Research Article **ISSN: 2321 – 0915**

Asian journal of Phytomedicine and Clinical Research

Journal home page: www.ajpcrjournal.com

EVALUATION OF THE ANTI-INFLAMMATORY ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *AEGLE MARMELOS* LEAVES

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ABSTRACT

The anti-inflammatory activity of the hydroalcoholic extract of *Aegle marmelos* leaves in experimental acute and chronic inflammatory animal models. The hydroalcoholic extract of *Aegle marmelos* leaves was prepared and tested for anti-inflammatory activity in albino rats weighing 150-280 grams. The animals were randomly divided into 3 groups of 6 each; one group served as control and other two groups received indomethacin and *Aegle marmelos* leaves orally 1 hour prior to experimentation. The *in vivo* anti-inflammatory activity was studied using the acute (Carrageenan induced paw edema) animal models. Anti-inflammatory activity was expressed as Percent inhibition (PI). Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. $P < 0.05$ was considered statistically significant. The PI with indomethacin and Bilwa in carrageenan induced paw edema were 49.50% and 34.02%. Indomethacin showed highly significant anti-inflammatory activity. However, *Aegle marmelos* leaves showed highly significant activity in acute model when compare to indomethacin.

KEY WORDS

Indomethacin, Carrageenan, Hydroalcohol, Percent inhibition and Anti-inflammatory.

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INTRODUCTION

Aegle marmelos is an Indian plant, which has enormous therapeutic value in traditional systems of medicine[1] *Aegle marmelos* belongs to the family Rutaceae and grows wild, in outer Himalayas and Shivaliks. *Aegle marmelos* is the only member of the monotypic genus *Aegle*[2]. It is a mid-sized, slender, aromatic, armed, gum-bearing tree growing up to 18 meters tall. It has a leaf with three leaflets. *Aegle marmelos* occurs in dry forests on hills and plains of

northern, central and southern India, southern Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Vietnam, Laos, Cambodia and Thailand. It is cultivated throughout India, as well as in Sri Lanka, the northern Malay Peninsula, Java, the Philippines, and Fiji. It has a reputation in India for being able to grow in places that other trees cannot. It copes with a wide range of soil conditions (pH range 5-10), is tolerant of water logging and has an unusually wide temperature tolerance (from -7°C to 48°C). It requires a pronounced dry season to give fruit. *Aegle marmelos* is a medium to large sized deciduous glabrous, armed tree with axillary and 2.5 cm long alternate trifoliate leaves, short flowers and globular fruits[3]. The crude extracts of *Aegle marmelos* have shown various activities including antidiabetic, antioxidant[4], anti-inflammatory[5], analgesic, antiulcer[7], antimicrobial, antihyperlipidaemic[8], anticancer, antiviral, radioprotective[9] and antispermatogenic properties[10].

Pain and inflammation is a common complaint in most patients suffering from disease conditions. Inflammation is a host defences mechanism to combat or overcome the invading pathogen or the foreign particles. Non-steroidal anti-inflammatory drugs (NSAIDs) make up one of the largest groups of drugs used for pain and inflammation[11]. Currently available anti-inflammatory agents are associated with unwanted side effects and have their own limitations. About 34-46% of the users of NSAIDs usually sustain some gastrointestinal damage due to the inhibition of the protective cyclooxygenase enzyme in gastric mucosa[12] The added advantages of indigenous medicinal treatment would include its complementary nature to the conventional treatment making latter safer, well tolerated and economical remedy for acute and chronic inflammatory conditions.

MATERIALS AND METHOD

Preparation of Extract

The leaves was separated from plant and it was washed with absolute ethanol to avoid the microbial growth, the leaves were dried at open air under the shade, cut in to small pieces and powdered mechanically, then 50 gm of powder *Aegle marmelos* leaves was extracted with 250ml of hydroalcohol (water 75%: ethanol 25%) in a soxhlet apparatus for 72 hrs. The extract obtained was concentrated by recovery of ethanol. The concentrated product was used as hydroalcoholic extract of *Aegle marmelos* leaves.

