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ORIGINAL ARTICLE

In vivo Toxicity Studies on Gall Extracts of *Terminalia chebula* (Gaertn.) Retz. (Combretaceae)

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ABSTRACT

The galls of *Terminala chebula* (Gaertn.) Retz. (Combretaceae) are used for the treatment of various diseases in folk medicine and has been found to posses anti-inflammatory, anti-bacterial, anti-helmintic, anti-tyrosinase, and anti-aging activities. Considering the ethano-botanical and diverse pharmacological applications of galls of *T. chebula*, in this study, we investigate the possible toxic effects of different gall extracts of *T. chebula* by Brine shrimp (*Artemia salina*) toxicity assay. The cytotoxicity test of leaf gall extracts (petroleum ether, chloroform, ethanol, and aqueous) of *T. chebula* was evaluated by Brine shrimp (*A. salina*) toxicity assay, which is based on the ability to kill laboratory cultured *Artemia nauplii* (animals eggs) and also total content of polyphenols, flavonoids with other qualitative phytochemical analysis of the extract were determined. It was observed that the petroleum ether extract was virtually nontoxic on the shrimps, and exhibited very low toxicity with LC_{50} value of 4356.76 µg/ml. Furthermore, the chloroform extract exhibited very low toxicity, giving LC_{50} value of 1462.2 µg/ml. On the other hand, the ethanol extract was very toxic to brine shrimps with LC_{50} value of 68.64 µg/ml. The ethanol extract had the highest total phenolic and flavonoid content of 136 ± 1.5 mg of gallic acid equivalent/g d.w and 113 ± 1.6 mg of quercetin equivalent/g d.w, respectively. The higher toxicity effect was positively correlated to the high content of total polyphenols/flavonoids in the extract. This significant lethality of different extracts to brine shrimp is an indicative of the presence of potent cytotoxic components which warrants further investigation.

Key words: Assay, brine shrimp assay, cytotoxic, drug, extract, galls, toxicity

SUMMARY

- The present study investigates the toxicity effect of different extracts of galls of *T. chebulla*, which would serve as an index for formulation of drugs for treatment of various diseases. Presumably, these activities could be attributed in part to the polyphenolic features of the extract, as there was a strong correlation of higher toxic effect with that of high total phenolic and flavonoids content in the ethanolic leaf gall extracts of *T. chebula*.

Table 1: Brine shrimp lethality test of *Terminalia chebula* leaf gall extracts

T. chebula	LC_{50} µg/ml	95% CI
Pet ether extract	4356.76	1273.9-14900.12
Chloroform extract	1462.2	684.6-3123.3
aqueous extract	180.37	97.44-385.74
Ethanol extract	68.64	55.80-84.43
Reference standard (potassium dichromate	20.65	13.96-27.63

The results are mean SD (n=6). The results are presented as LC_{50} values (µg/ml) and 95% CI. CI: Confidence interval; SD: Standard deviation; *T. chebula: Terminalia chebula*

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INTRODUCTION

Terminala chebula (Gaertn.) Retz. (Combretaceae), is an important medicinal plant native to tropical regions of southern Asia viz., India, Nepal, China, Sri Lanka, Malaysia, Vietnam. It is commonly known as black myrobalan and haritaki, and amply referred to as "King of medicines;" as it has been the component of many formulations for the treatment of various diseases in all the streams of Indian system of medicines such as Ayurveda, Siddha, Unani, and Homeopathy.[1,2] It consists of gall-like excrescences formed by plant insect *Dixothrips onerosus* (Thysanoptera) on the leaves, petioles, and branches.[3] These galls are commonly known as Karkatshringi, which is an important ayurvedic drug used in preparations of Dasamularista, Cyavanaprasa, and Shringyadi curna and used in the treatment of diseases such as swasa (asthma), yakshma (tuberculosis), ajeerna (indigestion), hydroga (heart diseases), jwara (fevers), and yakrt roga (liver disorders) to mention a few.[1,2] Karkatshringi also finds usage in the treatment of children's ear infections, suppress hemorrhage from gums and also used to suppress bleeding from nose.[4] Hakims consider galls as useful in pulmonary infections, diarrhea, and vomiting.[5] Although the accepted source of Karkatasringi is the galls of *Rhus succedanea L.*, however *Pistacia integerrima* and *T. chebula* are also generally used in preparations.[2,6] Gall extracts of *T. chebula* have been found to posses anti-inflammatory, anti-bacterial, anti-helmintic, anti-tyrosinase, and anti-aging activities.[7-13] Considering the ethno-botanical and various pharmacological applications of galls of *T. chebula*, the toxicity aspect of it has to be verified. Since until date, there are no scientific literature in these lines, the aim of this study was to investigate the possible toxic effects of gall extracts of *T. chebula* by brine shrimp (*Artemia salina*) toxicity assay, which is based on the ability to kill laboratory cultured *Artemia nauplii* (animals eggs).[14]

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MATERIALS AND METHODS

Chemicals

Potassium dichromate was purchased from SRL Chemicals, India. All other chemical reagents and solvents of analytical grade were purchased from SRL Chemicals, India.

