IOSR Journal Of Pharmacywww.iosrphr.org
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 8, Issue 2 Version. 1 (February 2018), PP. 10-17

Analysis of phytochemical profile and genotoxicity of crude methanolic extracts of *Quassia indica* (Gaertn.) with anti-inflammatory and antioxidant properties.

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Abstract: The use of traditional medicine is expanding to newer horizons and plants still remain as the novel source of structurally important compounds that lead to the development of innovative drugs. It is believed that about 80% of world's population use plants as their primary source of medicinal agents. In this juncture, the present study was undertaken to analyze the antioxidant, anti-inflammatory potentiality and genotoxicity of the medicinal herb *Quassia indica*. An attempt has also been made in the present study for supplementing evidence from *in-vitro* studies with information on phytochemical profiling, and genotoxic nature to establish the safe range of extract consumption. The results reveal potential anti inflammatory and anti oxidant activity when compared to the respective standards. In general, more activity was obtained when using more concentrated decoctions of the plant extract studied. The systematically performed *in vitro* assays revealed that the tested plant extract may find in therapy as agent with high pharmaceutical value. Treatment of extract of *Quassia indica* inhibited root growth and developed genotoxic effect in onion meristem cells. The bridges, breaks, lagging and multipolar anaphase chromosomes were observed at all concentrations studied. Various types of chromosomal abnormalities such as chromosomal lagging, disruptive anaphase, irregularity in the movement and arrangement of chromosome, chromosome stickiness, vagrant chromosomes, polar deviation and abnormal dispersed metaphase were recorded. As the human clinical evidence is still limited, future research needs to define the actual magnitude of health benefits, establishing the safe range of extract consumption with these benefits and elucidating the mechanism of action. In addition, the plant is a good candidate for further phytochemical and chromatographic studies to isolate and fully characterize the compound related to this *in vitro* biological activity.

Key words: *Quassia indica*, antioxidant, antiinflammatory, TLC, genotoxicity

Date of Submission: 06-02-2018

Date of acceptance: 24-02-2018

I. INTRODUCTOION

Natural products, mainly the plant-derived constituents, have long been sources of drugs, and a great part of the pharmaceuticals available in modern medicine are directly or indirectly derived from natural sources. Natural products are also of great interest in the process of drug discovery, due to their large diversity in nature, permitting the identification of lead molecules of greater interest for the development of new therapeutic agents, as well as biochemical and molecular tools needed to clarify complex cellular and molecular mechanisms of action involved in most physiological and pathological processes. Furthermore, a growing world-wide interest in the use of phytopharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, has been noted in recent years. Simaroubaceae includes 32 genera and more than 170 species of trees and bushes of pantropical distribution. It is characterized by its content of bitter substances, quassinoids mostly responsible for its pharmaceutical properties [1,2]. Due to the chemical diversity many species of simaroubaceae family, it is worth noting that it can be characterized as a promising source of bioactive molecules with remarkable research potential. An example of this is that since 1961, when the first quassinoid structure was elucidated, the growing interest on various species of simaroubaceae family resulted in the isolation and identification of the more than 200 currently-known quassinoids [3]. Nevertheless, many of its species have not been studied or remain unexplored. Quassia indica belonging to simaroubaceae family is one such species. It grows in the evergreen forest and along backwaters of the tropical belt. The tree can grow up to 11 m high with stout branches and pale yellow bark. The leaves are large, elliptic, shortly acuminate entire, shining. The flowers are pinkish yellow in axillary umbelsThe present study was conducted with the aim of making an indepth analysis of the phytochemical constituents and pharmacological potency of Quassia indica and also to determine the genotoxicty of the crude methanolic plant extract

II. MATERIALS AND METHODS

Preparation of the Sample:
The plant material, required for the entire study was collected in and around Karunagappally, of Kollam district. The collected material was shade dried and then powdered using a blender.