Phytochemical Test[13]

The freshly prepared hydroalcoholic extract of *Aegle marmelos* leaves was subjected to phytochemical screening tests for various constituents. The procedures are as follows

Test for saponins

Foam test

Take 2ml of drug solution in a test tube. To it add small amount of water, shake well, stable froth (foam) is formed.

Test for tannins

a. Ferric chloride test

A small amount of test solution treat with ferric chloride solution, blue colour appears if hydrolysable tannins are present and green colour appears if condensed tannins are present.

b. Phenazone test

To the 5ml of aqueous extract add 0.5 gm of sodium acid phosphate. Then warm it and filter. To the filtrate add 2% Phenazone solution, precipitate is formed which is often colured.

c. Gelatin test

To the test solution add 1 % gelatin solution containing 10 % sodium chloride. Precipitate is formed.

Test for amino acids

a. Millon's test

To the test solution add 2ml of millions reagent, white precipitate indicates presence of amino acid.

b. Ninhydrin test

To the test solution add ninhydrin solution, boil, violet colour indicates presence of amino acid.

Test for proteins

a. Warming test

The test solution take in a test tube and heat in boiling water bath, proteins get coagulated.

b. Test with trichloro acetic acid

To the test solution add Trichloroacetic acid, precipitate is formed.

c. Biuret test

To the test solution (2ml) add Biuret reagent (2ml), violet colour indicates presence of proteins.

d. Hydrolysis test

Hydrolyze the test solution with hydrochloric acid or sulphuric acid. Then carry out the ninhydrin test for amino acid.

e. Xanthoproteic test

To the 5ml of test solution, add 1ml of concentrated nitric acid and boil, yellow precipitate is formed. After cooling it, add 40 %sodium hydroxide solution, orange colour is formed.

Glycosides

a. Keller kiliani test

The test consists of boiling about 1 gm finely powered sample with 10 ml 70 % alcohol for 2 to 3 minutes. The extract is filtered. To the filtrate is added, 5 ml water and 0.5 ml strong solution of lead acetate. Shake well and separate the filtrate. The clear filtrate is treated with equal volume of chloroform and evaporated to yield the extractive. The extractive is dissolved in glacial acetic acid and after cooling, 2 drops ferric chloride solution is added to it. These contents are transferred to a test tube containing 2 ml concentrated sulphuric acid. A reddish brown layer acquiring bluish-green colour after standing is observed.

b. Legal test

The extract is dissolved in pyridine, sodium nitroprusside solution is added to it and made alkaline-pink or red colour is produced.

c. Baljet test

To the section of sample, sodium picrate solution is added. It shows yellow to orange colour.

Test for cardiac glycosides[14]

a. Keddes test

Extract the drug with chloroform, evaporate to dryness. Add one drop of 90 % alcohol and 2drops of 2 % 3, 5 Di nitro benzoic acid in 90 % alcohol. Make alkaline with 20 % sodium hydroxide solution, purple colour is produced. The colour reaction with 3, 5 Di nitro benzoic acid depends on the presence of alpha, beta unsaturated lactones in the aglycone.

b. Keller-killiani test (Test for deoxy sugars)

Extract the drug with chloroform and evaporate it to dryness. Add 0.4 ml of glacial acetic acid containing trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5 ml of concentrated sulphuric acid by the side of the test tube. Acetic acid layer shows blue colour.

c. Raymonds test

Treat the test solution with hot methanolic alkali, violet colour is produced.

d. Legals test

Treat the test solution with pyridine and alkaline sodium nitroprusside solution, blood red colour appears.

e. Baljet test

Treat the test solution with picric acid or sodium picrate, orange colur is formed.

Test for alkaloids

The qualitative chemical tests used for detection of alkaloids are dependent on the characters of alkaloids to give precipitates as salts of organic acids or with compounds of heavy metals, like mercury, gold, platinum, etc. The different reagents used are Mayer's reagent (potassium mercuric iodide solution) giving cream colored precipitate. Dragendorff's reagent (potassium bismuth iodide solution) giving reddish brown precipitate. Wagner's reagent (iodine-potassium iodide solution) yielding reddish brown precipitate. Some alkaloids also give yellow colured precipitates with picric acid called as Hagner's reagent and picrolonic acid. Individual alkaloid gives colour or precipitate with certain specific reagent.

a. Dragendorff's test[15]

To 2-3 ml filtrate, add few drops of dragendorff's reagent. Orange brown precipitate is formed.

b. Mayer's test

2-3 ml filtrate with few drops of Mayer's reagent gives precipitate.

c. Hager's test

2-3ml filtrate, add few drops of Hager's reagent gives yellow precipitate.

d. Wagner's test

2-3 ml filtrate with few drops of Wagner's reagent gives reddish brown precipitate.

