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EVALUATION OF *IN-VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT 0F *SYZYGIUM OLEOSUM* IN WISTAR RATS

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ABSTRACT

Natural products are always helpful in the maintenance of life and good health. *Syzygium oleosum* (*S. oleosum*). is a very large evergreen tropical tree belonging to the family Myrtaceae. The plant is also mentioned in literature as Jamun, synonym as black plum. The native home of the *Syzygium* is India and East Indies. This plant is also found in other countries like Thailand, Philippines, Madagascar. Extensive work was carried out on plant of *S. oleosum* for their pharmacological properties. The medicinal value is due to presence of malic acid, oxalic acid, gallic acid, tannins. Various works on tannin, flavonoids essential oil and betulinic acid was reported to have diverse pharmacological activities like gastroprotective, antiulcerogenic, antibacterial, anti-infective, antimalarial. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. Antioxidant activity was studied through DPPH assay, ABTS+ radical scavenging assay method using ascorbic acid as standards. The present study is aimed to evaluate the anti-inflammatory activity of *S. oleosum* on histamine induced rat paw edema method in rats as for controlling inflammatory disorders. Acute toxicity of the extract (2000 mg/kg) was examined in wistar rats for 14 days. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, glycosides, alkaloids. The total phenolics content of *S. oleosum* extract was (71.94 mg/g/100mg), followed by flavonoids (1.81 mg/g /100mg) respectively. The activities of ethanolic bark extract against DPPH assay, ABTS+ radical scavenging assay, were concentration dependent. Ethanolic extract up to 2000 mg/kg did not produce any toxic effects. The ethanolic extract of *S. oleosum* (200 and 400 mg/kg) inhibited the inflammation induced by histamine in rats in a dose dependent manner. The ethanolic extract of *S. oleosum* possesses a strong anti-inflammatory activity and may be considered an interesting source of effective anti-inflammatory compounds.

KEYWORDS: *Syzygium oleosum*, Antioxidant activity. Acute toxicity, Anti-inflammatory effect, Phytochemical screening, Flavonoid, paw edema.

INTRODUCTION

Herbal therapy, although still an unwritten science, is well established in some countries and traditions and has become a way of life in almost 80% of population in rural areas. Inflammation is one of the most important physiological reactions of a body to stimuli such as irritation, trauma, tissue injury and infection, but excessive or persistent inflammation results in a variety of pathological conditions or organ damage.[1] Usually, inflammation develops through infiltration of leukocytes to the injury sites and production of specific cytokines such as IL-1b and TNF-a. Reactive oxygen species (ROS) also are released during the inflammation process to exert a protective effect against invading pathogens.[2,3] Chronic anti-inflammatory diseases including rheumatoid arthritis are still one of the main health problems of the world's population. At present, although synthetic drugs are dominating the market but element of toxicity that these drugs entail, cannot be ruled out. Their prolonged use may cause severe adverse effects on chronic administration[4], the most common being gastrointestinal bleeding and peptic ulcers.[5] Consequently, there is a need to develop a new anti-inflammatory agent with minimum side effects. Search for safe and effective anti-inflammatory agents have been given priority in scientific research in herbal system of medicine. *S. oleosum* is a very large evergreen tropical tree belonging to the family Myrtaceae.[6] The plant is

