

An *in vitro* study assessed the antioxidant potencies of several dietary flavonoids compared with vitamin C. When ranked in order of potency, only apigenin, rutin and quercetin were more effective than vitamin C in reducing oxidative DNA damage [9]. Intraperitoneal administration of apigenin at 25 mg/kg body weight caused a significant reduction in the lung colonization of B16-BL6 cells in female 57BL/6N mice [10]. In the same experiment they observed no mortality or body weight changes at doses up to 50 mg/kg body weight.

Hence, for the present study, apigenin was chosen as a potential compound for cancer chemotherapy. In this present investigation, antioxidant and macromolecular protection of apigenin were assessed. The defense exhibited by apigenin against oxidative stress-induced macromolecular damage provide information on the anti-cancer efficacy of apigenin against experimental HCC.

MATERIALS AND METHODS

Materials

N-nitrosodiethylamine (DEN), normal melting and low melting agarose and apigenin were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used for the experiments were of analytical grade.

Animals

Male rats of Wistar strain weighing around 130–150g (procured from Tamil Nadu Veterinary College, Chennai, India) were divided into four groups of 6 rats each. All rats were housed in pure polypropylene cages and were maintained on a diurnal 12-hour light and 12-hour dark cycles with constant temperature and humidity.

The animals were fed a commercial rat diet. Animals had *ad libitum* access to food and water.

DEN induced and phenobarbital promoted rat liver tumorigenesis

Group I-Control animals received vehicle alone (saline containing 0.02% KOH). Liver cancer was induced in Group II and Group III animals by a single intraperitoneal injection of DEN (200 mg/kg body weight). After two weeks to allow for recovery, the carcinogenic effect was promoted in both the groups by phenobarbital (0.05%, PB). Promoter was supplemented to the animals through drinking water up to 14 successive weeks [11]. At the beginning of 15th week, Groups III and IV animals received intraperitoneal apigenin (25 mg/kg body weight/day) dissolved in alkaline (0.02%of KOH) physiological saline (0.89% of NaCl) daily for 14 consecutive days.

On completion of 14 days of apigenin administration, the animals were fasted overnight and sacrificed by cervical decapitation. Liver was excised immediately and immersed in physiological saline. Homogenate was prepared with fresh liver tissue in 0.1 M Tris-HCl buffer (pH 7.4). Tissue homogenates were used for further analysis.

Lipid peroxidation assay

The level of lipid peroxides was assayed by the method as previously described [12]. To 0.2 ml of liver homogenate/plasma, 0.2 ml of SDS, 1.5 ml of acetic acid and 1.5 ml of TBA were added. The mixture was made up to 4 ml with water and then heated in an oil bath at 95°C for 60 min using glass ball as a condenser. After cooling, 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The level of lipid peroxides was expressed as nmoles of MDA formed/ mg protein.

Assessment of DNA damage

DNA damage was assessed by using alkaline single cell gel electrophoresis (Comet assay). Comet assay was performed by the method as previously described [13]. 100 ml of 1% normal melting point agarose (NMPA) in phosphate-buffered saline was dropped

on to frosted slides, immediately covered with cover slip and kept for 10 min in a refrigerator to solidify. The cover slips were then removed and 100 ml of low melting point agarose (LMPA) containing cells (100 ml of cell suspension in 100 ml of LMPA) were added to the slides. The cover slips were replaced and the slides were kept in the refrigerator for another 10 min to solidify the LMPA. After this, the cover slips were removed and a top layer of 100 ml of LMPA was added and the slides were again cooled for 10 min.

After removal of cover slips, the slides were immersed in cold lysing solution. The slides were kept in dark at 4°C for at least 1 hr. To prevent the occurrence of additional DNA damage, the following steps were performed under dim light. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank. The unit was filled with a freshly made electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to alkali for 20 min to allow for DNA unwinding.

An electric current of 25 V and 300 mA was applied for 20 min of electrophoresis. After electrophoresis, the slides were placed horizontally, and neutralized with Tris-HCl. Finally, 50 ml of ethidium bromide was added to each slide and covered with a cover slip and analysed using a fluorescence microscope (Nikon, Japan) with a calibrated scale. Images of 50 randomly selected cells were analysed from each sample. For each cell, the length of the image (diameter of the nucleus plus migrated DNA) was measured.

Statistical analysis

Values are expressed in mean ± SD for six rats in each group. Significance of the differences between mean values was determined by Student's t- test. The levels of significance were evaluated with p-values. P-values less than 0.05 were considered to be statistically significant.

RESULTS

The levels of lipid peroxidation in the liver and plasma of control and experimental animals are depicted at Figure 1. It was found that HCC-bearing animals (Group II) showed a significant ($p<0.001$)

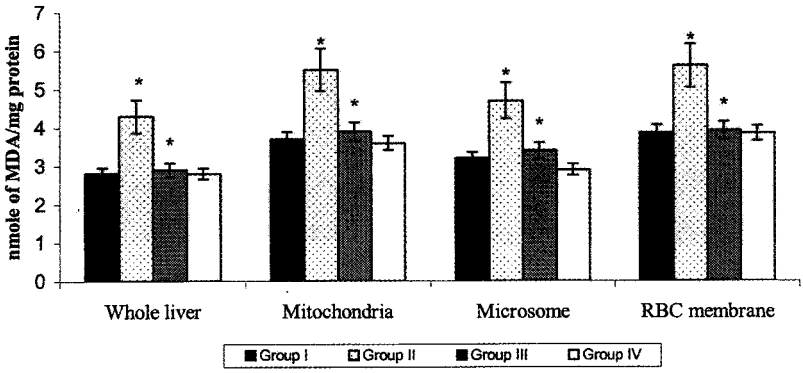

Figure 1. Levels of Lipid peroxidation.
Values are expressed as mean ± SD for six rats in each group. Comparisons were made between Group I and Group II; Group II and Group III; Group I and Group IV; *$p < 0.001$.

increase in the level of ipid peroxidation in both plasma and liver when compared with control animals (Group I). Apigenin-treated (Group III) animals showed a significant decrease ($p<0.001$) in the levels of lipid peroxidation in both liver and plasma when compared with HCC-bearing (Group II) animals. There was no significant difference in the lipid peroxidation between apigenin-treated (Group IV) and control animals (Group I).

The same results were found for DNA single strand breaks (Figure 2) with a significant increase ($p<0.001$) in Group II animals when compared with controls and a significant decrease ($p<0.001$) in Group III (apigenin-treated) animals when compared with Group II animals. There was no significant difference in the levels of protein carbonyls in plasma and liver and DNA strand breaks in leucocytes and liver between apigenin-treated (Group IV) and control animals. Figure 3 illustrates the levels of DNA strand breaks in control and experimental animals.

DISCUSSION

It is widely accepted that the mutagenic action of nitrosamines is mediated via their immediate metabolic product. The metabolism

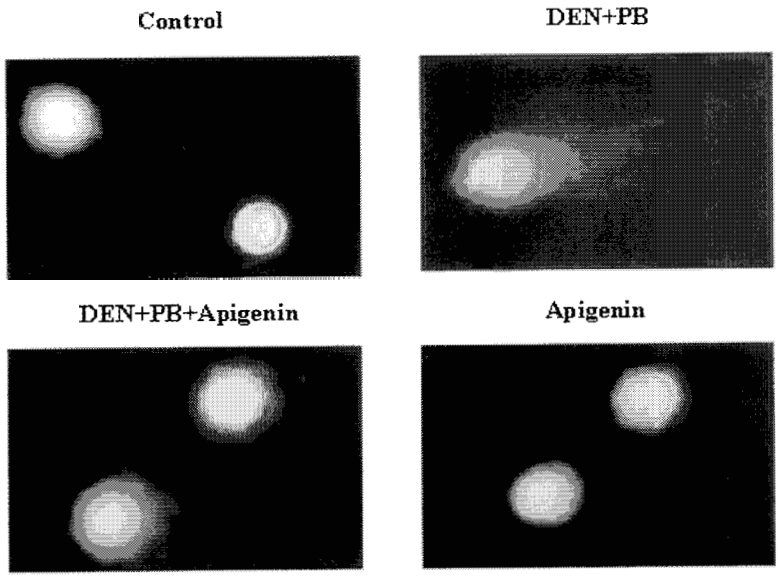

Figure 2. **Comet assay showing** leucocytic DNA single strand breaks of control and apigenin-treated rats.

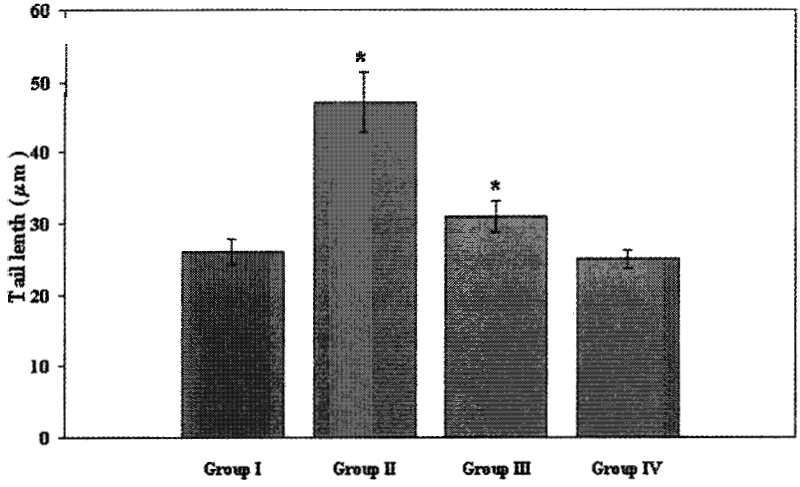

Figure 3. Levels of DNA single strand breaks in leucocytes of control and apigenin-treated animals.

Values are expressed as mean ± SD for six rats in each group. Comparisons were made between Group I and Group II; Group II and Group III; Group I and Group IV; *$p < 0.001$

of nitrosamines *in vivo* results in the formation of elecrophilic reactive intermediates, free radicals and associated oxidative stress, which are able to alkylate lipids, proteins and genetic materials.

Depletion of antioxidant defenses and/or rise in ROS production can tilt the ROS-antioxidant balance and cause oxidative stress [14]. Oxidative stress is associated with damage to a wide range of macromolecular species including lipids, proteins and nucleic acids [15] thereby producing major interrelated derangements of cellular metabolism including peroxidation of lipids and single strand breaks on DNA.

Lipid peroxidation (LPO) is the most studied biologically relevant free radical chain reaction. LPO is initiated by the attack of a free radical on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. LPO gives rise to a variety of products including short chain aldehydes, such as malondialdehyde, alkanes, conjugated dienes, a variety of hydroxides and hydroperoxides [16]. Many of these products can be measured as markers of lipid peroxidation. Increased level of lipid peroxidation was recently reported during DEN-induced and phenobarbital-promoted hepatocellular carcinogenesis [17].

Administration of apigenin to DEN-induced and phenobarbital-promoted animals decreased the level of LPO. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants [18]. It has been reported that the hydrogenation of the double bond between carbons two and three (C2-C3) of the C ring in apigenin decreased its antiperoxidative effects [19, 20]. This double bond may thus be responsible for the antioxidant potency of apigenin and be an effective inhibitor of MDA formation. Our observation that apigenin is able to interfere with the LPO chain suggests it is capable of shielding cell membranes from free radical-induced injuries.

Oxidation of DNA in human cells occurs as a consequence of attack by endogenous free radicals. Sources of free radicals in the body include reactive oxygen species released during respiration, or from leukocytes, as a part of the defense against foreign components like tobacco smoke, ionizing radiation and intermediates of xenobiotic metabolism. In our study an increased level of tail length of nucleus

was observed in group II DEN- and phenobarbital-treated rats. These results support the reports of Singh and Roscher [21], whose observation on DEN-induced DNA strand breaks in hepatoma cells correlated with aldrin epoxidation and could be augmented by phenobarbital treatment.

The $OH^{\bullet}$ radical is implicated in the oxidation of DNA bases, the most studied product being 8-oxo-7, 8-dihydroguanine. Oxygen radicals attack DNA bases and deoxyribose residues producing damaged bases and single strand breaks. It has recently been reported that the administration of phenobarbital to rat induces DNA oxidative base modifications in the nuclear membrane of hepatocytes via generation of free radical-associated oxidative stress [8].

In the present investigation the elevated levels of lipid peroxidation and single strand breaks on DNA contents were observed in Group II animals. This indicates the accumulation of free radicals associated oxidative stress caused by the administration of DEN and phenobarbital. It has been well established that the administration of DEN and phenobarbital will result in severe oxidative stress and associated imbalances in the antioxidant system. In contrast, diets rich in fruits and vegetables can decrease both DNA damage and cancer incidence [22].

Apigenin is a relatively abundant flavonoid present in several fruits and vegetables. Our findings indicate that apigenin inhibits the level of LPO and DNA single strand breaks significantly in DEN-induced and phenobarbital-promoted experimental hepatocellular carcinogenesis. Furthermore the study underlines the complex nature of the inhibition of LPO by apigenin and that the inhibitory effect may be dependent on the structure. With these findings, we conclude that apigenin can protect hepatocytes from the tumorigenic action of DEN and also from the tumor-promoting effect of phenobarbital. Hence we suggest that apigenin may be developed as a successful chemotherapeutic agent. However, further studies are required to elucidate the broader implication of apigenin *in vivo*.

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The Effects of Saponin from Sea Cucumber (*Holothuria atra* Jaeger) on CEM-SS T-Lymphoblastic Cells

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INTRODUCTION

Sea cucumber is a marine invertebrate from the phylum, *Echinodermata*. The sea cucumber has a specific organ called the cuvier gland [1] which produces a toxin known as holothurin or saponin [2]. Seasonal variation and distribution of holothurin occur in the animal where the content of holothurin in ovaries is higher than that in other organs during the breeding season [3]. Holothurin are mainly utilized in defense and have a wide range of pharmacological effects [4]. Toxicological studies of the saponin were pioneered in 1955 [2]. Saponin is a monosacharide complex with steroid or triterpenoid with/ without sulphuric acids [5]. Sea cucumber saponin is a triterpenoid type while starfish contains steroid type saponin [6]. Cytotoxic effects of extracts from sea cucumber were reported using sarcoma cells-180 as a model [7] followed by experiments using sarkina epidermal cells and Enrich cells [8]. Holothurin treatment has been proven to extend the survival of rats with sarcoma-180 but is only effective when administered slightly lower than the mortality dosage. Holothurin also inactivate ascites Kreb-2 tumor cells *in vitro* and prolonged the life of mice, which was previously induced with this type of tumour

[9]. Administration of holothurin can also reduce adenocarcinoma formation in BDF-1 cells but failed to suppress the growth of the B-16 tumor in rat [4]. Since most of the experiments were done using the crude extracts, the effect on cells could only be seen at higher dosage such as 50 mg/ml on sarkina cells and 150 mg/ml on Ehrlich cells.

In the biological system, holothurin plays an integral part in the metabolic process and like-low-molecular regulators, exhibit polyfunctional properties [10]. Mechanism of action of triterpenoid glycoside has been studied intensively since 1980. Chemical or functional changes in membranes are central to pathogenic processes in cells [11]. Using artificial membrane as a model, it was found that a low concentration of tritepenoid glycoside decrease the Ca-ATPase activity with no noticeable increase in the permeability of the cells membrane whereas at high concentration, a steep increase in permeability of membrane towards Ca^{2+} ions was observed [12]. Interaction of triterpenoid with membranes having different sterol composition has also been reported. It was shown that the membrane activity of the glycoside was defined by the levels of its affinity to sterol receptor [13]. The interaction of glycoside with sterol causes disturbance of selective permeability in plasma membranes. Triterpene glycoside affects the liposome ionic permeability and bilayer lipid membranes whereas the rate of glycoside effect depends on quantitative and qualitative sterol level in the membrane [14]. There is also a possibility of involvement of glycosylated and sulfated forms of sterol in the development of resistance in cell membranes and tissues of sea cucumber towards their own glycoside [15]. Using high-angel X-ray diffraction spectra, it was demonstrated that triterpenoid glycoside form crystalline complex with the cholesterol molecules in the membrane. Electron microscopy showed a decreased vesicle size of the membrane preparation from rat brain which is enriched in Na^+-K^+-ATPase by the triterpenoid glycoside [16].

There were intermittent reports on the effects of holothurin on cell membranes during the 1980s with more reports appearing from 1990 onwards. The triterpenic glycoside, holotoxin A of *Stichopus japonicus* has been shown to inhibit the Ca^{2+} flux of lipid bilayers from sea cucumber phospholipids as well as the Ca^{2+} flux induced in phosphotidylcholine bilayers by the calcium ionophore X-537 [17].

There was also a report on the cumulative action of holotoxin A and B which suppressed ovulation and stimulated the contractile activity of the rat's uterus [18]. Delayed mitosis in liver cells and compensational increase in mitotic activity using immunomodulatory doses of cucumarioside was also observed [19]. In this study, triterpene glycoside can be considered as compounds that regulate proliferative processes. In low concentration, the triterpene glycosides showed mitogenic activity and modulated the immune response. Comparison between the immunomodulating activity of triterpene glycoside of the holostan series and triterpene glicoside of the dammaran series was studied *in vitro*. The similarity in action of glycoside was observed with respect to dose dependent duality of their effects i.e. the diametrically opposite action of the high and low doses. The expression of the effects was most likely due to the chemical structure of the triterpene glycoside. Liberation of the soluble mediators served as a secondary signal to clonal expansion and differentiation of the cells [20].