Preparation of Methanolic Extract
The leaves of *Quassia indica* were washed in tap water, chopped into pieces and air dried .The dry leaves were powdered and used for extraction. 30 grams of the powdered plant leaf was filled in a soxhlet extractor and extracted with 200 ml methanol (boiling point - 64 to 65° C) for ten hours. The extract was transferred to a conical flask, concentrated and evaporated to dryness in vacuo in a rotary evaporator [4].

Phytochemicals analysis:
Phytochemical analysis of the test sample was carried out according to standard methods [5,6].

Salkowski reaction test for phytosterols: To 0.5 mL chloroform extract in a test tube add 1 mL of concentrated (conc.) H_2SO_4 from the sides of the test tube. Appearance of reddish brown colour in chloroform layer indicates presence of phytosterols.

Liebermann-Burchard's test for triterpenoids:
Extract was treated with few drops of acetic anhydride, boil and cool. Conc. sulfuric acid was added from the sides of the test tube which showed a brown ring at the junction of two layers, and formation of deep red color indicated the presence of triterpenoids.

Foam test for saponins:
Small amount of extract was taken in a test tube with little quantity of water and shake vigorously. Appearance of foam persisting for 10 min indicated presence of saponins.

Dragendroff's test for alkaloids:
Various extracts of the herbal drug were dissolved in chloroform. Chloroform was evaporated and the residue was acidified by adding few drops of Dragendroff's reagent (Potassium bismuth iodide). Appearance of orange red precipitate indicated presence of alkaloids.

Molisch's test for carbohydrates:
The extract was mixed with Molisch reagent, and then added conc. H_2SO_4 along the sides of the test tube to form layers. Appearance of reddish violet ring the interference indicated the presence of carbohydrates.

Lead acetate test for flavanoids:
To the alcoholic solution of the extract add few drops of 10% lead acetate solution. Appearance of yellow precipitate indicated presence of flavonoids.

Legal's test for lactones:
To the extract mixtures add sodium nitroprusside and pyridine. Then the mixture was treated with NaOH. Appearance of deep red colour indicated the presence of lactones.

Ferric chloride test for phenolic compounds and tannins:
Take 2 mL of extract in a test tube and add ferric chloride solution drop by drop. Appearance of bluish black precipitate indicated presence of phenolic compounds and tannins.

Ninhydrin test for proteins:
Few drops of ninhydrin added to the extract. Appearance of blue colour indicated presence of amino acid where as proteins may rarely give positive result.

Keller-Killiani test for glycosides:
A total of 1 ml of glacial acetic acid, few drops of ferric chloride solution and conc. H_2SO_4 (Slowly through the sides of the test tube) were added to the extract. Appearance of reddish brown ring at the junction of the liquids indicated the presence of de-oxysugars.

Thin layer chromatography (TLC) analysis for antioxidant constituents:

About 2 µg of extracts of *Q.indica* was loaded on TLC plates (Merck, 20 cm×20 cm). The plates were developed in methanol: chloroform: hexane (7:2:1, v/v/v) to separate various constituents of the extracts. The developed plates were air dried. Then the antioxidant constituents were analyzed by DPPH technique[7],[8]. For this 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant constituents of the *T. arjuna* extract was detected as yellow spots produced via reduction of DPPH by resolved bands against purple back ground on the TLC plates. Ascorbic acid was used as standard antioxidant[9].

Antioxidant activity determination by DPPH free radical scavenging assay

DPPH radical scavenging activity of the extract was measured by the method described by Barros *et al [19]*. For this, different concentrations of extract and ascorbic acid (standard) were prepared with methanol (Sigma-Aldrich) as the test solutions. About 1 mL of each prepared concentrations were placed into test tubes and 0.5 mL of 1 mmol/L DPPH solution in methanol was added. The test tubes were incubated for 15 min and the absorbance was read at 517 nm. A blank solution consisted of DPPH dissolved in same amount of methanol. The DPPH radical scavenging activity percentage was calculated by using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{extract}}{A_{control}}$$

Where $A_{control}$ is the absorbance of a DPPH solution without extract; $A_{extract}$ is the absorbance of the tested extract. All measurements were performed in triplicate.