also mentioned in literature as Jamun, synonym as black plum or jambolan, botanical name the plant are very well known for their pharmacological properties since ancient age. The native home of the *Syzygium* is India and East Indies. This plant is also found in other countries like Thailand, Philippines, Madagascar[7], extensive work were carried out on plant of *S. oleosum* for their pharmacological properties. The medicinalvalue is due to presence of malic acid, oxalic acid, gallic acid, tannins. Various works on tannin, flavonoids essential oil and betulinic acid was reported to have diverse pharmacological activities like gastroprotective, antiulcerogenic, antibacterial[8], anti-infective[9,10], antimalarial.[11] *S. oleosum* belongs to family Myrtaceae, which records the occurrence of taxonomically informative molecules, namely malic acid, oxalic acid, gallic acid, betulinic acid, tannins, flavonoids and essential oil. Review detailing the chemical constituents of the *S. oleosum* have been reported several researchers. The widespread uses of *S. oleosum* in traditional medicines have resulted in considerable chemical analysis of the plant, and active principles which attribute the plant its medicinal properties have been identified and isolated. The entire plant is used in the traditional medicine; however the leaves andstem bark is mentioned to be most powerful part. *S. oleosum* is rich in compounds containing anthocyanins, glucoside, ellagic acid, isoquercetin, kaemferol and myrecetin. The seeds are claimed to contain alkaloid, jambosine, and glycoside jambolin or antimellin, which halts the diastatic conversion of starch into sugar and seed extract has lowered blood pressure and thisaction is recognised to the ellagic acid content. The seeds have been reported to be rich in flavonoids, a well-known antioxidant, which accounts for the scavenging of free radicals and protective effect on antioxidant enzymes[12, 13] and also found to have high total phenolics with significant antioxidant activity[14] and are fairly rich in protein and calcium. Therefore, the aim of this study was phytochemical screening and evaluation of antioxidant activity and anti-inflammatory activities of ethanolic extract of bark of *S. oleosum* by using histamine-induced rat paw edema model.

MATERIALS AND METHODS

Plant material

S. oleosum barks were procured from local market. *Syzygium oleosum* barks was authenticated by Dr. Saba Naaz, Professor, Department of Botany, Safia College of Science, Bhopal. The barks were shade dried. It was then coarsely powdered and preserved in air tight glass container for further processing.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. Mumbai, India), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade. Histamine from Loba Chemie. Standard Diclofenac from Aventis Pharma. Ethanol (Analytical grade).

Extraction procedure

The barks were shade dried at room temperature for 10 days. Then these were milled into powder by mechanical grinder. This powder was sequentially extracted to their increasing polarity with Petroleum ether, Ethanol respectively. About 500gm of powdered leaves was uniformly packed into a thimble in a soxhlet apparatus and extracted with 1000ml petroleum ether and ethanol, respectively. Constant heat was provided by Mantox heater for recycling of the solvent. The process of extraction continues for 1-2 hours for each solvent. The excess solvent was evaporated and the dried extracts were kept in refrigerator at 4ºC for their future use in phytochemical analysis and pharmacological screenings.[16]

Qualitative phytochemical analysis of plant extract

The *S. oleosum* barks extract obtained was subjected to the preliminary phytochemical analysis following standard methods.[16] The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavanoids, glycosides, saponins, alkaloids, fats or fixed oils, protein, amino acid and tannins.

Total phenol determination

The total phenolic content was determined using the method of Olufunmiso *et al*.[17] A volume of 2ml of each extracts or standard was mixed with 1 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 10min for colour development. The absorbance was measured at 765 nm using a UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al*.[17] 1ml of 2% $AlCl_3$ solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer. The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Antioxidant activity

DPPH method

DPPH scavenging activity was measured by modified method. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the

absorbance in presence of sample extract at different concentration (50-250µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol. Three test samples were taken and each processed similarly. Finally, the mean was taken. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

ABTS radical cation decolorization assay

ABTS+ was dissolved in water at a concentration of 7mM. The stock solution was mixed with .45mM potassium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours prior to use in incomplete oxidation of ABTS+. The radical remained stable in this form for more than two days (in the dark at room temperature). The reagents were added in the following order: The incubation mixture in a total volume of 5 ml contained 0.54 ml of ABTS+, 0.5 ml of phosphate buffer and different concentrations of (all extracts of *S. oleosum* barks) (25-125µg/ml). The blank sample contained water instead of sample or the standard. Absorbance was measured spectra photometrically at 734 nm and compared with the standard (Ascorbic acid).[18]

Animals

Male Wistar rats of 6-8 weeks old and 180-200 g body weight were selected for study. All rats were housed and maintained under standard conditions of temperature (25 ^{0}C ± 5 ^{0}C), relative humidity (55 ± 10%), and 12/12 h light/dark cycle. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. Protocols for the study were approved by the Institutional Animal Ethical Committee (IAEC) for Animal Care and were in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

Acute toxicity test

Acute oral toxicity study was performed as per OECD-423 guidelines. The mice were fasted overnight with free excess of water and were grouped into four group consisting of 3 animals each, to which the extract was administered orally at the dose level of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg body weight. They were observed for mortality; toxic symptoms such as behavioral changes, locomotor activity, convulsions; direct observation parameters such as tremor, convulsion, salivation, diarrhoea, sleep, coma, changes in skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and CNS, somatomotor activity etc. periodically for 30 min during first 24 h. And specific attention given during first 4 hours daily for a total period of 14 days.