Toxins from sea cucumber act on Δ^5-sterol in the membrane with the formation of glycoside–sterol complexes followed by the disturbance of membrane permeability and inhibition of activities of some membrane enzymes [21]. Glycoside from sea cucumber is more cytotoxic and has liposomal activities as compared to ginsenoside [22]. Addition of cholesterol to the cell membrane enhances the cytotoxic effects of these glycosides [23]. A recent study demonstrated that apart from glycoside, novel pentasaccharides (the desulfated derivative of calcigerosides) also showed moderate cytotoxicity against human and mouse tumor cell lines [22]. Glycoside of *Mensamaria intercedens* showed significant cytotoxicity against mouse Lewis lung cancer and mouse S-180 sarcoma cell [24].

In this study, we evaluated the effects of extracts of a local sea cucumber species on CEM-SS T-lymphoblastic cells.

MATERIALS AND METHODS

The sea cucumber, *Holothuria atra* were sampled from Terengganu waters during low tide. The visceral organs were removed and the animal was then clean and cut into small pieces. These were blended and dried at 70–80°C for 24 hrs. The supernatant was

removed and tissues were further dried, then grounded and extracted twice in methanol. Saponin was obtained by rechromatographing the extracts three times. Saponin were then diluted into stock solution of 100 mg/ml in 5% DMSO.

CEM-SS T-lymphoblastic cell line (Universiti Putra Malaysia stock culture) were cultured into RPMI-1640 medium supplemented with 5% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin to become a complete growth medium. Cells were maintained in 25 cm^3 cultured flask with 10 ml CGM at 37°C for 72 hrs with 5% CO_2 to achieve confluency.

Confluent CEM-SS cells were transferred into 96-well cultured plate and treated with toxin ranging from 0 to 50 mg/ml. The cells at LC_{50} were then filtered, oven-dried at 50°C for 24 hr, transferred on a stub, gold-coated and observed under Philips XL30 SEM at 10kV.

RESULTS AND DISCUSSION

Morphological observation showed that the membrane of treated cell had an irregular shape (Figure 2) compared to untreated cell (Figure 1). Some membrane exhibited either cracks or invaginated processes. Besides the distorted shape of the cell membrane, openings or holes were also found. This indicated that the methanol extracts has disturbed the stability of the outer membrane of the cells [12] which allow more substances to enter the cell, thereby sustaining their damaging effects [10]. In this study, we suggested that the methanol extract physically acts on the membrane by replacing the compound with a basic structure which is almost similar. We believe that it was a steroid base material, which created openings into the cells or 'holes'. The second possibility is interaction between glycoside with the sterol membrane [14]. Since the extract is in crude form, combination of some glycoside might enhance the damaging effect on membrane [18].

The morphological changes of the membrane as observed under SEM is unique indicating that toxin acts physically on cell membrane and continuation of this damaging effects lead to either necrosis or

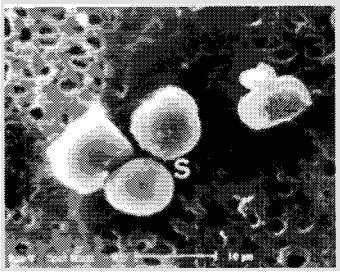

Figure 1. Normal growth of CEM-SS.

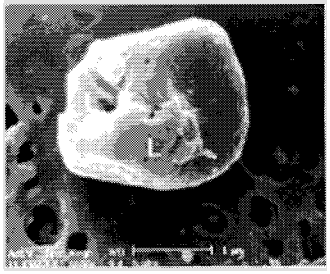

Figure 2. CMSS treated with *H atra*. ('L' indicates hole formation).

apoptosis of the cells. The damaging effect of *H. atra* effect on cell membrane is lower compared to the effect by saponin of *Stichopus badionotus*[25]. Further experiments are necessary to identify compounds in the crude saponin extract that produced this cytotoxic effect.

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Lentinan — A Medically Valuable Glucan

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INTRODUCTION

For many years, mushrooms have been consumed by man, not only as food but also as medicinal remedies. The use of the hot water extracts of fungi for treatment by the Chinese has a long history and the curative effects of mushrooms were recorded in many medicinal books, including the famous "*Shinnoh-Honsoh-Kyo*" (approx. 500 A.D), "*Honsoh-Kohhmoku*" (1552) and "*Ri Yong Ben Cao*" Vol. 3 (1620) [1,2]. In recent years, some medicinal fungi were even used to treat cancer in the USSR, USA, Canada, China and Japan. Among them, the shiitake mushroom, *Lentinus edodes,* has been extensively studied for its valuable medicinal traits.

The most important anti-tumour component in *Lentinus edodes* is Lentinan, a β-1,3-D glucan, which was extracted by Chihara and colleagues in 1969 [3-5]. Lentinan shows prominent anti-tumour activity against both allogenic tumors such as sarcoma 180, as well as synergic and autochthonous tumours, and it exerts its effect by stimulating the host's immune system. Many of the past studies [3-16] were carried out using the intravenous (i.v.) or intraperitoneal (i.p.) route of administration. Oral administration appeared to be a more attractive mode to introduce lentinan into the host's body since it is easier, more convenient, painless, requires no expertise and is also safer. From our previous study [17], pure lentinan was obtained and serves as the source for the experiments in this study. The aim is to investigate the effectiveness of oral administration of lentinan in inhibiting tumour development as well as to unravel the underlying mechanism of its actions in host.

MATERIALS AND METHODS

The Limulus Colorimetric Test for quantitating lentinan in murine blood after oral administration

The amount of lentinan consumed and absorbed could be a factor affecting its efficacy. The pharmacokinetics of Lentinan was studied by using the Limulus Colorimetric Test (LCT – Seikagaku Corporation, Japan) to measure blood levels of the compound. This test uses a synthetic chromogenic substrate, developed by Iwanaga and colleagues [18]. Lentinan was orally administered at 3 mg per mice and blood was extracted from the mice at 15 minutes, 30 minutes, 45 minutes, 1 hour and 2 hours after feeding.

Induction of tumours using human colon carcinoma cells

Six established human colon carcinoma cell lines (ATCC, USA) representing cancers at three stages of cell differentiation were used as tumour models in male AKR white mice, aged 5 – 6 weeks (Animal Holding Unit, National University of Singapore, Singapore). Group I comprises LoVo and SW48 cells, representing the most differentiated cells with gland and signet ring formation. Group II consists of SW480 and SW620 cells, representing moderated differentiated cells while Group III consists of SW403 and SW1116 cells, representing poorly differentiated cells with no gland or signet ring formation. Lentinan was orally administered at a dose of 3 mg per mouse for 7 days prior to the induction with the tumour cells. The same cell lines were also subsequently used for studies in the immunodeficient nude (athymic) and Severe Combined Immunodeficiency (SCID) mice (from the Animal Holding Unit, National University of Singapore, Singapore).

Inoculation of lentinan-primed T lymphocytes in nude and SCID mice

The actions of lentinan were proposed to be host-mediated in that the lymphocytes played important roles. The T lymphocytes from lentinan-, crude mushroom homogenate- and buffer-fed (controls) AKR mice were inoculated into the immunodeficient mice. Two groups of each type of mice (nude and SCID), consisting of ten mice in each group, were included in the study. Tumours were induced using the

six human colon carcinoma cell lines. Each of these tumour cell lines was inoculated into 20 athymic mice at the same time as the inoculation of lymphocytes. Ten of them were inoculated with T lymphocytes extracted from lentinan-fed mice while the other ten mice were inoculated with T lymphocytes from either crude mushroom homogenate or buffer-fed mice (controls). Only two cell lines (LoVo and SW620 cells) were used for the SCID mice experiments as there was a shortage of such mice.

Determination of cytokine profiles in mice

The levels of four cytokines, namely IL-1α (interleukin-1α), IL-2 (interleukin-2), TNF-α (tumour necrosis factor-α) and IFN-γ (interferon-gamma) were analysed using the ELISA (enzyme-linked immunosorbent assay) technique (OptEIA™ ELISA, BD Pharmingen, USA). Blood was extracted from the mice *via* tail bleeding at 2, 4, 6, 8, 10 and 24 hours after feeding with lentinan, crude mushroom extracts, mycoproteins, mycolipids or buffer solutions (negative controls) for the assays.

Programmed cell death (apoptosis)

Apoptotic activities of the tumour cells were studied via electron microscopy [17], TUNEL-staining of DNA fragmentation followed by immuno-fluorescence microscopy (ApoAlert DNA Fragmentation Assay Kit, Clontech, USA), flow cytometry (Coulter EL, Florida, USA), and the determination of the levels of caspase 3 and caspase 8 (ApoAlert, Caspase kits, BD Clontech, USA). Tumour cells were extirpated from both lentinan-fed mice or buffer solution-fed mice and analysed using the above techniques.

RESULTS

Quantitation of lentinan in murine blood using Limulus Colorimetric Test

Quantitative analyses of murine blood using Limulus Colorimetric Test at different timings after feeding demonstrated the presence of pure lentinan in murine blood. A peak was detected at 0.2 mg/ml (equivalent to the usual i.v. or i.p. blood level) 30 minutes

after feeding (Figure 1). As a dosage-dependent relationship was proposed, the most effective dosage of lentinan administered orally was previously determined as 3 mg per mouse [17].

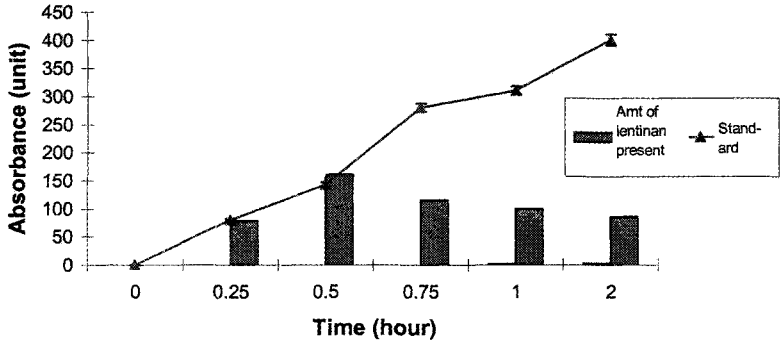

Figure 1. The histogram shows adsorbed Lentinan present in murine blood measured at different time interval (hour) after oral administration of lentinan. The quantitative analyses were done using the Limulus Colorimetric Test. A standard curve was also done to ensure the validity of the test data. The peak level of adsorbed Lentinan is observed at 0.5 hr (30 mins) after feeding and 0.2 mg/ml is detected.

Experiment on human colon carcinoma cells

The results obtained using human cancer cells (Table 1) were similar to those of earlier experiments [17] using K36 cells, a murine lymphoma cell line. The tumor inhibition rate (TIR) of lentinan in nude mice was over 90% for all six carcinoma cell lines. Tumour development in mice pre-fed with lentinan (Figure 2a) was inhibited and if formed, appeared to be relatively small compared to the buffer solution-fed mice (Figure 2b). An average TIR of about 90% (lentinan-fed) was much higher than that from mice pre-fed with crude mushroom homogenate (with average TIRs of 50% - data not shown).

Table 1. Tumor Inhibition Rates (TIRs) of lentinan on different human colon carcinoma cell lines

	Type of Human Colon Carcinoma Cells					
	LoVo	SW48	SW620	SW480	SW403	SW1116
Tumour Inhibition Rate (%)	**89.50**	**90.33**	**90.12**	**91.54**	**92.86**	**93.15**

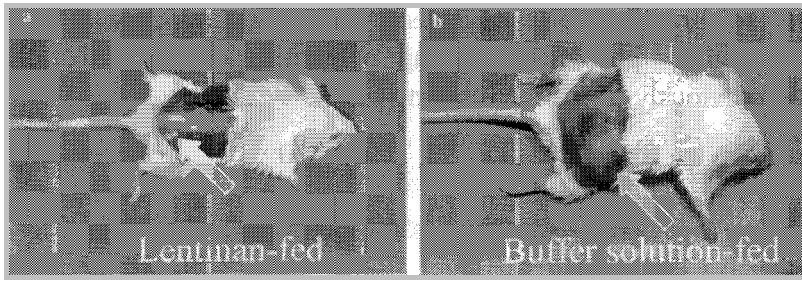

Figure 2. Tumors induced with human colon carcinoma cell lines
(a) Low numbers of mice that have been fed with Lentinan for seven days prior to the inoculation of the tumor cells developed tumors. In cases where tumors developed, the tumors are minute (arrow). (b) In contrast, mice that have been fed with buffer developed large tumors (arrow).

Inoculation of nude and SCID mice with lentinan-primed T lymphocytes

T lymphocytes were purified from AKR mice that were fed with either lentinan, crude mushroom extracts or buffer for 7 days. As shown in Figure 3, the tumour from the group that received the lentinan-primed T lymphocytes was minute in size (Figure 3a & b) compared to that in the groups that received T lymphocytes from either crude mushroom homogenate- (Figure 3c & 3d) or buffer-fed (Figure 3e & f) AKR mice.

Comparatively high TIRs (86% to 91%) were achieved in nude mice inoculated with the lentinan-primed T lymphocytes isolated from lentinan-fed mice (Table 2) as compared to moderate TIRs of 50% achieved in the cgroup inoculated with T lymphocytes isolated from crude mushroom homogenate-fed mice. Remarkable results were also achieved from the experiments with SCID mice. The group inoculated with primed T lymphocytes achieved an average TIR of 70% (Table 2). These results thus showed the beneficial effect of priming the lymphocytes of immunodeficient mice with lentinan.

Determination of cytokine profiles in mice

Cytokine analyses have shown that the four cytokines under study, namely IL-1α, IL-2, TNF-α and IFN-γ increased significantly after feeding with lentinan. The peaks were observed at different timings after lentinan administration. The IL-1α and IL-2 levels peaked at 2

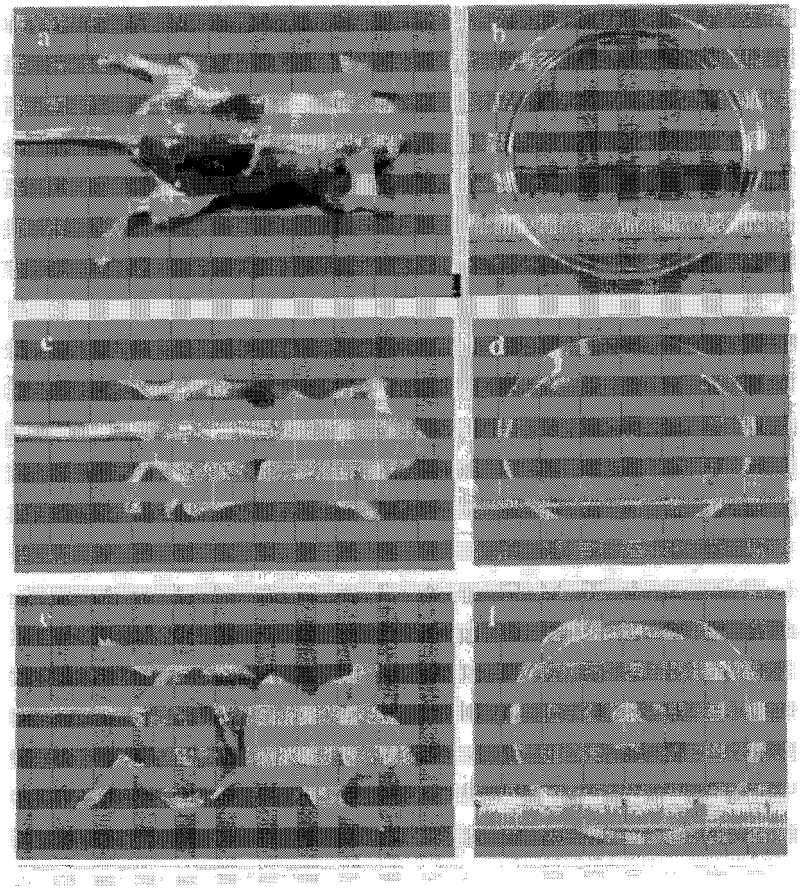

Figure 3. Tumors in nude mice inoculated with lentinan-primed T lymphocytes. (a & b) Tumors developed in nude mice inoculated with primed T lymphocytes extracted from lentinan-fed AKR mice appeared tiny in size. (c & d) Tumors developed in nude mice inoculated with primed T lymphocytes extracted from crude mushroom homogenate-fed AKR mice appeared moderate in size. (e & f) Tumors developed on nude mice inoculated with unprimed T lymphocytes extracted from buffer solution-fed AKR mice were largest when compared to (a to d).

hours whereas TNF-α and IFN-γ levels peaked at 4 hours after feeding with lentinan. The detailed results have been presented in [19]. All cytokine levels returned to baseline after 24 hours.