Test for carbohydrates

a. Molisch's test

The test is positive with soluble, as well as, insoluble carbohydrates. It consists of treating the compounds with alpha napthol and concentrated sulphuric acid which gives purple colour ring at the junctions of two layer.

b. Reduction of fehling's solution

To the solution of sample, equal quantity of Fehling's solutions A and B is added. After heating, brick red precipitate is obtained.

Test for flavonoids

a. Shinoda test

To dry powder or extract, add 5 ml 95 % ethanol, few drops concentrated Hcl and 0.5 gm magnesium turnings. Pink colour observed.

Acute toxicity study as per OECD guideline 425[16]

In the assessment and evaluation of the toxic characters of the substance, determination of acute oral toxicity is usually an initial step. It provides information of health hazards likely to arise from a short-term exposure by the oral route. Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24h. Data from an acute study may serve as a basis for classification and labeling. LD (medium lethal 50 doses), oral, is a statistically derived single dose of a substance that can be expected to cause death in 50% of animals when administered by the oral route. The LD_{50} value expressed in terms of test substance per unit weight of test animal (mg/kg). It is initial step in establishing a dosage regimen in sub chronic and other studies and may provide initial information on the mode of toxic action of a substance.

Statistical analysis[17]

Statistical analysis of the results was done using the statistical functions of the Graphpad Prism 5.0 software. The results were expressed in terms of mean ± SD. The significance of difference between mean values for the various treatments were tested using one way analysis of variance test (ANOVA test) followed by Dunnett Multiple Comparisons Test and the p values less than 0.05 were considered significant.

Chemicals

Indomethacin (Sigma), Carrageenan (Sigma) and all other chemicals were of analytical grade.

Experimental design

Animals

Adult albino rats of either sex weighing between 150 to 250 grams were randomly selected from central animal facility. Animals were housed in groups of 3, at an ambient temperature of 25±1°C with ad libitum access to food and water. The study protocol was approved by Institutional Animal Ethics Committee.

Methods

Animals were randomly divided into 3 groups of 6 rats each;

Group I: Control (1ml of Vehicle, 2% Gum acacia suspension);

Group II: Standard drug (Indomethacin 100mg/kg);

Group III: Test drug (hydroalcoholic extract of *Aegle marmelos* leaves 100 mg/kg).

Carrageenan Induced Rat Paw Edema Animal Model[18]

In this method, rats were divided in 3 groups of six animals each. The animals were pretreated with drugs orally 1 hr before the experiment. 0.05 ml of 1% carrageenan was injected aseptically into the subplantar surface of right hind paw of each rat. Paw edema was measured by Mercury Plethysmograph (UGO Basile, Italy) at '0' hour and at the end of '4' hours. The difference between the zero and 4 hours gives the actual edema. Percentage inhibition (protection) against edema formation was taken as an index of acute anti inflammatory activity.

It was calculated by:

The percent inhibition of edema = 100 × (1- Vt / Vc)

Where,

Vc = mean paw edema volume in the control group.

Vt = mean paw edema volume in the drug treated group.

RESULTS AND DISCUSSION

The preliminary phytochemical screening like Saponins, Tannins, Amino acids, Proteins, Glycosides, Cardiac glycosides, Alkaloids, Carbohydrates and Flavonoids was done with the hydroalcoholic extract of *Aegle marmelos* leaves according to the procedure. In the above chemical test the hydroalcoholic extract of *Aegle marmelos* leaves showed positive results for Amino acids, Proteins, Alkaloids, Flavonoids and glycosides. The results of preliminary test of hydroalcoholic extract of *Aegle marmelos* leaves were shown in Table No.1.

