

Anti-Lipoxygenase Activity of Leaf Gall Extracts of *Terminalia chebula* (Gaertn.) Retz. (Combretaceae)

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ABSTRACT

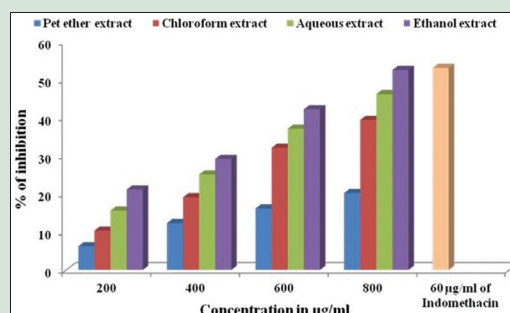
Lipoxygenase (LOX) inhibitors are the promising therapeutic target for treating a wide spectrum of inflammatory-related diseases such as cancer, asthma, lymphoma, leukemia, and autoimmune disorders. In the present study, the phytochemical constituents and the anti-LOX potential of leaf galls of *Terminalia chebula* are evaluated to exemplify its further potential development as medicine. Extracts of *T. chebula* galls were tested for anti-LOX activity using linoleic acid as substrate and lipoxygenase as an enzyme and also the total content of polyphenols with phytochemical analysis of the extract were determined. The presence of highest total phenolic and flavonoid content of 141 ± 2.2 mg of gallic acid equivalent/g d.w and 125 ± 1.4 mg of quercetin equivalent/g d.w and maximal LOX inhibitory activity (52.67%) at 800 $\mu\text{g}/\text{mL}$ concentrations were identified in the ethanolic extracts of leaf galls of *T. chebula*. The higher LOX inhibitory activity was positively correlated to the high content of total polyphenols/flavonoids. The results of this study confirm the folklore use of *T. chebula* leaves gall extracts as a natural anti-inflammatory agent and justify its ethnobotanical use. Therefore, the results encourage the use of *T. chebula* leaf gall extracts for medicinal health, functional food, and nutraceuticals applications.

Key words: Antioxidant, Anti-lipoxygenase, Drug, Gallic acid, Galls, Plants, *Terminalia chebula*

SUMMARY

The present investigation demonstrated promising anti-LOX properties of *T. chebula* leaves gall extracts. Presumably, these activities could be attributed in part to the polyphenolic features of the extract, as there was a strong correlation of higher LOX inhibiting activities with that of high total phenolic and flavonoid

content in the methanolic leaf gall extracts of *T. chebula*. The results of this study confirm the folklore use of *T. chebula* leaves gall extracts as a natural anti-inflammatory agent and justify the ethnobotanical approach in the search for novel bioactive compounds.



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INTRODUCTION

Lipoxygenases (LOXs) enzymes are reported to convert the arachidonic, linoleic, and other polyunsaturated fatty acid into biologically active metabolites that are involved in the inflammatory and immune responses.^[1] It is noted that 5-LOX and platelet type 12-LOX are generally considered as pro-carcinogenic, 15-LOX-2 suppresses carcinogenesis while 15-LOX-1 remains controversial.^[2] LOXs are the key enzymes in the biosynthesis of leukotrienes (LTs) that play an important role in several inflammation-related diseases such as arthritis, asthma, cancer, and allergic diseases.^[3,4] High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis, and colitis ulcerosa.^[5] Therefore, it is of the view that the production of LTs can be prevented via inhibition of the LOX pathway and targeting LOX with inhibitors is of a promising therapeutic target for treating a wide spectrum of human diseases. Pidgeon *et al.*,^[2] also suggested that LOX inhibitors may lead to the design of biologically and pharmacologically targeted therapeutic strategies inhibiting LOX isoforms and/or their biologically active metabolites which may be useful in cancer treatment.^[2] Plants and plant based herbal preparations have been used to treat ailments since

prehistoric times, and the treatment of various diseases with plant-based medicines has remained an integral part of many cultures across the globe. The World Health Organization estimates that 4 billion people (i.e., 80% of the world's population) use herbal medicines in some aspects of primary healthcare and there is a growing tendency to "go natural."^[6] In these aspects, all around the world, the medicinal properties of plants have been investigated and explored for their potent biological activities to counteract diseases which are with no side effects and with high economic viability.^[7-9]