Programmed cell death (apoptosis)

The tumour cells extirpated from lentinan-fed mice showed signs of fragmented condensed chromatin (black arrows) at the nuclei

Table 2. Tumour Inhibition Rates (TIRs) achieved with nude (athymic) and SCID mice after simultaneous inoculations of primed T lymphocytes and human colon carcinoma cells

Type of Human Colon Carcinoma Cell line	**LoVo**	**SW48**	**SW620**	**SW480**	**SW403**	**SW1116**
TIR obtained after inoculation with lentinan-primed T lymphocytes in nude mice	85.84	87.30	88.38	88.69	89.94	91.43
TIR obtained after inoculation with crude mushroom homogenate-primed T lympgocytes in nude mice	45.76	45.75	49.70	47.25	51.34	53.89
TIR (%) obtained by inoculation with primed T lymphocytes in SCID mice	74.69		70.18			
TIR (%) obtained by inoculation with unprimed T lymphocytes (buffer-fed) in SCID mice	11.73		10.12			

membrane under TEM (Figure 4a). The tumour cells from the buffer-fed mice had diffused chromatin (Figure 4b - white arrows).

TUNEL staining of the tumour cells from lentinan-fed mice were observed to have a majority of bright green fluorescein-dUTP-labeled apoptotic cells (Figure 5a). Very few cells were labeled with the counter-stain, propidium iodide (red colour). In comparison, tumour

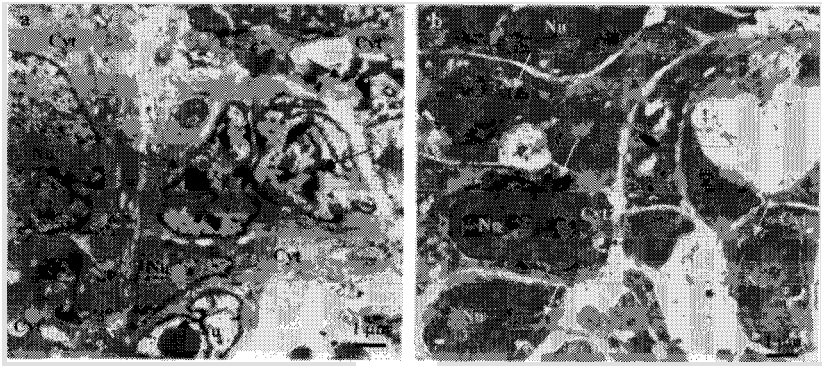

Figure 4. Ultrastructural morphologies of tumor cells extirpated from the lentinan- and buffer-fed mice.
(a) Tumour cells extirpated from lentinan-fed mice show highly vacuolated dying cells with pleomorphic-shaped nuclei containing condensed chromatin (black arrows) (b) Tumour cells extirpated from buffer-fed mice have generally rounded nuclei with diffused chromatin (white arrows). Cyt denotes cytoplasm and Nu denotes nuclei.

cells extirpated from crude mushroom homogenate-fed mice (Figure 5b) and buffer solution-fed mice were poorly labeled (Figure 5c) with the green fluorescein-dUTP. Many non-apoptotic cells were counter-stained with propidium iodide and appeared red in color.

Results obtained from the flow-cytometric analyses of apoptotic processes in the tumour cells indicated that cells from lentinan-fed mice underwent a high degree of apoptosis (average of 65%) in (Figure 6a – c) as compared to the relatively lower degree of apoptosis (~24%) in crude mushroom homogenate-fed mice (Figure 6d – f) and buffer solution-fed mice [(~7%) (Figure 6g - i)].

The levels of the two main cysteine proteases that mediate apoptosis, caspase 3 and 8, were raised up to three-fold upon feeding with lentinan (Figure 7a & b).

CONCLUSION

Based on these results, we conclude that the oral administration of lentinan is effective in inhibiting tumor development. Upon consumption, lentinan was able to activate the host's immune system

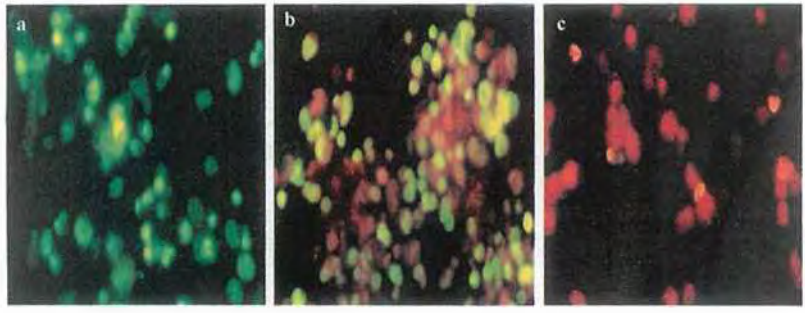

Figure 5. Immuno fluorescence micrographs showing TUNEL staining of apoptotic cells
(a) Tumor cells extracted from mice pre-fed with lentinan are brightly labeled with green fluorescein indicating high degree of apoptosis. (b) Tumor cells extracted from mice pre-fed with crude mushroom homogenate are labeled with fluorescein and the counter stain, propidium iodide, indicating moderate degree of apoptosis. There is a mixture of apoptotic (green) and healthy (red) cells (c) Tumor cells extracted from mice pre-fed with buffer solution (control) are scarcely labeled with fluorescein, but there is significantly labeling with propidium iodide, indicating low degree of apoptosis.

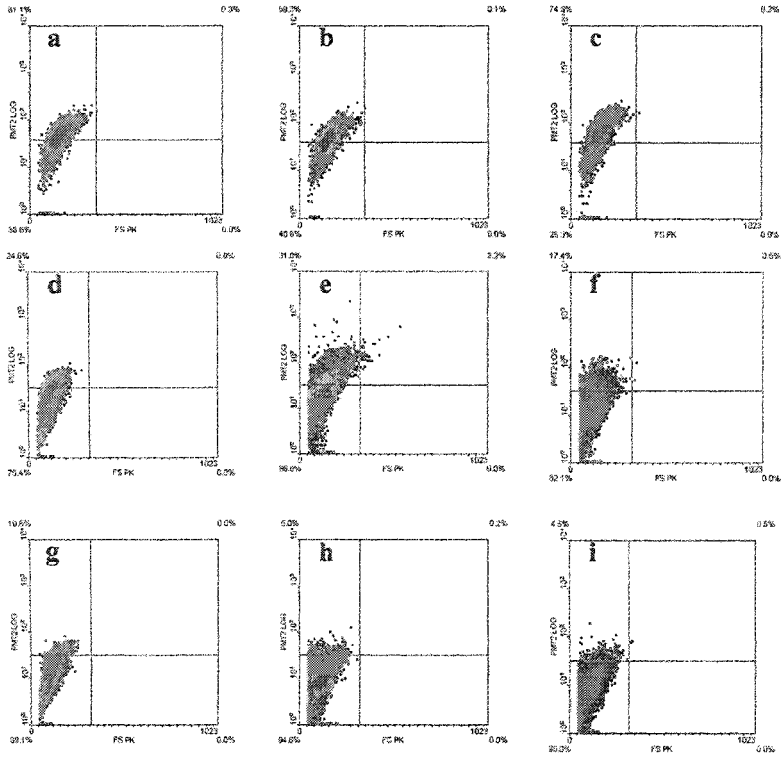

Figure 6. Flow-cytometry density chart showing the percentage of apoptotic cells.
(a to c) lentinan-fed cohort: Average percentage of apoptosis = 64.97%; (d to f) crude mushroom homogenate-fed mice: Average percentage of apoptosis = 24.33%; (g to i) buffer solution-fed batch: Average percentage of apoptosis = 6.77%.

and induce various immunological responses that were beneficial in helping to fight the cancerous cells. The lentinan that was absorbed into the bloodstream of the host activated the macrophages. The activated macrophages, in turn, could provoke the immune system through activation of T lymphocytes, particularly the T-helper cells and thus stimulate their actions.

As significant increment of various cytokines was observed, the raised cytokine levels could further activate the other sets of the immune system and provide additional immune responses against the tumour cells. The activated immune cascade would bring about the

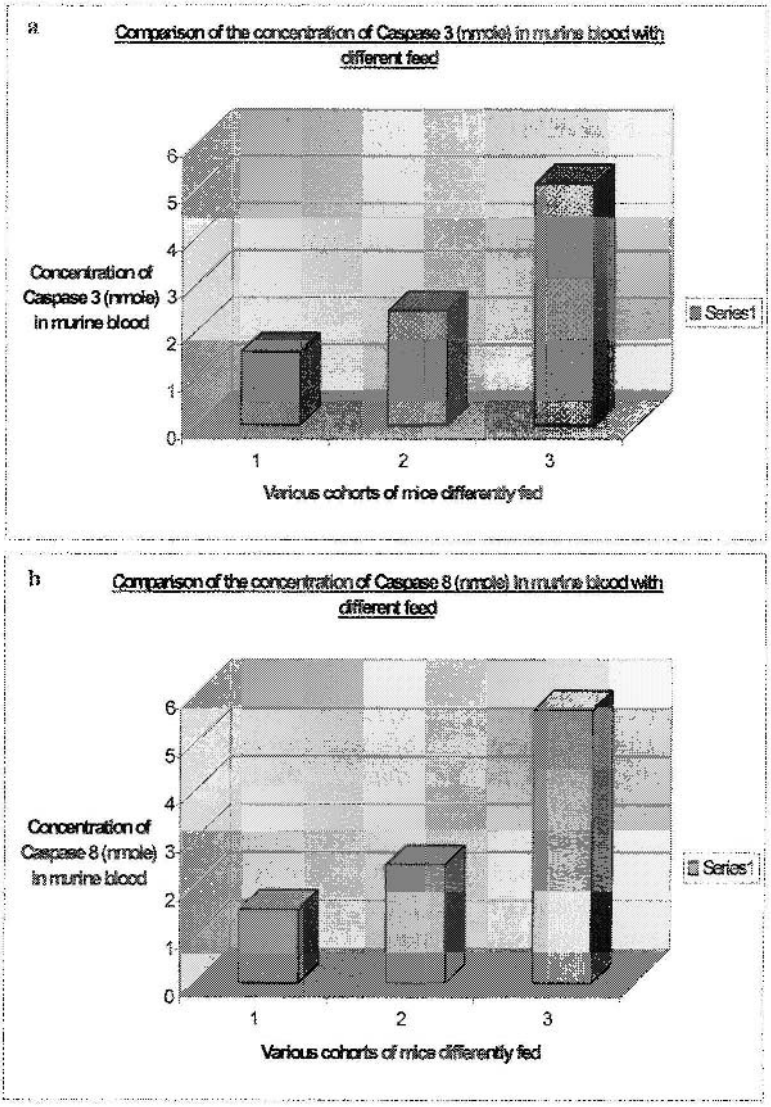

Figure 7. Levels of Caspase 3 and 8 in murine blood.
(a) The level of caspase 3 increases approximately 3.3 fold after feeding with lentinan (3rd column) while it increased approximately 1.6 fold after feeding with crude mushroom homogenate (2nd column) as compared to buffer solution-fed (1st column). (b) The level of caspase 8 increases approximately 3.7 fold after feeding with lentinan (3rd column) while it increases approximately 1.6 fold after feeding with crude mushroom homogenate (2nd column).

total destruction of the tumour cells through apoptosis – programmed cell death, getting rid of what could be harmful to the body.

ACKNOWLEDGEMENT

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Turmeric and Colon Cancer

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INTRODUCTION

Colon cancer is the third most common malignant neoplasm worldwide. Diet plays a major role in the etiology of colon cancer. Experimental and epidemiological studies have highlighted the role of high fat diet in increasing the risk of colon cancer. β-glucuronidase and mucinase are two important enzymes which are associated with the bacterial microflora [1]. Studies have also shown that high fat diet promotes dimethyl-hydrazine (DMH)-induced colon carcinogenesis by increasing the activities of β-glucuronidase and mucinase [1, 2]. Many procarcinogens, carcinogens, toxins and mutagens are detoxified by glucuronide conjugation in the liver. The intestinal bacterial enzyme β-glucuronidase can hydrolyse these derivatives, releasing the active carcinogens and allowing their re-entry into the enterohepatic circulation [3, 4]. Mucinase, another microfloral enzyme, hydrolyses the protective mucin coat in the intestinal wall and exposes the underlying mucosa to the luminal carcinogens that are released by β-glucuronidase activity [5]. Hence, β-glucuronidase and mucinase have assumed significance as colon cancer risk markers.

International dietary guidelines for the prevention of chronic diseases recommend increased consumption of plant foods, including cereal, legumes, fruits, vegetables and spices. These contain a wide variety of physiologically active phytochemicals such as enzyme inhibitors, phytosterols, indoles, flavones, fibre and saponins [6, 7]. Turmeric is a common spice used as a flavouring and coloring agent

in Indian food. Turmeric and its active phenolic curcuminoids namely, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) were reported to be chemopreventive against DMH-induced colon cancer [7]. However, there is to date no report on the effect of synthetic phenolic curcuminoid on experimental colon cancer. Hence, in the present study we have chosen a synthetic bisdemethoxycurcumin analog (BDMC-A) and investigated its effect on DMH-induced colon cancer by assessing (1) the histopathological changes and (2) the activities of β-glucuronidase and mucinase. The effects of BDMC-A are compared with those of turmeric and the naturally occurring curcuminoid, curcumin.

MATERIALS AND METHODS

Male Wistar rats of body weights ranging from 100g - 150g were obtained from the Central Animal House, Department of Experimental Medicine, Raja Muthiah Medical College, Annamalai University, Annamalai Nagar, Tamil Nadu, India. Standard pellet diet containing 5% fat (obtained from Hindustan Lever Ltd., Mumbai) were powdered and mixed with 20% peanut oil to make 20% fat in the daily diet. Animals were fed with the above diet and water was given *ad libitum.*

Experimental protocol

After 2 weeks of acclimatization, the rats were randomly divided into 8 groups as follows:

Group 1: Control rats

Group 2: Rats given a weekly subcutaneous injection of DMH (20 mg/kg body weight) for 15 weeks

Group 3: Rats administered turmeric daily (5g/kg body weight) orally through intragastric tube for 30 weeks

Group 4: Rats given DMH (as in group 2) + turmeric (as in group 3)

Group 5: Rats administered curcumin daily (80 mg/kg body weight) orally through intragastric tube for 30 weeks

Group 6: Rats administered DMH (as in group 2) + curcumin (as in group 5)
Group 7: Rats administered BDMC-A daily (80 mg/kg body weight) orally through intragastric tube for 30 weeks
Group 8: Rats administered DMH (as in group 2) + BDMC-A (as in group 7)

After the experimental period of 32 weeks (including 2 weeks of acclimatization), the rats were deprived of food overnight. 24-hour fecal samples were collected for the assay of mucinase. For the histopathological study, the animals were perfused with 10% formalin by means of cardiac puncture. The colon was then dissected and stored in formalin. It was later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). For enzyme assay, the animals were killed by cervical dislocation. The GI tract was opened carefully and the number of tumours/polyps were counted and the tissues transferred into ice-cold containers. Colon contents were scraped carefully without disturbing the tumours.

Biochemical investigation

β-glucuronidase activities in the colon, liver, colon and fecal contents were assayed by the method of Kawai and Anno [8]. Mucinase activity was assayed by the method of Shiau and Chung [9]. Values are expressed as mean ± SD. Group means were compared by Duncan's Multiple Range Test (DMRT). Values are considered statistically significant if p value was 0.05 or less.

RESULTS

Tables 1, 2 and 3 show the incidence of colonic neoplasms and histopathological changes in control and experimental animals. Normal mucosal and submucosal layers were noticed and no malignant neoplasms or pre-malignant lesions were seen in the colon of control animals (Figure 1). DMH-treated rat colon shows carcinomatous glands of varying shapes and sizes. Glands were filled with mucin and lined with pleomorphic cells (Figure 2). Figure 3 shows lining

Table 1. Incidence of colon and intestinal tumours/polyps.

Rat Group	No. of rats with tumours/polyps/ total no. of rats	Incidence of colon tumours/polyps (%)	No. of tumors/polyps in colon per tumour-bearing rat	No. of tumours/polyps in distal intestine per tumour-bearing rat
Control	-	-	-	-
DMH	28 / 30	93.3	24	10
Turmeric	-	-	-	-
Turmeric + DMH	20 / 30	66.6	7	3

Table 2. Incidence of colon and intestinal tumours/polyps.

Rat Group	No. of rats with tumours/polyps/ total no. of rats	Incidence of colon tumours/polyps (%)	No. of tumours/polyps in colon per tumour-bearing rat	No. of tumours/polyps in distal intestine per tumour-bearing rat
Control	-	-	-	-
DMH	30/32	93.75	25	10
BDMC-A	-	-	-	-
DMH+BDMC-A	19/32	59.4	5	4
Curcumin	-	-	-	-
DMH+Curcumin	20/32	62.5	6	4

cells of mucosal glands with marked dysplasia. In the colon of turmeric- (Figure 4), BDMC-A- (Figure 5) and curcumin-treated control animals (Figure 6), normal mucosal and submucosal layers were noted. In the colon of animals treated with DMH+turmeric, DMH+curcumin and DMH+BDMC-A (Figures 7, 8 & 9), well-organized dense lymphocyte infiltration in the form of aggregates was noticed.

Figures 10 and 11 show the activities of β-glucuronidase in the colon and colon contents in the different groups of animals. β-glucuronidase activity was significantly increased in all the tissues and colon content of the DMH group, compared to control group, whereas in the DMH+turmeric, DMH+curcumin and DMH+BDMC-A groups, the activity was significantly decreased when compared to the DMH group. There was no significant difference in the activity of

β-glucuronidase between DMH+BDMC-A and DMH+curcumin group. The values in BDMC-A- and curcumin-treated control groups do not differ significantly from untreated control.