The hydroalcoholic extract of *Aegle marmelos* leaves (100mg/kg) and indomethacin (100mg/kg) both significantly inhibited carrageenan induced rat paw edema ($P < 0.001$). The maximum inhibition of paw edema was observed in both indomethacin and the hydroalcoholic extract of *Aegle marmelos* leaves at the end of four hours when compared to the control group. Anti-inflammatory activity is expressed as Percent Inhibition (PI). The PI with the hydroalcoholic extract of *Aegle marmelos* leaves and indomethacin were 49.50% and 34.02% respectively shown in Table No.2.

Acute inflammation is characterized by vasodilatation, exudation of plasma, release of various inflammatory mediators, cytokines, growth factors and emigration of leukocytes. While the features of chronic inflammation includes infiltration of mononuclear cells, proliferation of fibroblasts, blood vessels and increased connective tissue formation. Tissue infection is a prototype of inflammatory response. Anti-inflammatory drugs inhibit different stages of inflammation.

Preliminary phytochemical screening of hydroalcoholic extract of *Aegle marmelos* leaves revealed the presence of alkaloids and glycosides. Studies have shown the evidence of the presence of various chemical constituents in the *Aegle marmelos* leaves. Oxidative stress and neutrophil infiltration are common for inflammatory diseases and it is now accepted that gastric ulcer is mainly caused by oxidative stress. Intra gastric administration of marmin at a dose of 25mg/kg body weight exhibited antiulcer activity in experimental ulcer models[20].

Carrageenan is regarded as an established phlogistic agent/oedemogen and edema induced by the subplantar injection of carrageenan in the rat hind paw is reported to have been inhibited by a number of steroidal and non-steroidal anti-inflammatory drugs[19]. It has a biphasic effect. The first phase is due to release of histamine and serotonin (5-HT) (0-2 hr), plateau phase is maintained by a kinin like substance (3hr) and second accelerating phase of swelling is attributed to PG release (>4hr). In our study the hydroalcoholic extract of *Aegle marmelos* leaves 100 mg/kg, p.o. significantly reduced edema induced by the carrageenan. The percent inhibition of paw edema by indomethacin was 49.5% while the hydroalcoholic extract of *Aegle marmelos* leaves is 34.02%.

Table No.1: Phytochemical screening results of hydroalcoholic extract of *Aegle marmelos* leaves

S.No	Phytoconstutient	Result
1	Saponins	-
2	Tannins	-
3	Amino acids	+
4	Proteins	+
5	Glycosides	+

6	Alkaloids	+
7	Carbohydrates	-
8	Flavonoids	+

Present = (+), Absent = (-)

Table No.2: Effect of anti-inflammatory activity hydroalcoholic extract of *Aegle marmelos* leaves

S.No	Groups	Paw Edema mean	Percentage inhibition
1	Control -1 ml gum acacia (vehicle)	18.22±2.04	0
2	Indomethacin (100 mg/kg)	9.24±2.64	49.50
3	*Aegle marmelos* leaves (100mg/kg)	12.02±4.02	34.02

CONCLUSION

The studies on the *Aegle marmelos* leaves are fewer and studies on the hydroalcoholic extract of *Aegle marmelos* leaves on anti-inflammatory models are sparse. Hence the present study has attempted to fill these lacunae of this invaluable drug. The standardization of the extracts, identification and isolation of active principles and pharmacological studies of these needs to studied further. To conclude, a large number of studies on the *Aegle marmelos* leaves have showed antidiabetic, antiulcer, anti-inflammatory, analgesic, antipyretic, antidiarrhoeal, antihyperlipidaemic, antioxidant, anticancer, antimicrobial and radioprotective properties. In the present study the hydroalcoholic extract of *Aegle marmelos* leaves has showed promising results in acute model however these were less effective in the chronic model of experimental inflammation when compared to the standard indomethacin. These studies are valuable for identifying lead compounds for anti-inflammatory drugs, keeping in mind the side effects of NSAIDs and corticosteroids. Further human studies are needed to prove the safety and efficacy of long term administration of hydroalcoholic extract of *Aegle marmelos* leaves as potential anti-inflammatory agent in routine clinical practice.

ACKNOWLEDGEMENT

The authors are sincerely thanks to Bharathi College of Pharmacy, Mandya, Karnataka, India for providing the facilities to complete this research work.

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