Anti-inflammatory activity determination by Hyaluronidase inhibition assay

The assay was performed according to Ling *et al.* [11]. The assay medium consisted of 3,5 U-hyaluronidase in 100 µl 20 mM sodium phosphate buffer pH 7.0 with 77 mM Sodium Chloride, 0.01% BSA pre-incubated with different concentrations of the test compound (in DMSO) for 15 min at 37° C. The assay was commenced by adding 100 µl hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) to the incubation mixture and incubated for a further 45 min at 37° C. The undigested hyaluronic acid was precipitated with 1 ml acid albumin solution made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid (pH 3.75). After keeping the reaction mixture at roomtemperaturefor10min,theabsorbance was measured at 600 nm. The absorbance in the absence of the enzyme was used asthereference value for maximum inhibition. The inhibitory activity of the test compound was calculated as the percentage ratio of the absorbance in the presence of test compounds vs absorbance in the absence of the enzyme. The enzyme activity was checked by control experiment run simultaneously, in which the enzyme was preincubated with 5 µl DMSO instead, and followed by the assay procedures described above. Compounds were tested in a range of 5 µg - 250 µg in the reaction mixture. Indomethacin was used as reference standard.

Genotoxic Studies:

Bulbs of *Allium cepa* (L.), purchased from the local market were used for the present investigation. 5 g of leaf tissue was weighed and grinded in distilled water. Extract made up to 100 ml and kept it as stock. From the stock 50 ml extract measured and added equal volume of distilled water and kept as stock 2. Again measured 50 ml extract from stock 2 and added equal volume of distilled water and kept as stock 3. Onion root in distilled water was taken as control. Root tip is fixed in 12. 30 pm. Root tip squashes were made by using, acetocarmine squash technique of Marimuthu and Subramanian [12]. To make micropreparations, the fixed root from each of the treatments and control, were transferred to some vials containing 1 N Hydrochloric acid, and kept at 60° C for 3-5 minutes in hot air oven. The hydrolyzed root tips (1-2) were transferred to a clean slide with 2-3 drops of acetocarmine stain, kept it for one minute and then squashed using a scalpel. The squash was covered with a cover slip and sealed using clear nail polish. Prepared slides were examined using a microscope. Chromosomal aberrations were examined and photomicrographs were taken.

Mitotic index: Mitotic index was calculated using the formula given below.

$$\text{Mitotic index (MI)} = \frac{\text{Total dividing cells}}{\text{Total dividing and non-dividing abnormal cells}} \times 100.$$

$$\text{Total percentage of abnormal cells} = \frac{\text{Total dividing and non-dividing abnormal cells}}{\text{Total dividing cells}} \times 100.$$

Statistical analysis:

Results were analyzed using One way Analysis of Variance (ANOVA) and expressed as mean ± SE. Data was further subjected to Duncan's post hoc analysis and differences between means were regarded significant at

$P<0.05$.

III. RESULTS

Preliminary phytochemical screening:
The preliminary phytochemical analysis in *Q.indica* methanolic extract showed the active compounds presence in high concentration, such as triterpenoids, alkaloids, flavonoids, phenolic compounds and tannins and glycosides(Table 1).

Determination of anti inflammatory activity
In the present study anti-inflammatory activity of the plant extract was assessed by hyaluronidase assay. Indomethacin was used as the positive control which showed significant anti inflammatory activity in reducing the hyaluronidase enzyme. Extract of *Q.indica* showed significant activity in reducing the enzyme.(Fig 2).In the present work, the plant extract at 5 µg/ml showed an excellent hyalurodinase enzyme scavenging activity (21.18%) and the activity increased with increasing concentrations Treatment with 20 µg/ml of the plant extract showed an inhibition percentage of 75.42% .The 50% inhibition of hyalurodinase enzyme by test samples (IC_{50}) was observed at a concentration of 13 µg/ml.