Figures 12 and 13 summarise the activities of mucinase in the colon and fecal contents in different groups. In the DMH group, the activity was significantly raised when compared to the control group. In the DMH+turmeric, DMH+curcumin as well as in the DMH+BDMC-A groups, the activity was significantly lowered when compared with the DMH group. Between the DMH+BDMC-A and the DMH+curcumin groups, the activity did not differ significantly. The values in the BDMC-A- and curcumin-treated control groups did not differ significantly from untreated control.

DISCUSSION

Histopathological observation shows the absence of tumour cells or adenocarcinoma in DMH+turmeric, DMH+curcumin and DMH+BDMC-A-treated rat colon. However, dense lymphocyte infiltrates, well organized in the form of aggregates, were noticed. These results substantiate the antitumourigenic effects of turmeric, curcumin and BDMC-A. It is interesting to note that the tumour inhibitory effect of BDMC-A is comparable to that of turmeric and curcumin (Tables 1 and 2). The presence of antimutagenic and anticarcinogenic phenolic constituents in turmeric may contribute to its tumour inhibitory action. Therefore, BDMC-A can act as a chemopreventive agent against DMH-induced colon carcinoma. Furthermore, the colon of the rats administered with BDMC-A or curcumin, exhibited normal mucosal layers without any pathological changes, showing that BDMC-A is non-toxic.

BDMC-A contains hydroxyl groups in the ortho-position of the aromatic ring system [13]. In a study involving a two-stage mouse skin tumour-promotion model, Anto et al. (1996) [14] suggested that the presence of hydroxyl groups in the ortho-position of the aromatic ring is critical for the anticarcinogenic property of BDMC-A [14]. They demonstrated that BDMC-A was the most effective tumour inhibitor among a series of curcuminoids [14]. Thus, the observed chemopreventive potential of BDMC-A in DMH-treated rats (as

evidenced by the absence of tumor and histopathological changes) may be attributed to the existence of ortho-hydroxyl groups in the aromatic ring. BDMC-A is a phenolic compound. It has been demonstrated that phenolic compounds have the ability to form adduct with the carcinogen and prevent its tumourigenic action [15]. Hence, the chemopreventive action of BDMC-A could also be due to its phenolic nature.

Liver is the major site of detoxification of many drugs, toxins, carcinogens and hormones. One of the main functions of liver is to conjugate these substances with glucuronides, thus making them soluble and at the same time detoxifying them. They are then excreted via the bile into the intestine. When they reach the colon, normally more than 90% is excreted and little is reabsorbed [16]. When the activity of colonic microfloral β-glucuronidase is stimulated due to the presence of the procarcinogen DMH, these glucuronide conjugates may undergo hydrolysis, leading to the liberation of toxic substances that can be harmful to the colonocytes. Entero-hepatic circulation of the active products formed by β-glucuronidase triggers neoplastic changes in the colonic and intestinal mucosa [17, 18]. Hence, we suggest that DMH enhances the conversion of normal colonic epithelial cells into cancerous ones by enhancing the activity of β-glucuronidase.

Mucins are glycoproteins consisting of a large number of carbohydrate side-chains attached to a protein core. Mucin serves as a barrier and protects the intestinal mucosa against bile acids, carcinogens and toxins present in the lumen [19]. Mucinase, on the other hand, hydrolyses/degrades the protective mucin and exposes the underlying cells to the toxic metabolites [5]. We have observed an increase in mucinase activity in the fecal and colon contents of DMH-treated rats. This may result in the degradation of mucin coat and exposure of the underlying tissues towards the bile acids and deconjugated carcinogens that are liberated into the colonic lumen by β-glucuronidase activity. Thus, we suggest that the carcinogenic DMH metabolites gain access to the colonocytes by increasing the microbial mucinase activity and initiate carcinogenesis.

Intragastric administration of turmeric, curcumin and BDMC-A decreased the activity of β-glucuronidase (in the colon, intestine, liver and colon contents) and mucinase (in the fecal and colon contents)

in DMH-treated rats. This shows that turmeric, curcumin and BDMC-A may decrease the hydrolysis of carcinogen-glucuronide conjugates, thereby decreasing the conversion of detoxified components into toxins in the intestine.

Decreased mucinase activities by turmeric may be due to its coloring agent, curcumin. Curcumin was shown to increase mucin secretion and prevent ulceration in the stomach of mice [20]. Mucins form gels that coat the intestinal mucosa and also function as a lubricant and probably as a chemical or mechanical barrier against bacteria, viruses and toxins [19]. The increase in mucin secretion induced by curcumin may prevent the underlying colonocytes from the attack of carcinogens. BDMC-A, being an analog of curcumin, may also enhance mucin secretion and prevent the epithelium from gaining access to the carcinogen.

BDMC-A has a phenolic structure [14]. According to Setchell et al. (1980) [21] and Jenab et al. (1996) [22], phenolic compounds exhibit antiproliferative and antiangiogenic activities in carcinogen-treated animals. Phenolic compounds have been suggested to protect rats against high-fat induced colon tumorigenesis by modulating gut microbial enzyme activity [23]. From these evidences, it could be demonstrated that BDMC-A, being a phenolic compound, exerts anticarcinogenic effect by decreasing the activities of β-glucuronidase and mucinase in the DMH-treated rats fed with high fat diet. The decreasing effect is comparable with that of turmeric and curcumin. To conclude, modulation of gut microfloral enzyme activities by BDMC-A, in the presence high fat diet, may play a critical role in the chemoprevention of DMH-induced colon carcinogenesis.

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Table 3. Histopathological Changes in Colon

	Control	DMH	T	T + D	BDMC-A	DMH + BDMC-A	Curcumin	Curcumin+DMH
MACROSCOPY	-	2 cm	-	0.5 cm	-	<0.5 cm	-	<0.5 cm
Size	-	Pedunculated	-	Sessile	-	Sessile	-	Sessile
Nature	-	Well defined	-	Ill defined	-	Ill defined	-	Ill defined
Margin								
MICROSCOPY	-	+++	-	-	-	-	-	-
Transitional zone with foci of dysplasia	-	Well differentiated	Signifi-cant	Significant	-	-	-	-
Lymphoid aggregates in the submucosa								
Papillary pattern	-	Large number of papillae	-	Occasional papillae	-	Few papillae	-	Few papillae
Mucinous secretion	-	Lumen is dialated and filled with mucin in which clumps of tumor cells float	-	-	-	-	-	-
Infiltration in the submucosa	Normal submucosa without infiltration	-	-	-	Normal submucosa	Dense lymphocyte infiltration in the submucosa organized in the form of aggregates	Normal submucosa	Dense lymphocyte infiltration in the submucosa organized in the form of aggregates
CELL MORPHOLOGY	-	Marked	-	Less severe	-	-	-	-
Nuclear pleomorphism	-	Prominent	-	Less prominent	-	Less prominent	-	Less prominent
Nucleoli	-	Scanty	-	Scanty	-	Moderate	-	Moderate
Cytoplasm	-	Numerous	-	Few present	-	-	-	-
Mitotic figures	-	Focal areas of fibroblastic reaction	-	-	-	-	-	-
Vascular granulation								