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Terminalia chebula (Gaertn.) Retz. (Combretaceae) commonly known as black myrobalan and haritaki, is an important medicinal plant native to tropical regions of southern Asia viz., India, Nepal, China, Sri Lanka, Malaysia, and Vietnam. It is amply referred to as “king of medicines” as it has been the component of many formulations for treatment of various diseases in all the streams of Indian system of medicines such as Ayurveda, Siddha, Unani, and Homeopathy.^[10,11] It consists of gall-like excrescences formed by insects on the leaves, petioles, and branches of the plant-insect *Dixothrips onerosus* (Thysanoptera). The galls are vasiform, lobed, greenish yellow, fleshy, truncated gall, 25–33 mm long, smooth when immature, and longitudinally striated or ridged when old.^[12] These galls are commonly known as Karkatshringi and is an important Ayurvedic drug used in preparations such as the dasamularista, Chyvanaprash and shringyadi churna which are used in the treatment of diseases like swasa (asthma), yakshma (tuberculosis), ajeerna (indigestion), hydroga (heart diseases), jwara (fevers), and yakrt roga (liver disorders) to mention a few.^[10,11] Karkatshringi also finds usage in the treatment of children’s ear infections, suppress hemorrhage from gums, and also used to suppress bleeding from the nose.^[9,13] Hakims consider galls are useful in pulmonary infections, diarrhea, and vomiting.^[14] The accepted source of Karkatshringi is the galls of *Rhus Succedanea L.*, but *Pistacia integerrima* and *T. chebula* are also generally used in preparations.^[11,15] Gall extracts of *T. chebula* have been found to possess anti-inflammatory, anti-bacterial, anti-tyrosinase, anti-cancer, and anti-aging activities.^[16-21] In the present study, the photochemical constituents and the anti-LOX potential of leaf galls of *T. chebula* are evaluated to exemplify its further potential development and use as a drug.

MATERIALS AND METHODS

Materials

Lipoxidase (E.C.1.13.12.12), indomethacin, ascorbic acid, and gallic acid were purchased from Sigma, USA. Linoleic acid was purchased from Himedia. Ascorbic acid, gallic acid, quercetin, were procured from SRL Chemicals, India. All the other reagents and solvents were of analytical grade.

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 Vrikshayurveda, Jain University, Bengaluru. Further letter of authentication of the plant material was provided by Vrikshayurveda Centre, Jain University dated 24th May 2014. The galls were cleaned with distilled water, dried and crushed into fine powder by using an electric grinder.

Preparation of extract

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 anti-LOX assay.

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.^[9,22-25]

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-Ciocalteu reagent method, employing gallic acid as a standard.^[26] Briefly, 200 mL of both methanol and aqueous extracts (2 mg/mL) were made up to 3 mL with distilled water, and 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.*^[27] In brief, 50 μ L 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% NaNO₂ 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.

Anti-lipoxygenase activity

Anti-LOX assay was studied using linoleic acid as substrate and lipoxidase as enzyme purchased from Sigma, USA.^[28] The plant extract sample (200–800 μ g/mL) was dissolved in 0.25 mL of 2 M borate buffer pH 9.0 and added 0.25 mL of soybean lipoxidase enzyme solution (final concentration of 20,000 U/mL). This mixture was incubated for 5 min at 25°C. After which, 1.0 mL of linoleic acid solution (0.6 mM) was added, mixed well, and absorbance was measured at 234 nm. Indomethacin (60 μ g/mL) was used as reference standard. The percent inhibition was calculated from the following equation:

$$\% \text{ inhibition} = \frac{([\text{Absorbance of control} - \text{Absorbance of test sample}] / \text{Absorbance of control}) \times 100}$$

A dose-response curve was plotted to determine the IC₅₀ values. IC₅₀ is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Statistical analysis

The experiments were carried out in triplicate and results are given as the mean \pm standard deviation. Statistical analysis of all data was carried out using Microsoft Excel 2007 (Microsoft®, Inc., USA) statistical software. Student’s *t*-test was used to determine a significant difference between the experimental groups. Statistical significance was assumed at *P* = 0.05.

RESULTS AND DISCUSSION

As LOXs are implicated in the biosynthesis of LTs that play an important role in several inflammation-related diseases such as arthritis, asthma, cancer, and allergic diseases;^[3,4] therefore, it is of the view that the production of LTs can be prevented via inhibition of the LOX pathway and targeting LOX with inhibitors is of a promising therapeutic target.

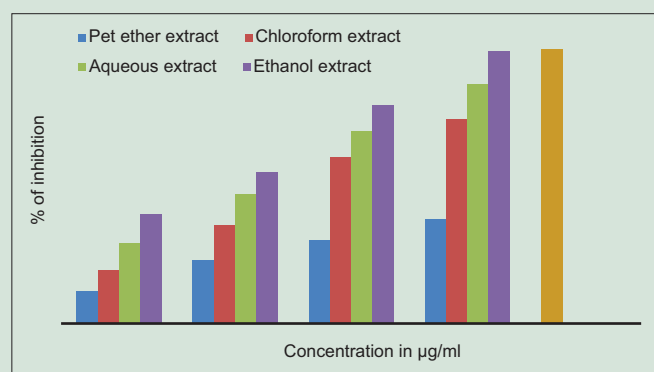


Figure 1: Lipoxigenase inhibitory activities of different extracts of leaf gall of *Terminalia chebula*. The plant extract sample (200–800 µg/mL) was preincubated with soybean lipoxidase enzyme (20,000 U/mL) for 5 min at 25°C. Linoleic acid solution (0.6 mM) was added, mixed well, and absorbance was measured at 234 nm. Indomethacin (60 µg/mL) was used as reference standard. Activity was measured and expressed as % inhibition. Each value is expressed as the mean ± standard deviation