Plant material

The gall induced leaves of *T. chebula* were purchased from local market of Bengaluru, India. The plant materials were certified and authenticated by Dr. S. Sundara Rajan and the voucher specimen (JU-RUV-52) were deposited at Research centre of vrkshayurveda, Jain University, Bangalore. Further letter of authentication of the plant material was provided by vrkshayurveda centre, Jain University dated May 24, 2014. The galls were cleaned with distilled water, dried and crushed into fine powder by using electric grinder.

Preparation of extracts

The coarsely powdered gall materials were sequentially extracted with ethanol, petroleum ether, chloroform and aqueous solvents in Soxhlet apparatus for 24 h. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (BuchiFlawil, Switzerland) and a portion of the residue was used for the Brine Shrimp Toxicity assays.

Phytochemical analysis

The preliminary qualitative phytochemical analyses of carbohydrates, saponins, alkaloids, flavonoids, fixed oils and fats, phenolic and tannins, glycosides, phytosterols and triterpenoids in the extracts were carried out using the standard methods as described.[13]

Quantitative analysis

Determination of total phenolic content

The total phenolics were determined in the *T. chebula* leaf gall extracts (ethanol, petroleum ether, chloroform and aqueous) using Folin - Ciocalteau reagent method, employing Gallic acid as standard.[15] Briefly, 200 ml of both methanol and aqueous extracts (2 mg/ml) were made up to 3 ml with distilled water, then mixed thoroughly with 0.5 ml of Folin - Ciocalteu reagent. After mixing for 3 min, 2 ml of 20% (w/v) sodium carbonate was added and allowed to stand for a further 60 min in the dark. The absorbance of the reaction mixtures was measured at 650 nm, and the results were expressed as mg of gallic acid equivalent (GAE)/g of dry weight.

Determination of total flavonoid content

Total flavonoid content of the extracts (ethanol, petroleum ether, chloroform and aqueous) was determined using the aluminum chloride colorimetric method as described by Chang *et al.*[16] In brief, 50 µl of methanol and aqueous extracts (2 mg/ml) were made up to 1 ml with methanol then mixed with 4 ml of distilled water and subsequently with 0.3 ml of 5% $NaNO_2$ solution. After 5 min of incubation, 0.3 ml of 10% AlCl3 solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 1 M NaOH solution to the mixture. Then water was added to the mixture to bring the final volume to 10 ml and the mixture was allowed to stand for 15 min. The absorbance was measured at 510 nm. Total flavonoid content was calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5–100 mg/ml in methanol. The result was expressed as mg quercetin equivalent (QUE)/g of dry weight.

Brine shrimp lethality bioassay (cytotoxicity)

The cytotoxicity test of leaf gall extracts (petroleum ether, chloroform, ethanol, and aqueous) of *T. chebula* was evaluated using the standard procedure as described by Meyer *et al.*,[17] McLaughlin,[18] and Logarto Parra *et al.*[19] The test was conducted by using brine shrimp (*A. salina*). Brine shrimp eggs (*A. salina* Leach) were hatched in a hatching chamber, filled with fresh sea water. Plant extracts are tested at a concentration of 10, 100 and 1000 µg/ml in the multi-welled culture plates containing brine and 15 shrimps in each replicate. They are then incubated at a temperature of 25°C for 24 h. Potassium dichromate was used as reference standard. Survivors were counted after 24 h, and the percentage death at each concentration was determined.[17-21] The LC_{50} value at 95% confidence interval was determined from the count using the statistical method of probit analysis.[19-21]

Statistical analysis

Statistical analysis was performed using SPSS (Windows version 10.0.1; SPSS Inc., Chicago, IL, USA) using a one-way Student's *t*-test; $P < 0.05$ was considered as statistically significant, when comparing with relevant controls. All results refer to mean ± standard deviation.