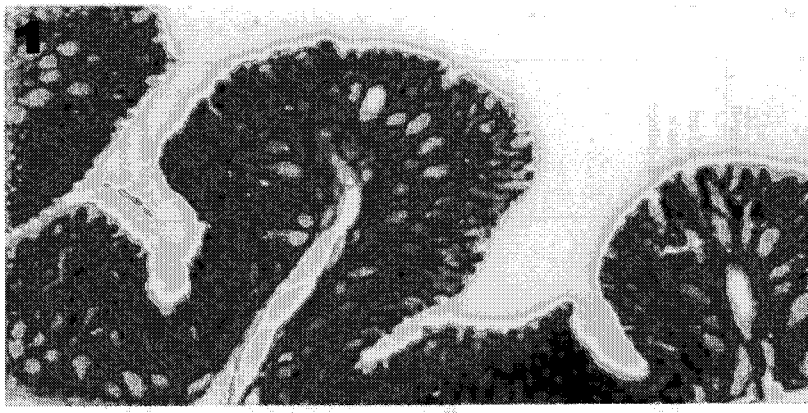

Figure 1. Colon of control rat with normal mucosa and submucosa

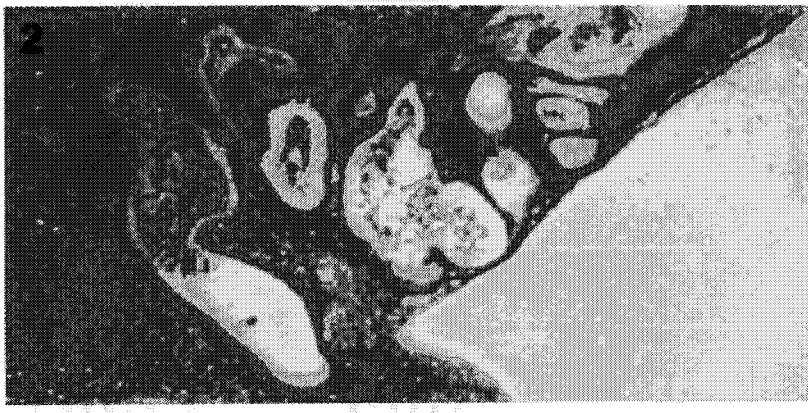

Figure 2. Colon of DMH-treated rat with carcinomatous glands

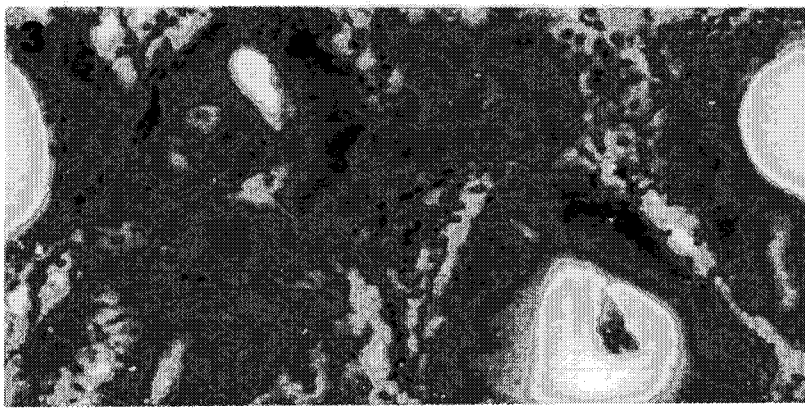

Figure 3. Colon of DMH-treated rat showing mucosal glands with dysplasia

Figure 4. Colon of turmeric-treated rat with normal mucosa and submucosa

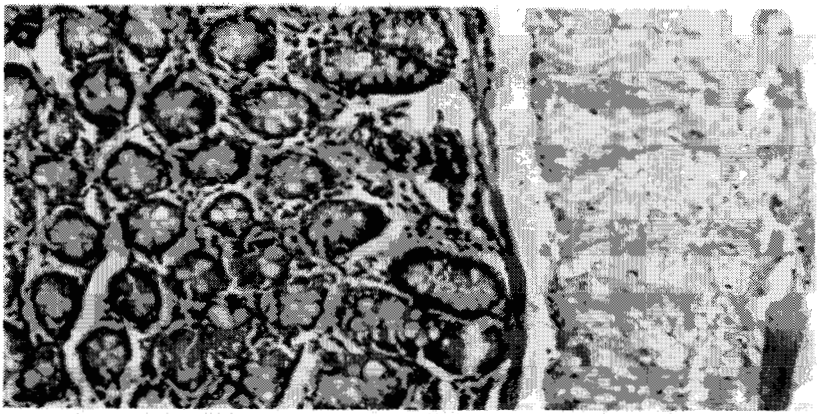

Figure 5. Colon of BDMC-A-treated rat with normal mucosa and submucosa

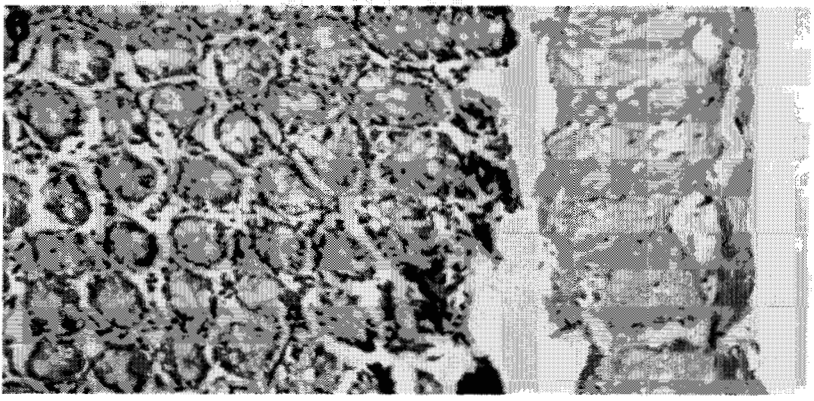

Figure 6. Colon of curcumin-treated rat with normal mucosa and submucosa

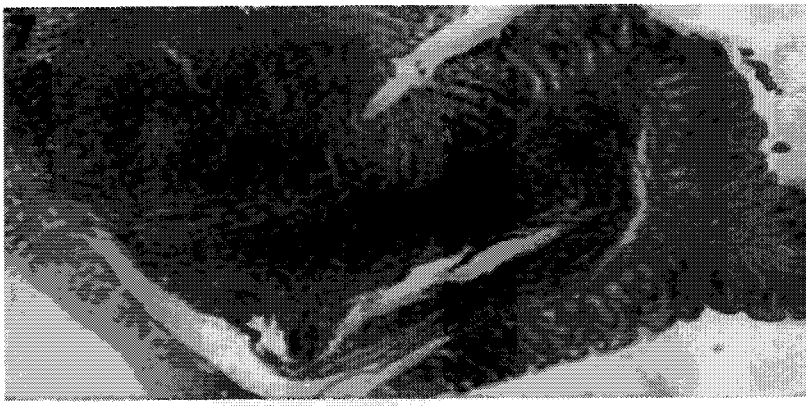

Figure 7. Colon of DMH + Turmeric-treated rat with lymphocyte infiltration

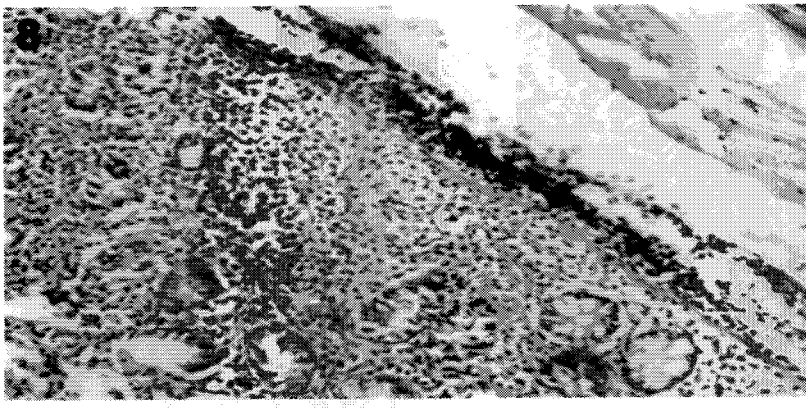

Figure 8. Colon of DMH + Curcumin-treated rat with lymphocyte infiltration

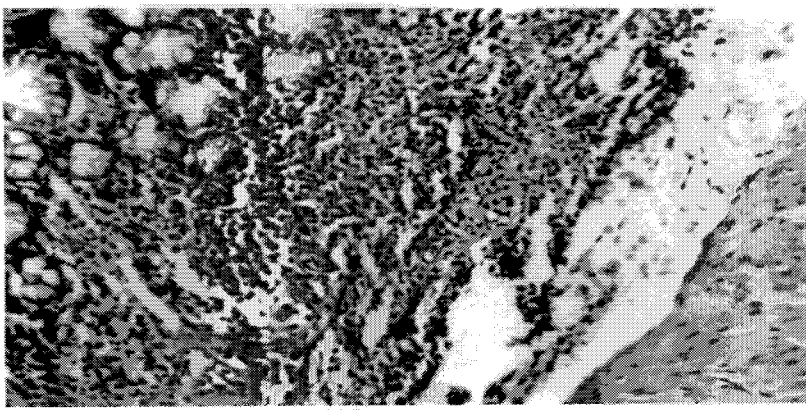

Figure 9. Colon of DMH + BDMC-A-treated rat with lymphocyte infiltration

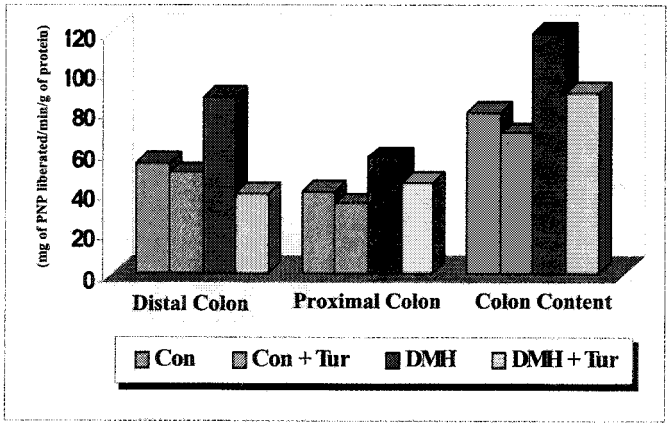

Figure 10. Activity of β - Glucuronidase

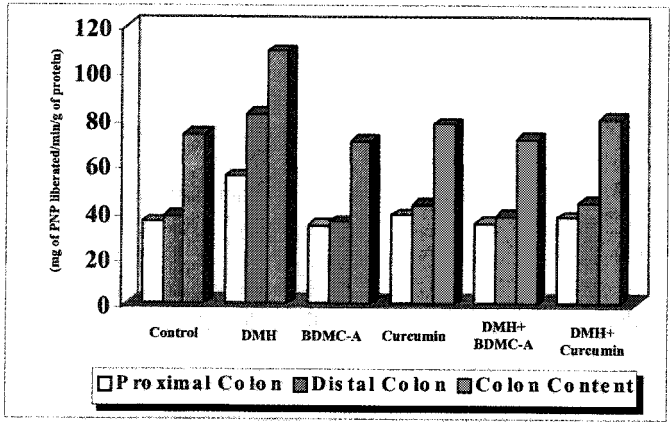

Figure 11. Activity of β - Glucuronidase

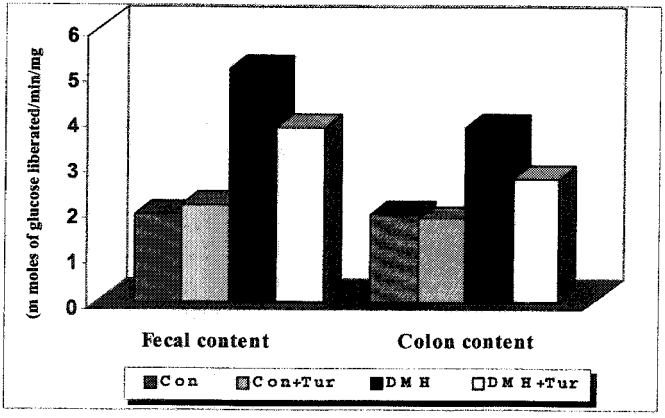

Figure 12. Activity of Mucinase

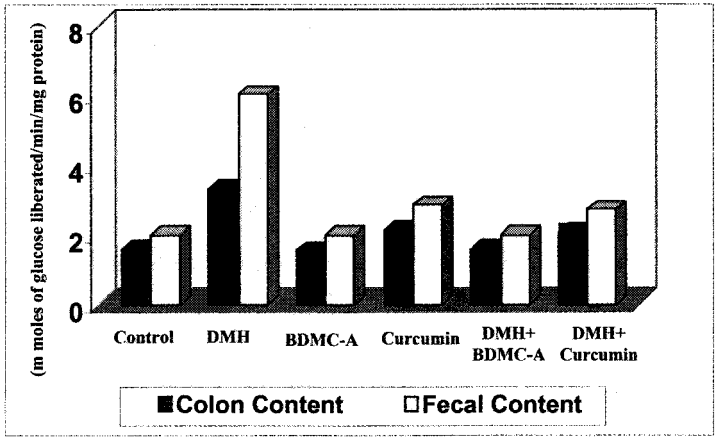

Figure 13. Activity of Mucinase

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Immunotherapy of Bladder Cancer Using Microbes

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INTRODUCTION

Bladder cancer is the ninth most common cancer in Singapore [1] and the seventh most common cancer amongst males worldwide [2]. The frequency in men is three times that in women.

More than 90% of bladder cancers are transitional cell carcinomas and the majority present as superficial (80%) rather than invasive (20%). Though not 'life threatening' bladder cancer is characterized by frequent recurrences which may progress to more malignant muscle invasive disease (10-30% of patients). Some 50% of patients with muscle invasive disease die of metastases within 5 years [3].

BCG has been used as a vaccine for tuberculosis from 1921 but it was only in 1935 that it was first used to treat cancer. However, BCG gained popularity as a therapeutic agent for cancer from the late 1950's to early 1960's mainly due to the work of Coe and Feldman [4] who showed that the bladder was capable of mounting an immune response. Zbar et al. [5] also showed that intra-tumoural BCG could cure animals of implanted tumours. In 1976, Morales et al. [6] reported that BCG eliminated visible tumours and prevented recurrences in all but 1 out of 9 patients with superficial bladder cancer. Subsequently a number of prospective trials confirmed this result. The average response rate between transurethral resection (TUR) and TUR+BCG based on 5 studies involving 437 patients carried out between 1985-1991 was 31% for TUR alone and 50-70% for TUR followed by BCG [7].

At present TUR followed by BCG immunotherapy is the treatment of choice for bladder cancer. BCG is believed to act by inducing a non-specific immune response that results in the removal of remnant cancer cells by sloughing off both normal as well as abnormal urothelial cells [8]. An intact immune system is required as shown by animal studies where the response to BCG could be abrogated in nude mice indicating a need for T lymphocytes [9, 10]. NK cells are also required for BCG immunotherapy to work [11].

Some limitations do exist, namely a significant proportion of patients do not respond to BCG therapy; furthermore, local side effects such as cystitis, giving rise to symptoms like frequency and urgency, dysuria, and occasional haematuria as well as systemic effects such as fever, transient influenza-like illness, do occur.

Lactobacilli have established beneficial effects, for example, in the treatment of lactose-intolerant individuals, prevention of diarrhoea and urogenital infections, lowering blood cholesterol levels and enhancing the immune system [12 -16]. *Lactobacillus* spp. have been used as probiotics and are found in food supplements eg. fermented milk drink (Yakult) containing *L. casei* strain Shirota – LcS, and Culturelle probiotic pills containing *L. rhamnosus* strain GG - LGG.

The antitumour activity of *Lactobacillus casei* (LC 9018) against experimental mouse bladder tumours (MBT-2 cells in C3H mice) has been reported by Asano et al. [17]. Bladder cancer patients who received a Lactobacillus preparation, BLP (biolactis powder), had a significantly longer disease-free interval compared to patients who did not receive *L. casei* [18]. Aso et al. [19] reported that in a double-blind trial to test the efficacy of a *Lactobacillus casei* preparation given orally to patients, *L casei* had a prophylactic effect in those patients who either had primary multiple tumours or a single recurrent tumour.

MATERIALS AND METHODS

Anti-proliferative effects

Cell lines used are the human bladder cancer cell lines — RT112, MGH and HUCT-2 and HUC-1, which is the SV40-transformed normal human bladder epithelial cell line. The murine bladder cancer

cell, MB49, and the SV40-transformed kidney cells, TMCK, were used as well. All cells were plated at a density of 1×10^5 and after 18 hours were exposed to either *Lactobacillus casei* strain Shirota (LcS) or the *Lactobacillus rhamnosus* GG (LGG) at a concentration of 1×10^7 or 1×10^8 cfu/ml or *Mycobacterium bovis* (Bacillus Calmette Guerin, BCG) at 1×10^7, for 24, 48 and 72 hours in the presence (PGS) and absence (PG) of streptomycin. The cells that were alive after the period of exposure were counted with a haemocytometer or analyzed by Annexin V staining.

Dose and time of exposure

Uroepithelial cells were incubated with LGG (1×10^3–1×10^9) for 2 hours and 48 hours, after which the bacteria were washed off before addition of ^{3}H-thymidine to the media. 24 hours later, the amount of ^{3}H-thymidine incorporated into DNA was determined by total cell lysis.

In vivo therapy

Murine MB49 bladder tumours cells (1×10^5 cells) were implanted subcutaneously on the flank of C57BL/6 mice. The mice were then divided into three groups. The control group was fed saline daily. The second group, the 'Fed immediate group", was given LGG daily while the last group was fed LGG daily from the 7th post-impant day. All mice except for the last group were fed for a period of 48 days. The tumour volume and the weight of mice were monitored every 3-4 days [20].

RESULTS

A comparison of the effects of Lactobacillus species and BCG on bladder cancer cells

As shown in Table 1, both *Lactobacillus species* significantly suppressed proliferation [21]. With 1×10^8 cfu *L. casei Shirota,* a significant cytotoxic effect was observed as early as 24 hours after exposure. While the effect of BCG was comparable with that of *L. rhamnosus GG* on MGH cells at the same dosage, BCG only reduced the growth of RT112 cells by 12% after 72 hours of exposure.

Table 1. The effect of *Lactobacillus sp.* and BCG on MGH and RT112 cell proliferation in PG media

Cell Line	MGH ($x10^5$)			RT112 ($x10^5$)		
Hours	24	48	72	24	48	72
Control	2.11 ±0.98	7.27 ±0.6	14.76 ±5.92	0.31 ± 0.18	2.34 ±1.32	4.12 ±1.21
LGG $x10^7$	1.65 ±1.27	3.75 ±1.18[a]	3.8 ± 1.45[a]	0.21 ± 0.12	0.18 ±0.06[a]	0.76 ±0.79[a]
LGG$x10^8$	1.07 ±0.43	0.99 ±1.76[a]	1.9 ± 0.9[a]	0.07 ±0.08	0.26 ±0.44[a]	0.01 ±0.13[a]
Control	0.95 ±0.37	2.0 ± 0.59	6.04 ±2.26	0.59 ±0.19	1.57 ±0.56	3.72 ±1.04
LcS$x10^7$	0.64 ±0.35	0.91 ±0.81	0.58 ±0.85[a]	0.41 ± 0.03	0.06 ±0.04[a]	0.01[a]
LcS$x10^8$	0.04 ±0.06[a]	0.07 ±0.05[a]	0.03 ±0.04[a]	0.01[a]	0.01[a]	0.004 ±0.01[a]
Control	1.12 ± 0.27	3.35 ± 0.99	7.12 ±1.89	1.19 ± 0.17	2.28 ± 0.10	4.69 ± 0.02
BCG$x10^7$	0.64 ± 0.06	1.53 ± 0.94	1.54 ±0.57[a]	0.91 ± 0.14	1.86 ± 0.17	4.13 ± 0.56

Cell numbers are the average of three experiments each done in duplicate ± SD. [a] denotes values that are significantly different from control ($p<0.05$) LGG represents *Lactobacillus rhamnosus GG* and LcS represents *Lactobacillus casei strain Shirota*

The anti-proliferative effects of BCG coincided with its internalisation by the bladder cancer cell lines in contrast to the Lactobacilli.

The bacterial culture components — lactic acid, the pH of the medium after cells were exposed to the Lactobacilli — were excluded as possible causes of the anti-proliferative effects. Rather the abrogation of the cytotoxic effect by streptomycin indicates that a bacterial protein product may be necessary for the inhibition observed (Table 2). This protein product could act either directly on the bladder cancer cells or indirectly by inducing the cancer cells to produce other proteins that are in turn responsible for inducing the anti-proliferative effects.

The supernatant from cells cultured with Lactobacilli after passage through a 0.2 μm filter to remove bacteria was able to induce

Table 2. The effect of *Lactobacillus sp.* and BCG on MGH and RT112 cell proliferation in Pen GS media.

Cell Line	MGH ($x10^5$)			RT112 ($x10^5$)		
Hours	24	48	72	24	48	72
Control	0.69 ± 0.56	4.58 ± 1.84	10.47 ±3.15	0.71 ±0.37	1.75 ± 0.58	6.11 ± 2.35
LGG $x10^7$	0.77 ±0.77	4.43 ±2.11	9.36 ±2.32	0.67 ± 0.32	1.96 ± 0.97	5.4 ±1.84
LGG$x10^8$	0.91 ±0.89	2.92 ±1.66	7.53 ±4.83	0.71 ±0.35	1.76 ± 1.14	3.73 ± 2.98
Control	1.02 ±0.83	1.68 ±0.96	6.41 ±4.21	0.29 ±0.06	1.96 ± 0.51	3.78 ± 1.79
LcS$x10^7$	0.88 ±0.6	1.72 ±1.29	6.62±4.79	0.32 ±0.06	1.62 ± 0.36	3.64 ± 0.74
LcS$x10^8$	0.35 ±0.5	0.69 ±1.02	2.78 ±4.69	0.16 ±0.18	1.11 ± 2.29	1.55 ±3.22
Control	1.04 ± 0.54	2.99 ± 2.03	7.10± 1.53	1.00 ± 0.02	2.3 ± 0.32	4.58± 0.38
BCG$x10^7$	0.56 ± 0.29	1.27 ± 0.90	1.76± 0.86 [a]	0.89 ± 0.03	1.96 ± 0.2	3.82 ± 0.1

Cell numbers are the average of three experiments each done in duplicate ± SD. [a] denotes values that are significantly different from control ($p<0.05$). LGG represents *Lactobacillus rhamnosus GG* and LcS represents *Lactobacillus casei* strain *Shirota.*

similar anti-proliferative effects as that observed when bladder cancer cells were directly exposed to Lactobacilli. In these experiments, spent media was mixed with an equal volume of fresh media. Similar spent media obtained from control cells (not exposed to bacteria) did not have any inhibitory effect, this indicates that the observed results were not due to any lack of components in the spent media (Figure 1).

A comparison of the effects of Lactobacillus species on normal and tumourigenic cells

As shown in Figure 2, there was a differential decrease in the tumourigenic HUCT-2 cells as compared to the non-tumourigenic HUC-1 cells. Further, as we have previously reported for bladder cancer cells, this effect of LGG was attenuated by the presence of streptomycin in the media.

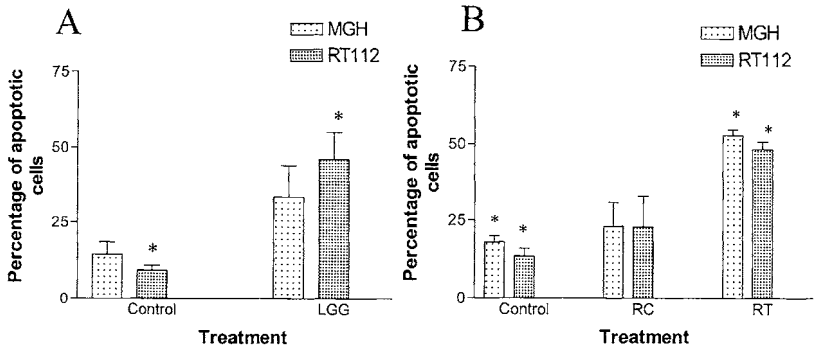

Figure 1. LGG-induced apoptosis of human bladder cancer cells.
LGG induces apoptosis in both MGH and RT112 cells (A).
Spent media from MGH and RT112 cells treated with LGG were able to induce apoptosis on RT112 cells (B).

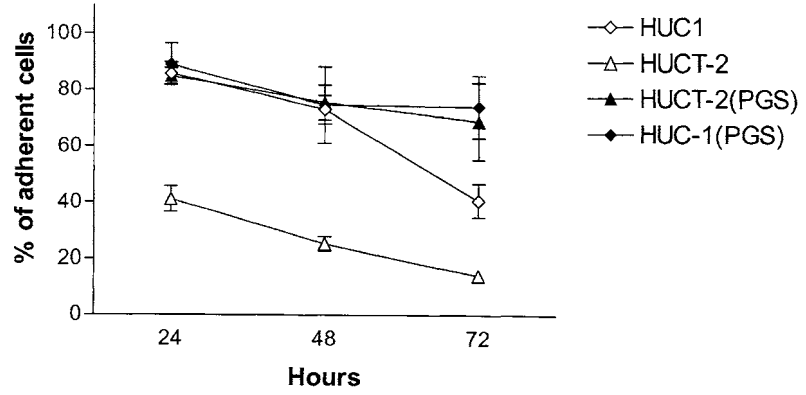

Figure 2. Anti-proliferative effects of LGG on murine bladder cancer cells and a SV40- transformed cell line.
Cells were exposed to LGG in the presence (PGS) and absence of streptomycin.

In order to mimic *in vivo* intravesical therapy where BCG is instilled for 2 hours, uroepithelial cells were incubated for as little as 2 hours with LGG. 1×10^7 to 1×10^9 viable LGG induced inhibition of ^{3}H-thymidine incorporation in both cell lines (Figures 3 A and B). In contrast with a 48 hour exposure, there was reduced ^{3}H-thymidine incorporation in both cell lines in the presence of lower concentrations of LGG at 1×10^3 to 1×10^9 viable bacteria (Figures 3 C and D).

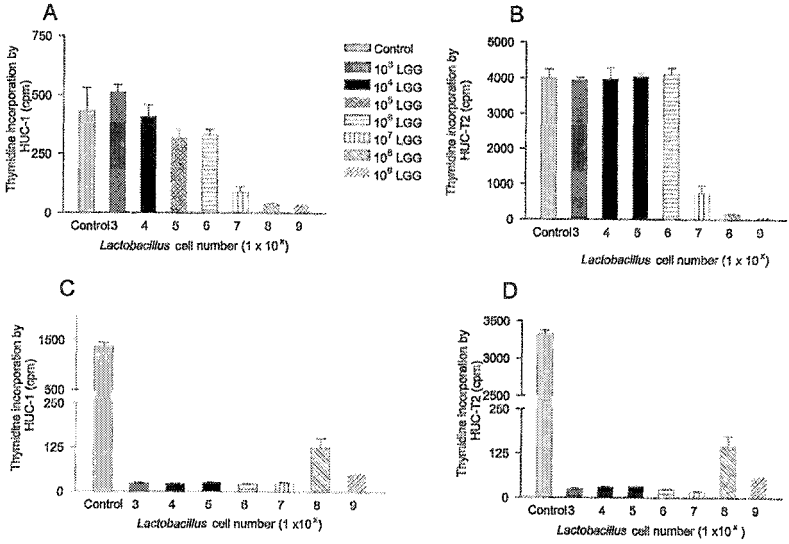

Figure 3. Dose and time-related anti-proliferative effects of LGG.
HUC-1 and HUCT-2 cells were exposed to LGG (1×10^3–1×10^9) for 2 (A and B) and 48 (C and D) hours and after removal of the bacteria, the proliferative potential of these cells were measured by labeling cycling cells with ^{3}H-thymidine.