TLC analysis for antioxidant constituents:
The plates TLC were developed in methanol: chloroform: hexane (7:2:1, v/v/v) and sprayed with 0.05% DPPH reagent. Purple colour of DPPH reagent was bleached by yellow spots which was the indication of positive antioxidant activity. The bark extract of *Q.indica* in terms of DPPH free radical scavenging activity showed one resolved TLC band with strong antioxidant activity and another spot with weak antioxidant activity as compared to standard antioxidant ascorbic acid.

Determination of antioxidant activity:
The change in colour of DPPH from deep violet to yellow is observed in all the concentrations used which is an indication of antioxidant activity. Fig 2 shows the decrease in the concentration of DPPH radical due to the scavenging ability of the methanolic plant extracts and the commercial standards. In this studywe used BHA as commercial standard. In the present work, the plant extract at 100µg/ml showed an excellent DPPH radical scavenging activity(43.8%) which was greater than that of the commercial standard used and the activity increased with increasing concentrations (Fig 1). Under same experimental conditions, positive control counterpart, BHA at 100µg/ml showed 53.4% DPPH radical scavenging activity. The 50% inhibition of DPPH radical by test samples (IC_{50}) was observed at a concentration of 115µg/ml.

Cytotoxic studies:
Cell divisions in the control materials of *Allium cepa* were normal. But aberrations were present in the root tips treated with different concentrations of *Quassia indica* leaf extract. The mitotic index reflects the frequency of cell division and it is regarded as an important parameter. The mitotic index showed very significant variations with leaf extract treatment of different concentration. Leaf extract treatment induced progressive decrease in mitotic index as function of increased concentration. The highest mitotic index value in treated group was 14.2 at very low concentration. The mitotic index decreased with increase in concentration of leaf extract treatment. Lowest value of mitotic index (9.5) was found in very high concentration of leaf extract.

Percentage of abnormal cells:
In the present study, it was observed that the percentage of abnormal cells increased with increase in concentration of leaf extract. The highest value of percentage of abnormal cells (42. 86%) was obtained in highest concentration of leaf extract. The lowest value of percentage of abnormal cells (20. 1%) was obtained in lowest concentration of leaf extract.

Chromosomal aberrations:
Various types of chromosomal abnormalities such as chromosomal lagging, disruptive anaphase, irregularity in the movement and arrangement of chromosome, chromosome stickiness, vagrant chromosomes, polar deviation and abnormal dispersed metaphase were recorded.

Lagging chromosomes resultant from chromosomal fragmentation and failure of chromosomes to move to either of the poles were also observed. However maximum laggard formation was observed in 20% concentration of the leaf extract, with the frequency decreasing at higher concentrations of the extract. In all the concentrations single, double and multiple chromatin bridges were found.