RESULTS AND DISCUSSION

The galls of *T. chebula* have been used in many ayurvedic formulations in the name of Karkatasringi for treating various diseases.[1,2,13] These studies have revealed its potential to provide novel drug candidates for various diseases, however its toxic effect if any have not been evaluated until date. Henceforth, the galls of *T. chebula* was analysed for its cytotoxicity by brine shrimp assay. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity, which in most cases correlates reasonably well with cytotoxic properties. Brine shrimp assay results are presented in Table 1, which shows that the petroleum ether extract was virtually nontoxic on the shrimps, and exhibited very low toxicity with LC_{50} value of 4356.76 µg/ml. Furthermore, the chloroform extract exhibited very low toxicity, giving LC_{50} value of 1462.2 µg/ml. On the other hand, the ethanol extract was very toxic to brine shrimps with LC_{50} value of 68.64 µg/ml. The aqueous

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The results are mean SD (n=6). The results are presented as LC_{50} values (µg/ml) and 95% CI. CI: Confidence interval; SD: Standard deviation; *T. chebula: Terminalia chebula*

Table 2: Preliminary phytochemical analysis of leaf gall extracts of *Terminalia chebula*

Chemical constituents	Aqueous	Petroleum ether	Chloroform	Ethanol
Carbohydrates	+	–	–	–
Alkaloids	+	+	+	+
Flavonoids	–	–	–	+
Saponins	+	–	–	+
Steroids	–	+	+	+
Tannins	–	+	+	+
Triterpenes	+	–	+	+
Glycosides	+	+	–	+

+: Presence; –: Absence; *T. chebula: Terminalia chebula*

extract exhibited the moderate toxicity with an LC_{50} of 180.37 μg/ml. Further, it was found that the degree of lethality was found to be directly proportional to the concentration of the extract used.

In the preliminary phytochemical studies, the qualitative presence of phenolics, flavonoids, triterpenes, saponins, glycosides, phytosterols, reducing sugars were identified in the extracts [Table 2]. The total amount of phenolic and flavonoid content of extracts of leaf galls of *T. chebula* is presented in Table 3. The results obtained indicate that in comparison with all the exact, the ethanol extract had the highest total phenolic and flavonoid content of 136 ± 1.5 mg of GAE/g d.w and 113 ± 1.6 mg of QUE/g d.w, respectively. It was observed that there was a correlation between the total phenolics and flavonoids content in the extract and toxicity exhibited by different extracts [Table 3], thus the higher toxicity exhibited by ethanolic extract of *T. chebula* might be related to the significantly high polyphenolic and flavonoids content. The leaf gall of *T. chebula* are reported to be very rich in tannins, triterpenoids, flavonoids, essential oils, and others phenolic constituents.[13] The results also demonstrates that the ethanol extract possessed significant activity in releasing most of the secondary metabolites from leave galls of *T. chebula*. This may be due to the fact that phenolic and flavonoid compounds are often extracted in higher amounts by using polar solvents such as aqueous methanol/ethanol.[13,22,23] It is reported that differences in the polarity of the extracting solvents could result in a wide variation in the polyphenolic and flavonoid contents of the extract.[13,22,23] Some of the phyto-constituents present in the gall extracts such as alkaloids, tannins, and phenols may be accountable to have this significant cytotoxic activity. The significant lethality of different extracts to brine shrimp is an indicative of the presence of potent cytotoxic components which warrants further investigation.

CONCLUSION

The results of this study shows the toxicity effect of different extracts of galls of *T. chebulla*, which would serve as an index for formulation of drugs for treatment of various diseases. Presumably, these activities could be attributed in part to the polyphenolic features of the extract, as there was a strong correlation of higher toxic effect with that of high total phenolic and flavonoid content in the ethanolic leaf gall extracts of *T. chebula*. Our study shows that although the brine shrimp lethality assay is rather inadequate regarding the elucidation of the mechanism of action, it is very useful to assess the bioactivity of the plant extracts. In the course of our studies, the brine shrimp lethality assay actually has proven to be a convenient system for monitoring biological activities of the plants that are used in the Indian traditional medicine.

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Conflicts of interest

There are no conflicts of interest.

Table 3: Total phenolic and total flavonoid content of *Terminalia chebula* leaf gall extracts

Gall extracts	Total phenolic (mg of GAE/g d.w)	Total flavonoids (mg of QUE/g d.w)
Aqueous extract	95±1.5	53±1.2
Petroleum ether extract	107±1.4	96±2.1
Chloroform extract	51±3.2	39±2.6
Ethanolic extract	136±1.5	113±1.6

Values are presented as mean±SD (*n*=6). SD: Standard deviation, GAE: Gallic acid equivalent; QUE: Quercetin equivalent; *T. chebula: Terminalia chebula*

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