Differential effects of LGG and LcS on uroepithelial cancer and normal cells

The murine bladder cancer cells MB49 and the SV40 transformed TMCK line were exposed to 10^8 LGG or LcS for 24, 48 and 72 hours. While exposure to LGG resulted in the same differential response observed with the matched human bladder cancer and normal cells, exposure to LcS resulted in both cell lines being equally inhibited (Figure 4A). The presence of streptomycin again inhibited this effect (Figure 4B).

Lactobacillus and bladder tumours in vivo

The "Fed immediate" mice had smaller average tumour volume than the "Control" mice while two "Fed immediate" mice had no tumour. There were no differences in the spleen cell populations between the 3 groups of mice. However, there were more granulocytes and a significant increase in lymphocytes at the tumour site in the "Fed immediate" group compared to the control group.

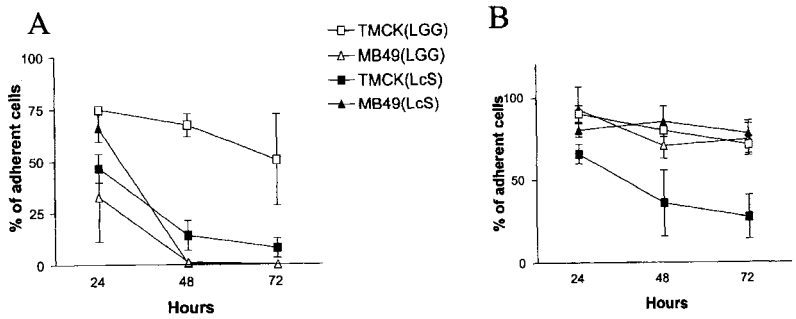

Figure 4. Anti-proliferative effect of Lactobacillus species on murine normal and cancer cell lines.
Murine cell lines were exposed to LGG in the absence (A) and the presence (B) of streptomycin.

When a perceptible tumour mass was present before treatment, the microbe was unable to cure mice. Takahashi et al. [22], who reported the effect of *L. casei* strain Shirota on orthotopically-implanted murine bladder tumours obtained from MBT-2 tumour cells implanted in the C3H/He mouse model, used intravesical *L. casei* and BCG to compare the efficacy of these 2 microbes. They reported that *L. casei* was more efficacious than BCG. However, this finding is non-conclusive as the MBT-2 murine bladder cancer model is known to be non-responsive to BCG therapy.

CONCLUSION

Both *Lactobacillus* strains had anti-proliferative effects on bladder cancer cells lines. The effects were not as pronounced with SV40-transformed normal bladder cells. This anti-proliferative effect seemed to be dependent on bacterial protein synthesis. It is not clear from our results whether it is a bacterial protein or a mammalian cell protein that induces the anti-proliferative effects and apoptosis in cancer cells exposed to Lactobacilli. We were able to cure some tumour-bearing mice by feeding them LGG while mice fed LGG also generally had smaller tumours. Our data thus indicate that Lactobacilli may be useful in the therapy of bladder cancer.

ACKNOWLEDGMENT

Some data was reproduced from "Lim BK, Mahendran R, Lee YK, Bay BH. Chemopreventive effect of Lactobacillus rhamnosus on growth of a subcutaneously implanted bladder cancer cell line in the mouse. Jpn J Cancer Res. 2002; 93(1): 36-41" with permission of Oxford University Press. Tables 1 and 2 and Figure 1 were reproduced from "Seow SW, Rahmat JN, Mohamed AA, Mahendran R, Lee YK, Bay BH. Lactobacillus species is more cytotoxic to human bladder cancer cells than Mycobacterium Bovis (bacillus Calmette-Guerin). J. Urol. 168:2236-22397, 2002" with permission of Lippincott Williams and Wilkins. This work was carried out with funding from the National Medical Research Council, Singapore.

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Modulation of Hypericin-Based Phototoxicity in Nasopharyngeal Cancer

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INTRODUCTION

Photodynamic therapy (PDT) is a relatively new treatment modality for cancer. The effects of PDT are mediated by light activation of a photosensitiser, which preferentially accumulates in tumour tissues [1]. Hypericin is a polycyclic aromatic naphthodianthrone extracted from plants of the *Hypericum* genus. It is described as one of the most powerful photosensitisers found in nature [2] and has been investigated as a promising photosensitiser for both PDT and photodynamic diagnosis. In previous studies, we have shown that hypericin-PDT induces lipid peroxidation [3] and modulate the expression of cytokines [4] in nasopharyngeal cancer (NPC) cells.

Photoactivated hypericin is known to generate a high quantum yield of singlet oxygen ($^1O_2$) through energy transfer (type II process) [5]. In addition, reduced oxygen species, including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are also produced (type I process) [6-8]. Reactive oxygen species (ROS) generated in this process is responsible for mediating the phototoxicity of hypericin. As unsaturated lipids in cell membrane, lipoproteins and other lipid-containing structures are prominent targets of ROS, lipid peroxidation is an important deleterious event associated with photooxidative stress. Lipid hydroperoxide (LOOH) produced by $^1O_2$ or $^{\cdot}OH$ may generate epoxy-allylic peroxyl radical ($OLOO^{\cdot}$), thereby triggering free radical chain peroxidation [9]. Photoactivated hypericin has been observed to induce an increase of malonaldehyde

content in NPC tumour tissue, an established index of lipid peroxidation [3].

There is strong evidence that antioxidants delay significantly or prevent the formation of ROS. Antioxidant enzymes such as superoxide dismutase are known to be increased in response to hypericin photoactivation [10]. Another approach that has been employed to modulate photodynamic effects is the utilization of scavengers of $^1O_2$ and free radicals. In this study, the protective efficacy of sodium azide, mannitol, butylated hydroxytoluene (BHT), and α-tocopherol were evaluated in hypericin-PDT treated nasopharyngeal (NPC) cells. Sodium azide, a $^1O_2$ scavenger, has been shown to effectively quench $^1O_2$ produced during PDT [11,12]. Mannitol, a hydroxyl radical scavenger, was reported to exert protection against ALA-based PDT damage [13]. Butylated hydroxytoluene (BHT) has been shown to intercept lipid peroxyl radical (LOO·) and lipid oxyl radical (LO·) [14]. BHT is particularly effective in suppressing oxidation of animal fats and has been used as an additive in the food industry [15]. α-tocopherol, an important chain breaking antioxidant which scavenges lipid radicals [16,17], has been reported to offer protection against protoporphyrin IX (PP IX) phototoxicity [18].

MATERIALS AND METHODS

Cell culture and treatment

CNE-2 cells were established from a poorly differentiated nasopharyngeal carcinoma and HK1 cells were obtained from a recurrent well-differentiated squamous carcinoma of the nasopharynx [19]. The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), supplemented with streptomycin/penicillin (100 U/ml), glutamine (2 mM) and sodium pyruvate (2 mM) under a water-saturated sterile atmosphere containing 5% CO_2. Logarithmically growing NPC cells were incubated with hypericin (Molecular Probes, Eugene OR, USA) prepared in FBS free medium with the desired concentration (0–0.5 μM) for 4 hours. Prior to light illumination, cells were washed with PBS and medium containing FBS was added to the cells. Light irradiation was performed with a light dose of 0.5 J/cm^2. A bank of fluorescent tubes (Philips Type OSRAM

L30w11-860, 30W) filtered with red acetate filter (No. 17 Roscolux, Rosco, CA, USA) was used to obtain wide band illumination with wavelength above 585 nm. The light intensity of the illuminator was about 1.1 mW/cm^2.

Photocytotoxicity assay

Viability of cells after hypericin-PDT treatment was assessed by the 3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, MO, USA). After light irradiation, cells were incubated in the dark for 20 hours. MTT was added to wells at a concentration of 0.5 mg/ml for 2 hours to allow MTT metabolism. Following the incubation, the blue formazan crystals were dissolved in DMSO, and the absorbance at 570 nm was determined using a spectrometer (Tecan, Australia). The cell survival was calculated with the formula: Survival (%) = O.D. value of treated cells/ O.D. value of control cell $\times$100%.

Scavengers and incubation

BHT was dissolved in DMSO and α-tocopherol in absolute ethanol and diluted in PBS to yield concentrations from 0.1–10 μM. The final concentration of DMSO or absolute ethanol in the culture medium was < 0.1%. Mannitol and sodium azide were prepared in PBS and used at 0–10 mM. The scavengers in FBS free medium were added to the cells and incubated for 4 hours. Immediately following incubation, cells were further incubated with hypericin for 4 hours. After washing with PBS, the cells were treated with light in fresh medium in the presence of the scavengers. Subsequently, the cells were incubated for 20 hours to evaluate viability by MTT colorimetric assay.

RESULTS

Effect of scavengers on hypericin PDT-induced cytotoxicity

PDT induced approximately 45%–50% cell death in HK1 cells, whereas 30%–40% cell death was obtained in CNE-2 cells (Figures 1–4). The effect of scavengers employed in this study was evaluated under the hypericin dose and light dose that produce this

Figure 1

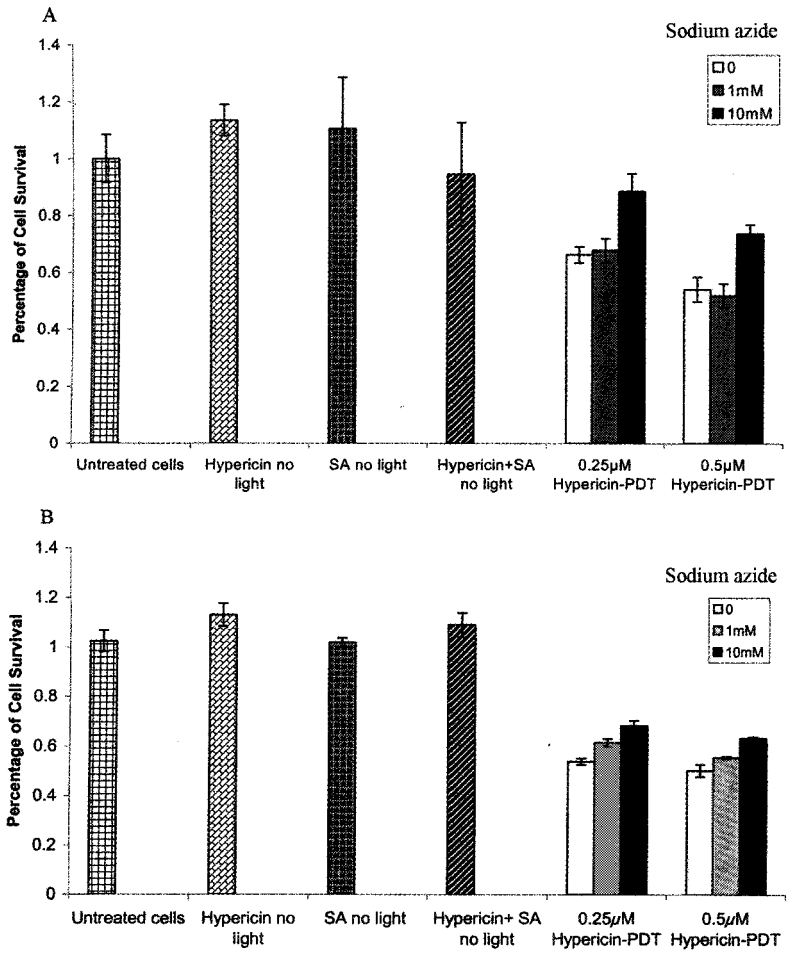

Figure 1. Effect of sodium azide on hypericin-PDT induced cytotoxicity in CNE-2 (A) and HK1 (B) cells.

1mM or 10mM sodium azide (SA) in FBS free medium was added to the cells and incubated for 4 hours. The cells were further incubated with 0.25μM or 0.5μM hypericin for 4 hours. After being treated with light at a dose of 0.5J/cm^2 in the presence of the scavengers, the cells were incubated for 20 hours to evaluate viability by MTT colorimetric assay. Untreated cells ⊞,cells incubated with hypericin alone ▨, cells incubated with SA alone ■, cells incubated with hypericin and SA without light treatment ▨, hypericin-PDT-treated cells □, hypercin-PDT-treated cells with 1mM SA ▤, hypercin-PDT-treated cells with 10mM SA ■. Each value represents the average of three determinations. Error bar shows standard error.

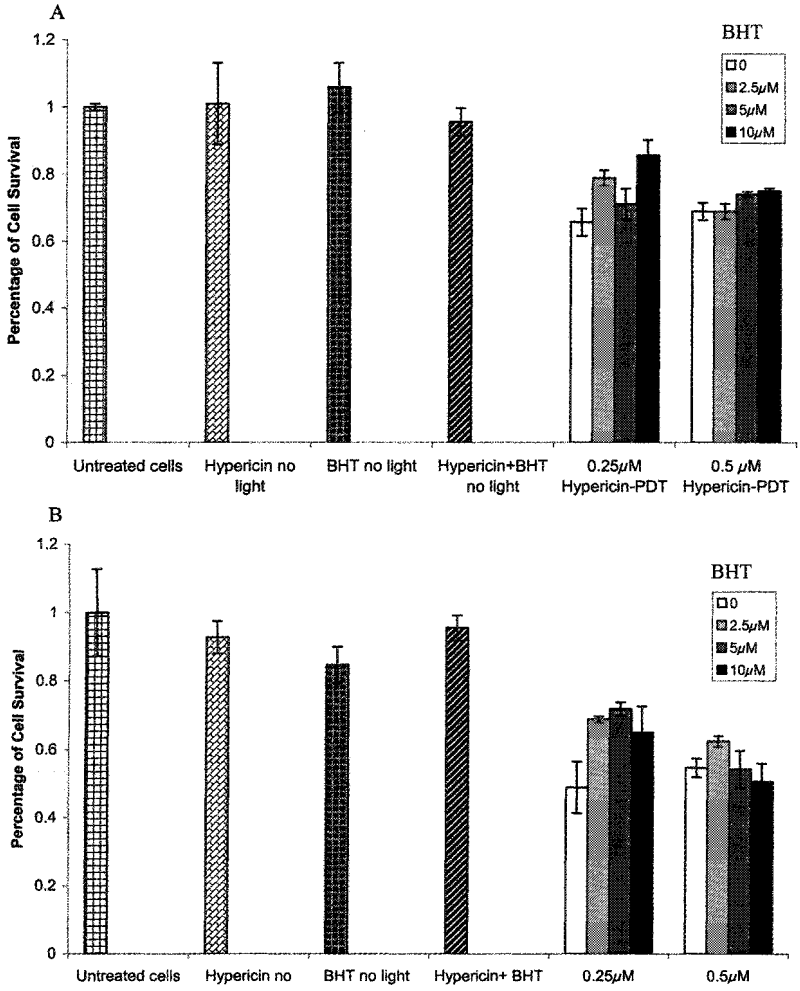

Figure 2. Effect of BHT on hypericin-PDT-induced cytotoxicity in CNE-2 (A) and HK1 (B) cells.

2.5 μM, 5 μM or 10 μM BHT in FBS free medium was added to the cells and incubated for 4 hours. The cells were further incubated with 0.25 μM or 0.5 μM hypericin for 4 hours. After being treated with light at a dose of 0.5J/cm^2 in the presence of the scavengers, the cells were incubated for 20 hours to evaluate viability by MTT colorimetric assay. Untreated cells ▦,Cells incubated with hypericin alone ▨, cells incubated with BHT alone ▩, cells incubated with hypericin and BHT without light treatment ▨, hypericin-PDT-treated cells □, hypericin-PDT-treated cells with 2.5 μM BHT ▩, hypericin-PDT-treated cells with 5 μM BHT ▩, hypericin-PDT-treated cells with 10 ìM BHT ■.