IV. DISCUSSION

Plants have become the focus of increasing attention from many researchgroups in recent years, owing to their exciting chemistry and their wide spectrum of pharmacological activities. Pharmacological screening is performed to allow targeted isolation of new or useful constituents with potential activities. Epidemiological studies over the last three decades have consistently correlated certain diets, specific foods and disease expressions. At the same time, number of bioactive compounds has increased dramatically and a new diet-health paradigm has evolved that emphasizes the positive aspects of diet. The terms ''phytochemical'', ''nutraceutical'' and 'functional food'' have been introduced to describe various aspects of this development[13],[14].In this present study, preliminary phytochemical analysis revealed a large amount of alkaloids, triterpenoids, flavonoids, phenolic compounds and tannins and glycosides present in methanol extract of *Q.indica*. The word 'inflammation' comes from the Latin '*inflammar*' (to set on fire), and it is defined as a complex biological response of vascular tissues against aggressive agents, involving a cascade of biochemical events comprising the local vascular system, the immune system and different cell types found in the injured tissue [15]. For the treatment of various inflammatory diseases, the nonsteroidal anti-inflammatory drugs (NSAIDs) are most widely prescribed, but the gastrointestinal, renal and cardiovascular toxicity associated with common NSAIDs limits their usefulness [16]. Because of this, the potential therapeutic evaluation of the medicinal plants has been the subject of incessant studies, which have proved pharmacological actions, such as the anti-inflammatory, of some plants and their constituents, including the terpenes [17]. Terpenes, which make up a very large family of natural products, contain more than 50,000 structurally diverse compounds, which are categorized by a number of C_5 isoprene units. Terpenes have been described as having important biological activities, such as analgesic [18,19] and cardiovascular [20]. Despite the existing technology in organic chemistry for the synthesis of a new drug, the natural products, including terpenes, serve as a source of raw material for innovative drug discovery [21]. Thus, in an attempt to improve the efficacy profile of new antiinflammatory drugs, including those of natural origin, the structure–activity relationship has been extensively studied, taking into account up-to-date knowledge on the mechanism of inflammation[22]. Our present study indicated high anti inflammatory capacity of the plant extract (fig 2).Structural modification of natural products showed promising activities that must be seen as an interesting source of new structure , with the possibility of presenting a better biological activity[23].Natural antioxidants mainly come from plants in the form of phenolic compounds, such as flavonoids, phenolic acids, tocopherols etc[24]. Reactive oxygen species produced by ultraviolet light, ionizing radiations, chemical reactions, and metabolic processes have numerous pathogenic effects such as causing lipid and protein peroxidation, DNA damage, and cellular degradation related to cardiovascular disease, aging, cancer, inflammatory capacity to scavenge free radicals. They are commonly found in both edible and non edible parts and have multiple biological effects and have anti oxidant activity [25]. During the present work the antioxidant activity of the plant extract was estimated, the result (fig 1) showed it as a potential source of anti oxidant. Previous studies on anti oxidant activity of Simaroubaceae members were carried out on *Quassia amara* and *Alvaradoa amorphoides* [26]. Further phytochemical studies are required to determine the types of compounds responsible for the anti oxidant effects of this species.Though the anti oxidant activity of plant extracts can be correlated to it's phenolic composition, non- phenolic and other unidentified compounds also contribute to the anti oxidant activity of plant extracts [27,28]. Anti -oxidant activity of polyphenols have been suggested to exert beneficial pharmacological effects on neurological disorders on the basis of *in vitro* observations. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals quenching singlet and triplet oxygen, or decomposing peroxides [29]. Many more studies on this subject would be greatly beneficial for the development of potential anti oxidant drugs.The *Allium cepa* test is important test *in vivo*, where the roots grow in direct contact with the substance of interest enabling possible damage to DNA of humans to be predicted [30]. In this study, the cytotoxic and genotoxic effects of leaf extract of *Quassia indica* is studied. Treatment of extract of *Quassia indica* inhibited root growth and developed genotoxic effect in onion meristem cells. The bridges, breaks, lagging and multipolar anaphase chromosomes were observed at all concentrations. Stickiness is due to inter-chromosomal linkages of sub-chromatid strands coupled with excessive formation of nucleoproteins and inappropriate protein-protein interaction.The results showed that higher concentrations were able to inhibit significantly cell division. The number of cytological aberrations increased with increasing concentration. The present study suggests a need for safe dose administration of the constituents in human medicine [31,32].The therapeutic potential of *Quassia indica* in terms of its efficiency and versatility needs further critical evaluation. In addition to all aforesaid pharmacological studies, herb- drug and herb- herb interactions of *Quassia indica* need to be taken further in depth. As the human clinical evidence is still limited, further research need to define the actual magnitude of health benefits, establishing the safe range of extract consumption with these benefits and elucidating the mechanism of action.