Results for LOX inhibitory activities of extracts (ethanol, petroleum ether, chloroform, and aqueous) of leaf galls of *T. chebula* are shown graphically in Figure 1. It is observed that the ethanol extract showed highest LOX inhibitory activity of 52.67% at 800 µg/mL concentration, whereas aqueous, chloroform, and petroleum ether extracts showed 46.31%, 39.51%, and 20.25% inhibition at the same concentration, respectively. The minimum inhibitory concentrations of the ethanol, petroleum ether, chloroform, and aqueous extracts of leaf galls of *T. chebula*, as well as those of standard antibiotics, are shown in Table 1. The results indicate that the ethanolic extract had potent inhibitory activities. The IC_{50} value of ethanolic extract was found to be 560 ± 02 µg/mL. The reference standard indomethacin showed a 53.20% inhibition at a concentration of 60 µg/mL [Figure 1]. Therefore, in the present study, the methanol extract exhibited potent LOX inhibitory activity, when compared to all the other extracts and was equal to the standard used. These results suggest that leaf galls of *T. chebula* have a potentially high anti-inflammatory effect. Reactive oxygen species is known to propagate inflammation by stimulating the release of cytokines and also by activation of enzymes such as LOXs from inflammatory cells. Therefore, plants rich in antioxidant constituents with potential antioxidant activity are found to be beneficial to counteract inflammatory reactions. The antioxidant activities of plant/herb extracts are often explained by their total phenolic and flavonoid contents. The qualitative presence of phenolics, flavonoids, triterpenes, saponins, glycosides, phytosterols, and reducing sugars identified in the extracts are shown in 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 indicated that in comparison with all the exact, the ethanol extract had the highest total phenolic and flavonoid of 141 ± 2.2 mg of GAE/g d.w and 125 ± 1.4 mg of QUE/g d.w, respectively. These results show 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.^[29] 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.^[4,30] Phenolic antioxidants are products of secondary metabolism in plants, and their antioxidant activity is mainly due to their redox properties and chemical structure, which can play an

Table 1: The MIC (µg/ml) of the petroleum ether, chloroform, aqueous, and ethanolic extracts of galls of *Terminalia chebula* and standard indomethacin against LOX enzyme activities

	Petroleum ether gall extract	Chloroform gall extract	Aqueous extract gall	Ethanolic gall extract
LOX enzyme	847±3	726±2	673±4	560±02

Values are presented as mean±SD ($n=5$). SD: Standard deviation; MIC: Minimum inhibitory concentrations; LOX: Lipoxygenase

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

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

+ : Presence; – : Absence

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

Gall extracts	Total flavonoids (mg of QUE/g d.w)	Total phenolic (mg of GAE/g d.w)
Petroleum ether extract	96±2.1	107±1.4
Chloroform extract	39±2.6	51±3.2
Aqueous extract	53±1.2	95±1.5
Ethanolic extract	125±1.4	141±2.2

Values are presented as mean±SD ($n=5$). QUE: Quercetin equivalent; GAE: Gallic acid equivalent; SD: Standard deviation

important role in chelating transitional metals and scavenging free radicals.^[31] Similarly, the mechanisms of action of flavonoids are also through scavenging or chelating processes.^[32] In addition, compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for the antioxidant effects of plants.^[33] The higher LOX inhibitory activity exhibited by ethanolic extract of *T. chebula* might be related to the significantly high polyphenolic content and antioxidant property. The leaf gall of *T. chebula* is reported to be very rich in tannins, triterpenoids, flavonoids, essential oils, and others phenolic constituents.^[12,21] The results given in this investigation showed that the phenolic and flavonoid content was higher in polar extracts (ethanol) and subsequently its LOX inhibitory potential. Therefore, the inhibition might be due to the synergistic effect of the compounds from the extract. These observations confirm the folklore use of *T. chebula* leaves and gall extracts as a natural antioxidant and justify the ethnobotanical approach in the search for novel bioactive compounds.

CONCLUSIONS

The present investigation demonstrated promising anti-LOX properties of *T. chebula* leaves gall extracts. Presumably, these activities could be attributed in part to the polyphenolic features of the extract, as there was a strong correlation of higher LOX inhibiting activities with that of high total phenolic and flavonoid content in the methanolic leaf gall extracts of *T. chebula*. The results of this study confirm the folklore use of *T. chebula* leaves gall extracts as a natural anti-inflammatory agent and justify the ethnobotanical approach in the search for novel bioactive compounds. Further, the results support the use of gall extracts as a promising source

that may be effective as preventive agents in the pathogenesis of some inflammatory diseases. Therefore, the results encourage the use of *T. chebula* leaf gall extracts for medicinal health, functional food, and nutraceuticals applications. More *in vivo* and *in vitro* studies along with detailed phytochemical investigations are needed in order to potentially use this plant in the prevention and therapies of inflammatory-related diseases. In short, the present study provides the biochemical foundation for further chemical analysis and develop it as a drug for therapeutic application.

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Conflicts of interest

There are no conflicts of interest.

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