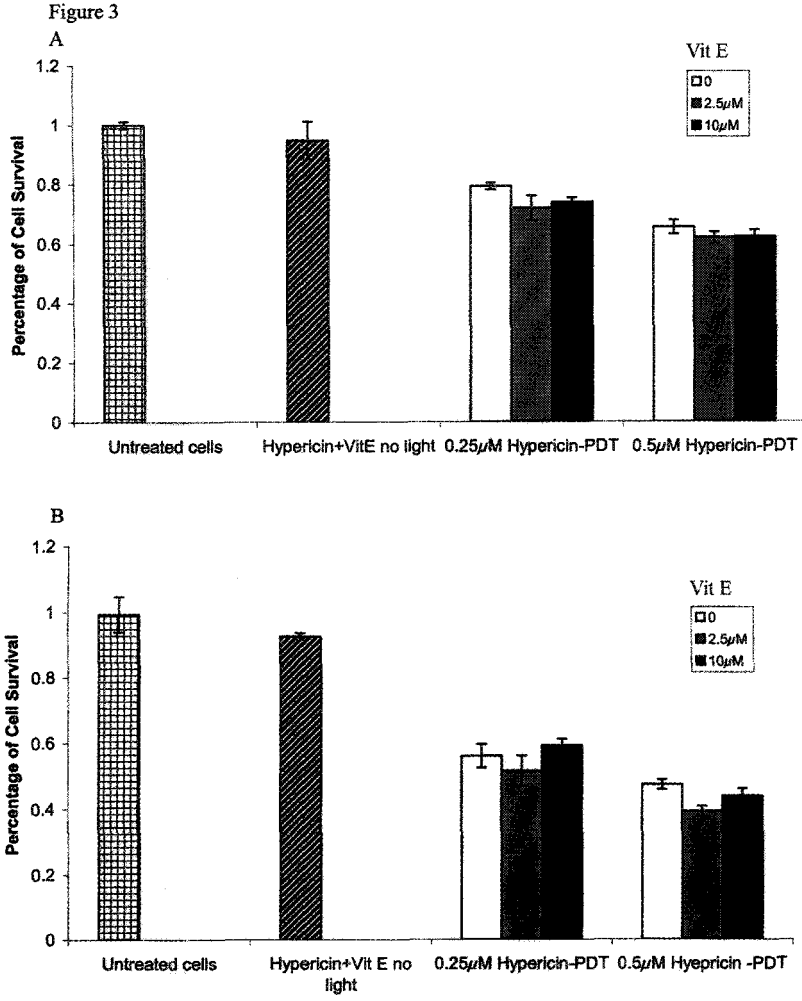

Figure 3. Effect of α-tocopherol (Vit E) on hypericin-PDT-induced cytotoxicity in CNE-2 (A) and HK1 (B) cells.
2.5μM. or 10 μM α-tocopherol in FBS free medium was added to the cells and incubated for 4 hours. The cells were further incubated with 0.25μM or 0.5μM hypericin for 4 hours. After being treated with light at a dose of 0.5J/cm^2 in the presence of the scavengers, the cells were incubated for 20 hours to evaluate viability by MTT colorimetric assay. Untreated cells ⊞, cells incubated with hypericin and α-tocopherol without light treatment ▨, hypericin-PDT-treated cells □, hypericin-PDT-treated cells with 2.5 μM α-tocopherol ▩,hypericin-PDT-treated with 10 μM α-tocopherol ■. Each value represents the average of three determinations. Error bar shows standard error.

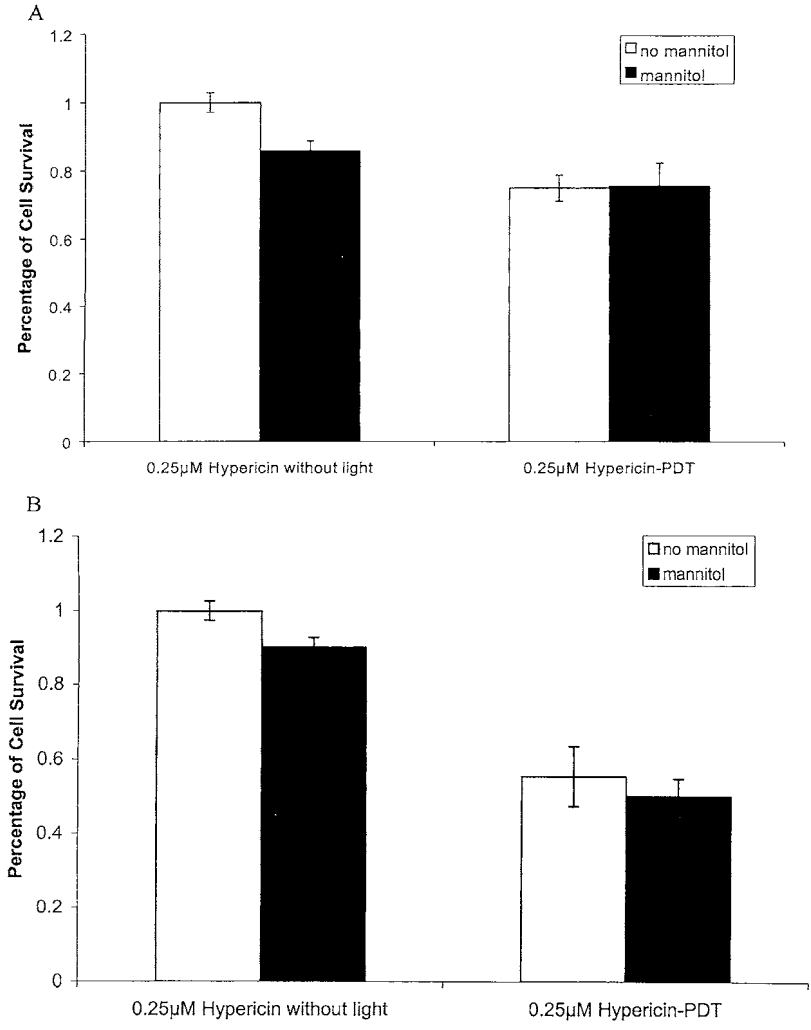

Figure 4. Effect of mannitol on hypericin-PDT-induced cytotoxicity in NPC/HK1 cells.

1mM mannitol in FBS free medium was added to the cells and incubated for 4 hours. The cells were further incubated with 0.25μM hypericin for 4 hours. After being treated with light at a dose of 0.5J/cm^2 in the presence of the scavengers, the cells were incubated for 20 hours to evaluate viability by MTT colorimetric assay. Cells without mannitol treatment □, cells with mannitol treatment ■. Each value represents the average of three determinations. Error bar shows standard error.

range of cytotoxicity. As shown in Figure 1A, 1mM sodium azide had minimal effect on cell survival in hypericin-PDT treated CNE-2 cells. However, cell viability of CNE-2 cells was significantly improved by pre-incubation with 10mM sodium azide. In fact, pre-treatment with 10 mM sodium azide produced a 22% increase in survival ($p=0.013$) when cells were incubated with 0.25μM hypericin followed by photoactivation, and approximately, 20% enhanced survival ($p=0.011$) with 0.5μM hypericin. As shown in Figure 1B, cell viability in HK1 cells pre-treated with 1 mM sodium azide was improved by 9% with 0.5μM hypericin incubation ($p=0.007$), and by 5% with 1mM hypericin ($p=0.048$). A higher concentration of sodium azide (10mM) provided a more significant protective effect, by boosting the survival rate to 15% with 0.5mM hypericin incubation ($p=0.0013$) and 13% with 1mM hypericin incubation ($p=0.0022$).

Co-incubation of cells with BTH also provided protection against hypericin-PDT induced cytotoxicity (Figure 2). The percentage of viable CNE-2 cells incubated with 0.25 μM hypericin followed by light irradiation was 65.6% ± 4% (mean ± SE; Figure 2A). Cell viability increased to 78.7% ± 2.3% ($p =0.024$) and to 85.5 % ± 4.6% ($p=0.015$) when cells were incubated with 5 μM and 10 μM BHT respectively. When cells were incubated with 0.5μM hypericin, the percentage of cell viability was enhanced by 4% with pretreatment of 10 μM BHT, as compared to without BHT pre-incubation ($p=0.04$). Similarly, the protective effect of BHT against hypericin phototoxicity was also observed in HK1 cells (Figure 2B), with an increase in survival rate of 20% after 0.5μM hypericin-PDT and 6% after 1 μM hypericin-PDT ($p <0.05$).

Sodium azide exerted a higher level of protection than BHT in CNE-2 cells (19.6% vs 3.9% when cells were treated with 0.5 μM hypericin-PDT). The protective effect of sodium azide was also more obvious in CNE-2 cells than in its more differentiated counterpart. However, incubation of cells with α-tocopherol or mannitol did not inhibit hypericin phototoxicity (as shown by Figure 3 and Figure 4).

It can be seen that the scavengers *per se* were not cytotoxic to NPC cells at the concentrations used. Co-incubation of cells with the scavengers and hypericin without light treatment did not influence cell viability. Furthermore, light illumination of the scavengers alone did not produce any deleterious effects. However, mannitol at higher concentrations (1 mM), decreased cell viability by 15% for CNE-2 cells ($p = 0.011$) and 10% for HK1 cells ($p = 0.027$).

DISCUSSION

In this study, we have shown that photoactivation of hypericin has the capacity to induce cell death. The inhibitory effects of scavengers have helped to provide information on the mechanism of photoperoxidation, including the relative importance of ROS in the process of hypericin photoactivation. It should also be borne in mind that the effectiveness of antioxidant protection against cytotoxicity may depend on the lipophilicity of the antioxidant which influences the incorporation into cells [18]. Sodium azide, a well-known and efficient singlet oxygen interceptor, was shown to be a good protector against hypericin-induced phototoxicity. On the other hand, the superoxide anion and hydroxyl radical quencher, mannitol, failed to protect. Since both sodium azide and mannitol are highly hydrophilic scavengers, we can conclude that singlet oxygen plays a predominant role in hypericin-PDT induced cell killing.

The production of $LO^{\cdot}$ and $LOO^{\cdot}$ is a downstream event in the process of lipid peroxidation [20]. LOOH, which accumulates via both type I reactions or type II reactions, would undergo iron-catalyzed reduction to $LO^{\cdot}$, giving rise to $LOO^{\cdot}$ [21]. It is therefore not surprising that BHT, a cell-permeant antioxidant against $LO^{\cdot}$ and $LOO^{\cdot}$, had a protective effect against cytotoxicity induced by hypericin photoactivation. On the other hand, Mark et al. [22] has previously reported that this lipid soluble chemical was unable to prevent para-aminophenol (PAP)-induced cytotoxicity in LLC-PK1 cells. In our study, it was also observed that sodium azide afforded more effective protection against hypericin-PDT than BHT. This may indicate that scavenging of upstream ROS is more efficient than clearing

downstream peroxidation products to reduce the photodynamic effects of hypericin.

In our study, α-tocopherol, a lipophilic chemical which acts as a chain breaking antioxidant to inhibit the chain peroxidation cascade, did not confer protection against cytotoxicity upon hypericin photoactivation. This is in accord with earlier studies which have also shown that α-tocopherol did not inhibit porphyrin-induced cellular phototoxicity in human Jurkat cells and fibroblast cells [18]. It has also been reported that á-tocopherol did not protect MRC-5 normal fibroblasts and HT29 adenocarcinoma cells from meta-tetra(hydroxyphenyl)chlorine-sensitized photodamage [23]. The fact that tumour cells are known to contain higher level of α-tocopherol than the cells of origin [24, 25] may provide a possible explanation for the low sensitivity of the NPC cells to á-tocopherol induced protection against hypericin phototoxicity.

In summary, we have shown that photoactivation of hypericin induces cytotoxcity in NPC cells and would therefore be a potential modality of treatment for this enigmatic cancer. Singlet oxygen is probably the main mechanism involved in hypericin-mediated phototoxicity as demonstrated by pre-treatment of cells with sodium azide. Incubating cells with BHT also afforded protection against hypericin-PDT. The findings gleaned from this study would also be useful in designing PDT strategies to protect normal cells against undesirable effects. Thus, it would be worthwhile to explore the findings further in an *in vivo* model.

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Research and Development of Natural Products in Developing Countries: Need for Venture Capital

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INTRODUCTION

Given the increasing interest in Natural Products as a source of raw material for use in the pharmaceutical nutraceutical, functional foods and dermaceutical industries, more resources are now being devoted to research and development, product development and commercialization of products derived from this source. However, for the move towards natural products to realize its full potential more and more resources must be introduced in a structured manner. Investment in research and development and product development are seen in the business arena, as very risky, and investors tend to seek safer, more predictable areas for investing their funds despite the great potential for profitability.

MARKET GROWTH AND POTENTIAL

The Global Nutrition Industry was estimated at $140 billion in the year 2000, based on sales [1]. The percentage breakdown of the industry based on the various product lines is shown in Figure 1.

The data showing growth by product on a global basis is also very revealing. Leading the way is Natural/Organic Food, which grew by 12% over the previous year (see Figure 2). Interestingly, total nutrition sales grew by 7.5% over the previous year.

The percentage breakdown of global sales, based on country and/or region, is shown in Figure 3. The Global Growth by region or country tells a very interesting story. The growth rate recorded has Latin America leading the way with 10% over the previous year,

Figure 1.

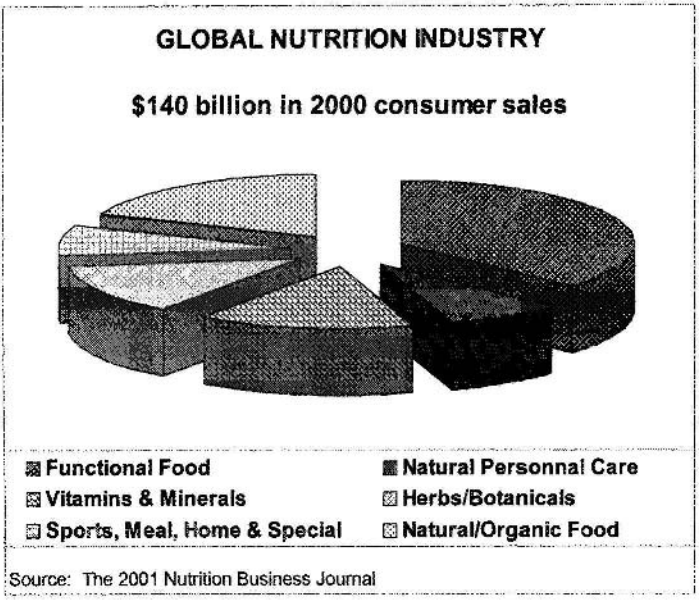


Figure 2.

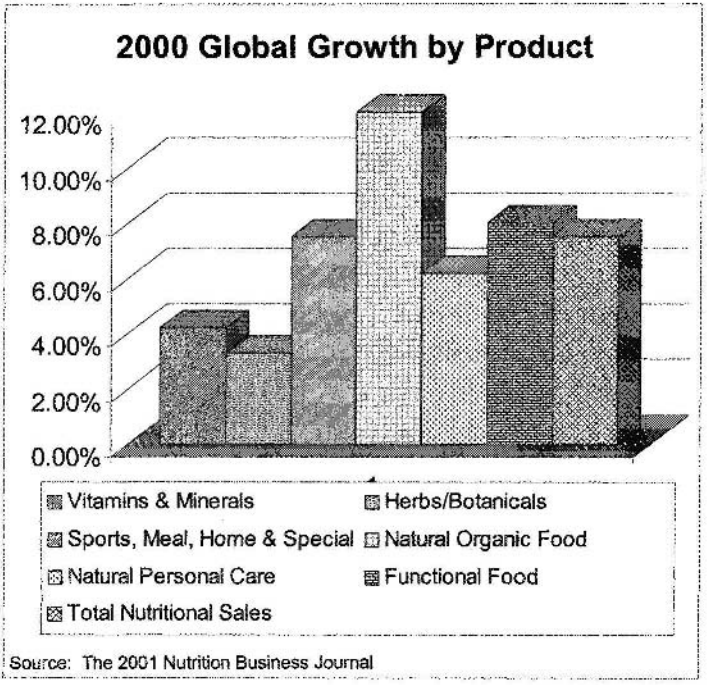
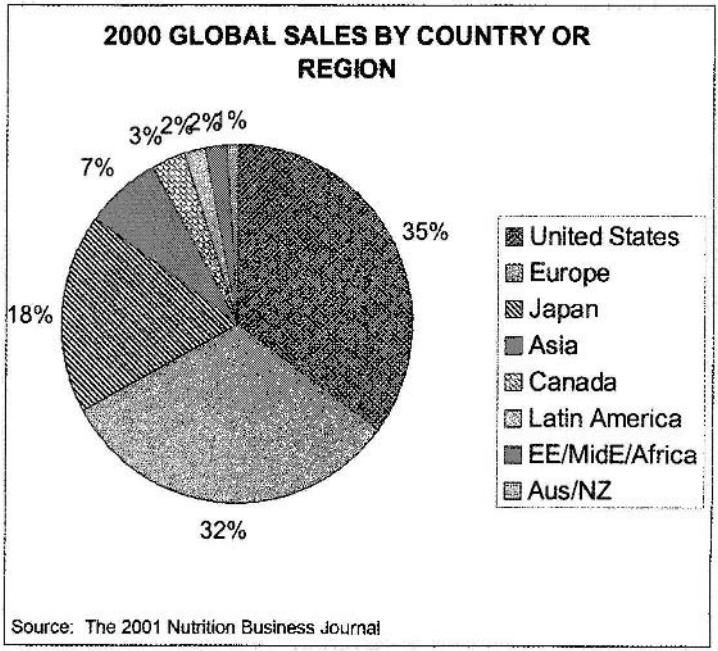


Figure 3.



followed by Europe, Canada, Asia, Australia/New Zealand and Africa, all showing 8% growth. The United States registered a 7% growth, Japan 6%, EE/FSU 5% and the Middle East 5%.