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Phytoconstituents	Tests	Conclusion
Phytosterols	Salkowski reaction	+
Triterpenoids	Liebermann-Burchard's test	++
Saponins	Foam test	+
Alkaloids	Dragendroff's test	++
Carbohydrates	Molisch's test	+
Flavanoids	Lead Acetate test	++
Lactones	Legal's test	
Phenolic Compounds and Tannins	5% $Fecl_3$ Test	++
Proteins	Ninhydrin test	+
Glycosides	Keller-Killiani test	++

TABLE-I: PRELIMINARY PHYTOCHEMICAL ANALYSIS OF QUASSIA INDICA EXTRACT.
(+: Present in low concentration; ++: Present in high concentration; - absent)

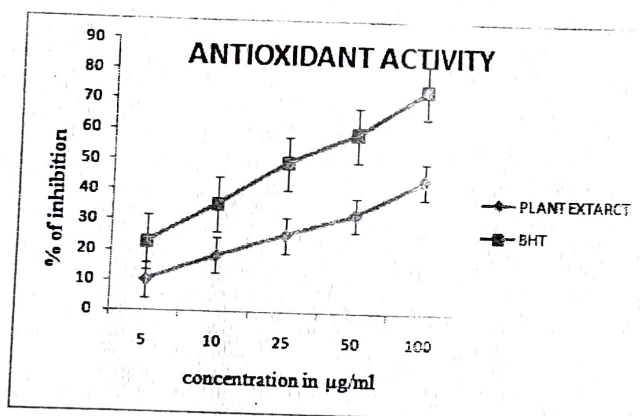

Fig. 1. DPPH radical scavenging activities of methanolic extract of *Q.indica* and the reference compounds BHA The data represent the percentage inhibition of DPPH radical. The results are mean ± S.D of five parallel measurements

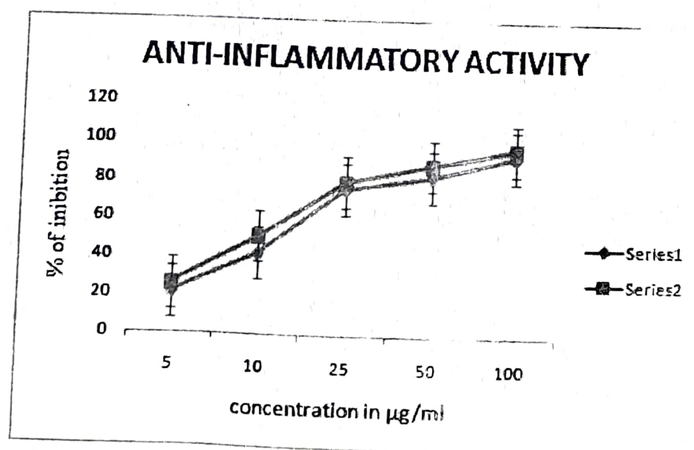

Fig: 2 Data expressed as effect of crude plant extract on hyaluronidase enzyme mean ±SE, n=4 (Anova post-test Duncan; P< 0.005)

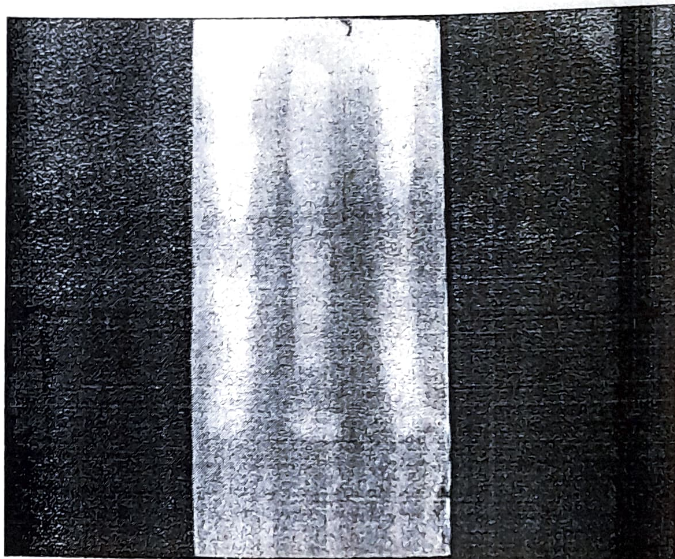

Fig: 3 TLC- antioxidant activity analysis of *Q.indica* constituents.

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