Overall, the growth rates are very promising and indicate excellent market strength. Of particular interest is the performance of Latin America, a region with a population of some 400 million; Asia, a region with a population in excess of 3 billion, and Europe, a region with a population of some 400 million. Should these countries/regions maintain this growth rate over the next five years, we could witness spectacular growth in market opportunities. This however is a quick and rough assessment. In order to get a firm grasp and understanding of the market, we would of necessity have to develop better trend analyses of the niche products.

Suggested Strategies for the Development of a Natural Products Industry

The question which must be addressed is, "How do we capitalise on the tremendous business potential from the growing

interest in Natural Products"? This section of the presentation will seek to focus on the elements that are critical to the transformation from attractive business potential to a vibrant, fast-growing industry.

The first element is: *Inculcation of Business Outlook in Science and Technology Leadership.* This element involves and includes:

- The development of a method of selecting market-oriented Research & Development (R&D) projects. This approach would not in any way compromise the integrity of the R&D process, which would continue unimpeded. What is advocated is that, in addition to the normal R&D programme, a special unit would be developed to pursue the market-driven approach.
- The market-oriented approach would be strengthened by a sharpened focus on the ultimate objective, which is the efficient commercial production of the products developed, yielding attractive Return on Investment (ROI).
- Taking the necessary steps to develop sound and affordable research plans, for generating essential data.
- Developing standard products and technology for manufacturing.

The second element focuses on ***Development of Business Processes.*** This includes:

- Identification and analysis of the market. Some of the areas to be considered are the estimated size of the market; the major components; the status – whether it is growing or declining; and future prospects.
- Identification of attractive market niches with competitive advantage.
- Development of a detailed Business Plan for:
  - R & D
  - accessing funds
  - manufacturing
  - marketing.

The third element is ***Creating Awareness***. The objective here is to inform and educate all the major players of the untapped potential,

and the need to pursue these opportunities. The purpose would be to:

- garner Government support
- foster Venture Capitalism
- foster an entrepreneurial approach to R&D.

The fourth element is the ***Fostering of Venture Capitalism.*** What is Venture Capital? Venture capital is generally considered to be early-stage financing, invested in highly risky ventures. These ventures are invariably new and young businesses pursuing rapid growth and profitability. It should be pointed out, however, that the field of Venture Capital (VC) financing is much more complex and far-reaching than this narrow definition would suggest. William Wetzel of the University of Hampshire argues that Venture Capital "is the cutting edge that, together with entrepreneurs, exploits opportunities to put together apparently neutral or sterile resources, to create firms with capitalised earning power on market value, well in excess of the cost of invested fund".

The venture capital process is defined by Steven James Lee [2] as consisting of six generally accepted stages:

- *Seed stage.* This is seen as an investment, which carries the highest risk and focuses primarily on R & D and product development.
- *First stage* investment is contingent on the successful performance of prototype. Further market studies may be pursued, business plan refined and modest manufacturing processes set up. This stage involves high to medium risk.
- *Second stage* shows some market success and potential profitability. More investments will now be devoted to equipment purchases, inventories and receivable financing.
- *Third* stage involves rapid development of the business and the generation of positive cash flow. Business shows features of being bankable.
- *Mezzanine financing stage* demonstrates sustained profitability. Possible financing instruments involve subordinated debt and quasi equity (preference shares) plus an equity component.

- *Initial Public Offering* (IPO) or sale of Company Stage. At this stage, the venture capitalist seeks an appropriate return on his investment either by selling his shares to the public or large corporations.

The fifth element is ***Policy Development***. The major policy development would be involved with two areas:

- Government Policies, with the aims
  - to attract local and international venture capital investments and
  - to support cooperative VC for cottage industries and small businesses of particular relevance to developing economies.
- Private sector policies with the aim
  - to promote corporate venture capitalism.

The critical importance of small and medium-sized businesses to economic growth and entrepreneurial development underlines the primacy of Policy Development.

The passage of the Small Business Investment Act in the United States in 1958 led to the creation of small business investment companies (SBIC) as vehicles of small business financing [3]. Jane Koloski Morris, in tracing the historical development of venture capitalism in the U.S., points out that "with tax advantages and potential government lending for leverage, SBICs were the first vestige of an organized venture capital industry".

Another example of the transforming impact of small and medium-sized business on economic stability is provided by South Korea's economic recovery. One of South Korea's policy initiatives was support for small and medium-sized businesses, including start-ups. Since 1998 (the time of the country's big economic crisis), 11,396 new companies have sprung up. This is largely attributable to the Government's initiatives to restructure the economy, in response to IMF's support and the demand for economic reform.

The sixth major element is the ***Development of Strategic Alliances***. The focus would be in two major areas:

- Government – establish relationship with key Government agencies
- International – special areas of interest would be investors and R&D leaders.

The seventh element addresses the ***Development of a Business Plan.***

Business Plan objectives include the:

- setting of criteria and bench marks
- charting of the course to be pursued
- attraction of and access to funds
- promotion and fostering of relationships with the business community.

The eighth element focuses on the ***Critical Features of the Business Plan.***

David Titus and Peter Bernadoni (4) stress the need to modify a Business Plan to fit the audience. The key audience includes:

- Investors — The most frequent recipients of business plans, which is the document for selling the long-term vision and capabilities of the company.
- Banks — The reasons for providing banks with business plans are to request a loan, request for special services, or to introduce them to the company.
- Customers — It is necessary to convince customers of the company's viability as a supplier, particularly at the start-up stage.
- Corporate Partners — By showing the company's vision, the Business Plan can convince large companies to share the risk.
- Employees — The plan would provide sufficient detail to experienced employees to establish credibility.

Titus and Bernadoni [4] placed special importance on the features of the plan, which should be emphasized. These were:

- product viability. The technical issues facing the company, and how they will be addressed, should be clearly identified.
- market opportunity. What is the market opportunity for the product? What will the competition be? What are the competitive

advantages? Why will the product have and maintain a place in the market?

- distribution strategy. How will revenues be generated? How effective is the strategy?
- financial projections. Projections over at least a five-year horizon should be prepared. Cash-flow, profit & loss and balance sheets should be prepared with the assumptions available for review.
- capital requirements. Estimates of total capital requirements for the business, timing of future capital infusions and potential sources of capital funds should be made.
- management. A brief summary of the backgrounds of key personnel with emphasis on why they are qualified to implement the business plan should be presented.
- exit scenarios. How does the investor become liquid on his investment? Will the ROI be at an acceptable level?

Another element, which could be added to these seven elements is the **Strengths, Weaknesses, Opportunities, Threats (SWOT)** analysis, which provides a critical assessment of the venture.

The ninth element is the ***Fostering of Strategic Partnerships***. The over-riding philosophy is that sustainable business successes in these partnerships can be derived only from win-win scenarios. The aim would be to convince established corporations to allocate funds as venture capital for the development and manufacture of new products. James Morris, in tracing the historical perspective and current trends of venture capitalism, observed that "strategic partner relationships, typically involve large corporations that have a minority equity investment in the small company, coupled with a side business agreement. These may include a research and development contract, a sales or marketing agreement, a suppliers' contract, a licensing agreement, a government product development effort, or the creation of a joint-venture company" [5].

The tenth element is the ***Due Diligence Process***. It is recommended that you do your own due diligence on the Venture Fund. Some of the issues to be addressed include:

- What is their record?
- Are there any conflicts of interest?
- Are they flexible?
- Do they understand the business?
- What will they bring to the table besides cash? Will they provide knowledge and support?
- Is the "chemistry" right?

It should be understood, as explained by Theodore Theodores, that the Venture Capitalist will perform a detailed and exhaustive analysis of the project proposal in order to make that vital go or no-go decision [6]. This process, as explained by Theodores, is very important to the success of the venture as it is the time when individuals are learning to work together, establish relationships and stake their turf.

CONCLUDING REMARKS

- Investors are profit-oriented but impatient with respect to results. A fairly recent example of this is one where biotech investors were impatient with Imclone Systems Inc's failure to come up with a cancer cure. Many investors seek to manage their risk by employing staged capital commitments whereby money is committed for a 3-18 month phase followed by subsequent commitments based on results and promise [7].
- It is important to establish a close working relationship with would-be investors to educate them and win their confidence.
- The name of the game is "seek an early winner". This success will allay fears, establish confidence and set the stage for a mutually rewarding relationship.
- The importance of a well-structured business plan cannot be stressed too strongly.
- Do not be turned off by rejection. Many successful entrepreneurs were turned down on many occasions but continued to press on.
- Effective cash management is vital. Some of the frugal investors have managed their resources so well that they have spurned overtures from IPOs and other Venture Capital sources.

- Business needs more and more innovation in order to maintain growth and profitability.

This paper has sought to give some understanding and appreciation of the many issues involved in transforming the output of R & D into business opportunities. The issues raised have not been treated in detail. It is hoped, however, that they may stimulate interest in further research and also investment into the future development and marketing of natural products.

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Protection of Ideas, Inventions, Trade Marks, Copyrights and Products under the Intellectual Property Law

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INTRODUCTION

After much research and development, you have a new product or service on the market. You would like to protect it from being copied. Should you patent it? Rely on trade secret protection? Resort to design registration? Or leave it to copyright?

Intellectual property is a product of creativity and intellectual effort which can be protected against theft by other people. The owner of intellectual property has certain exclusive rights to control or exploit them in the global market by licensing or franchising. Intellectual property rights are intangible rights and are considered assets of a company which can be bought, sold or transferred. Intellectual property rights include patents, trade marks, service marks, registered designs, copyright and confidential information.

Intellectual property transcends national boundaries. The General Agreement on Tariffs and Trade (GATT) under the aegis of the World Trade Organization (WTO) resulted in an Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS). As a result of GATT, the Uruguay Round Table of Multilateral Trade Negotiations is one of the major breakthroughs in international intellectual property protection. TRIPS imposes minimum standards and measures that have to be implemented by its members to prevent piracy in the global market place. TRIPS, which is binding on more than 120 members of the WTO, provides for trade concessions for intellectual property protection. Developing countries are likely to benefit greatly from adhering to TRIPS as it encourages foreign investments. The availability of adequate intellectual property protection is a vital pre-requisite to investment.

PATENTS

Patent protect ideas. With a patent, you can assert your rights and stop competitors from copying your product, especially if it is an attractive one. A patent grants the exclusive right to an inventor to allow him to enjoy the fruits of his inventions for 20 years and to sue others if they infringe his rights. But there is the cost and benefit scale of patent protection to consider. It should be patented if the invention has the potential to be commercially viable and is likely to be copied. A patent is not worthwhile if you have a niche market where others will not enter or when you can keep the invention a secret, e.g. the formula for the Coca-Cola drink remains a trade secret for over a hundred years.

Patent are granted by the government to protect new articles. The inventor gets a monopoly in return for a full disclosure of the invention in the patent specification.

There are two ways to apply for patent protection in Singapore. The first is to file a domestic application with the Intellectual Property Office of Singapore. The second is to file an international application under the Patent Co-operation Treaty (PCT) at the receiving office in Singapore.

The PCT is a multilateral treaty for the co-ordination and co-operation of the filing, searching and examination of patent applications. It is administered by the International Bureau of the World Intellectual Property Organization (WIPO) in Geneva. The PCT does not provide for the grant of patents which remains the responsibility of the national patent offices in the individual countries. Singapore citizens and residents can obtain patent protection in any of the signatory countries to the Treaty by filing a single application with the Registry and designating those countries in which protection is required.

TRADE MARKS

Brand loyalty can develop with patent protection. A trade mark may be kept and used long after a patent ends. The rights of trade marks and service marks protect logos, names and the get-up (the appearance) used for identifying the business products and services

e.g. the famous golden arch in the'M' of McDonald's. A trade mark is a valuable asset which can be pledged, sold or bought like any other property.

It is not compulsory to register a trade mark under the Trade Marks Act in Singapore. But registration has its advantages and serves as a notice to others that the owner has claims to the name. The owner of a registered trade mark has the exclusive right to use the mark which can be enforced by an injunction and damages. If a trade mark is not registered or if marks are pending registration, then the owner may acquire rights to his mark under an action of passing off.

Non-confidential disclosures by your employees can destroy patentability. Therefore getting confidentiality terms in the employment contracts of your employees is an important means of safeguarding your secrets.

The inventor may prefer trade secrecy to patenting because patents require the invention to be published. Business secrets such as chemical formulae and marketing techniques also constitute an interest that merits protection. A secret manufacturing process can be commercially advantageous. Trade secrets last indefinitely provided others do not know about them.

Misappropriation of trade secrets often occurs when an employee, after learning the trade secrets, leaves the job and discloses the information to his new employer. Trade secrecy may be maintained by entering into a confidentiality agreement with the employee obliging him not to disclose the business or trade secrets to subsequent employers.

INDUSTRIAL DESIGNS

The registration of a design is worth pursuing if it is certain that competitors would reach the same design independently. But the registration of a design must be done before the design is disclosed to anyone. The registered proprietor of the design has monopoly over its use. A registered design is infringed by any person using the same design even if he had created it independently.

COPYRIGHT ISSUES

Copyright prevents copying, and so protect the form of expression. It does not protect ideas *per se*. Copyright protects literary, artistic, dramatic, musical works as well as "entrepreneurial" products such as sound recordings, cinematographic films, television and sound broadcasts, cable programmes and publisher's rights in published editions of work. Copyright protection is provided by the Copyright Act protecting all authors of published and unpublished work. A copyright owner has the exclusive right to reproduce, publish, perform or broadcast his work and to include his work in a cable programme or make an adaptation of it. Singapore has no registration system for copyright which arises automatically on creation.

There is a requirement of originality for a work to be protected by copyright. A work is original if it is not copied from elsewhere and is the author's own creation. As long as some intellectual effort and skill is put into creating the work, the form of expression will be original.

LAW OF CONFIDENCE AND TRADE SECRETS

Confidence protects both the idea and its form. The law of confidence is about the right to stop persons from making use of the information while the copyright law gives the right to stop others from reproducing a work.

The confidential information to be protected must have the necessary quality of confidence about it and also have been imparted in circumstances importing an obligation of confidence. However, information relating to customers, prices and business methods obtained by an employee and which becomes part of his general skills and business methods obtained by an employee and which becomes part of his general skills and knowledge is not subject to an obligation of confidentiality. But the court will intervene when there is the use of any trade secret of the employer. Trade secret refers to some proprietary right of the employer such as some secret process of manufacture.

ENFORCEMENT ACTIONS

Singapore has its Patents, Trade Marks and Copyright Acts. Patent protection lasts 20 years, while copyright protection lasts 50 years (soon to be increased to 70 years by US-Singapore Free Trade Agreement) after the death of the author. In an infringement action, the issue to consider is whether the patent registration is valid and if the monopoly granted to it covers the alleged infringement. In a copyright infringement, a defendant who has copied will usually lose the action unless he comes with defences. The specific interest group defences include libraries, educational institutions and broadcasting organizations, while the general public interest defences include fair dealing for private study or research, for criticism or review, for reporting current events and back-ups for computer software.

Only civil remedies are available for patent and design infringement and passing off. Both civil and criminal remedies are available for trade marks and copyright infringement.

With the tremendous growth in international trade in recent years, intellectual property rights have gained greater prominence in international trade negotiations, as in the US-Singapore Free Trade Agreement. At the global level, there is the need for an effective system of international intellectual property rights protection and enforcement. The usefulness of any intellectual property system depends largely on the enforcement of intellectual property rights against counterfeiters and pirates.

Well-known cases of intellectual property infringement include pirated compact and laser discs, computer software and counterfeit aircraft and pharmaceutical chemicals. To ensure that international intellectual property protection remains viable and relevant in a rapidly changing world, TRIPS has laid the foundation for common border control measures and for enforcement of criminal procedures. TRIPS sets out the minimum framework needed for the protection and enforcement of intellectual property rights. Both the Berne Convention and the Paris Convention contain less detailed enforcement obligations than those in TRIPS.

The enforcement procedures under Part 3 of TRIPS permitting effective action against infringement include expeditious remedies to

prevent infringement, where such remedies and penalties are to deter further infringement. These enforcement procedures are not to be costly and unnecessarily complicated as to impede enforcement.

One of TRIPS' main provisions in the enforcement of intellectual property rights is the awarding of damages to compensate for loss caused by an infringement. Apart from damages, the infringer is to pay the right holder's expenses and legal fees. Recovery of profits is appropriate where an infringer does not knowingly engage in infringing activity.

The threat of litigation can sometimes be an obstacle to a potential infringer. Litigation in the intellectual property field can be very costly and should be avoided. There are also the internal costs to the client's business such as lost employee time to consider. Depending on the seriousness of the infringement, direct approaches to infringers may sometimes offer a better solution. These include sending "cease and desist" letters or visiting the infringers directly.

However an important part of the enforcement process is education. Informing intellectual property owners of their rights to products is not only an effective way of discouraging infringement but also makes it difficult for an infringer to put up a defence of innocence. Alternative dispute resolution procedures and arbitration are other convenient ways of resolving intellectual property rights cases in Singapore.

CONCLUSION

Singapore has quite adequately provided for criminal procedures and penalties for most cases of infringement on intellectual property rights. This is clearly in line with current global trends and with the increasing value of intellectual property and its vulnerability. Singapore's intellectual property laws and enforcement are in conformity with the TRIPS Agreement.

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