Experimental design

Wistar albino rats were divided into four groups of 6 animals each.

Table 1: Experimental Design for Histamine Induced Paw Edema.

GROUPS	TREATMENT
Group I	0.1 ml of freshly prepared histamine (0.1%)
Group II	Diclofenac (20 mg/kg), p.o
Group III	Extract (200 mg/kg), p.o
Group IV	Extract (400 mg/kg), p.o

One hour after the drug treatment, inflammation was induced by injection of 0.1 ml of freshly prepared histamine (1%) in normal saline underneath the plantar tissue of the right hind paw of rats. Paw volume, measured using a plythesmometer before histamine administration and at 1, 2, 3 h after histamine injection. The percentage inhibition (PI) of paw edema was calculated by using the following formula;

Percentage of edema inhibition = $(V_C - V_t/V_C) \times 100$

V_C = Mean edema volume in control, V_t = Mean edema volume in group treated with standard or extract.

Statistical analysis

Data were analyzed by one way ANOVA followed by Tukey's multiple comparison test using Graph pad 5.0 software. The values were expressed as Mean ± SEM.

RESULTS AND DISCUSSION

The crude extracts so obtained after the soxhlation process; extract was further concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of ethanolic extract was found to be 22. 5%. Phytochemical analysis of ethanolic extracts of *S. oleosum* barks showed the presence of phenols, flavonoids, tannins, saponins, glycosides, alkaloids Table 2. The presence of phenolic compounds (TPC) is expressed as mg/g of gallic acid

equivalent of dry concentrate test utilizing the mathematical expression obtained from the calibration curve: Y = 0.0095X + 0.0305, R2= 0.991, where Y is the absorbance and X is the Gallic acid equivalent (GAE). In the present study, the ethanolic bark concentrate of *S. Oleosum* displays 71.94 mg/g of gallic acid equivalent phenols Figure 1. Absolute concentration of flavonoids was computed as quercetin proportionate (mg/g) utilizing the mathematical statement in light of the calibration curve: Y=0.0041 X- 0.0015, R2=0.998, where Y is the absorbance and X is the quercetin comparable (QE). The result reveals that the ethanolic bark concentrate of *S. Oleosum* exhibits 1.81 mg/g of quercetin equivalent of flavonoids Figure 2. Antioxidant activity of the samples was calculated through DPPH assay, ABTS radical cation decolorization assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard and the values were comparable with concentration ranging from 50to 250µg/ml and 25-125 µg/ml. A dose dependent activity with respect to concentration was observed Table 3, 4. The acute toxicity test was performed by using the extract at concentrations of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg. Toxicity study was performed as per OECD guidelines 423. It was observed that the extract was not lethal to the rats at 2000 mg/kg dose. Hence the dose fixed for study as 200 mg/kg as low dose and 400 mg/kg as high dose. The observations are summarized in Table 5. Both doses of extract (200 mg/kg and 400 mg/kg) exerted a significant inhibition of 15.97% and 36.83% at 1 h, 48.11% and 55.89% at 2 h, 58.46% and 69.09% at 3 h respectively in the histamine induced rat paw edema model. It was observed that the extract was capable of inhibiting edema induced by histamine and the effectiveness for suppression of edema might be due to the ability of extract to inhibit the synthesis, release or action of histamine involved in the inflammation Table 6. To improve the safety of this traditional herbal remedy, additional research is needed to define the stability and bioactivity of this product. Therefore, further studies are needed for the isolation and characterization of the active constituents.

Table2: Phytochemical analysis of ethanolic barks extract of *S. oleosum*.

SL. No	Phytochemical Constituents	ethanolic barks extract of *S. oleosum*
1	Carbohydrates	_
2	Alkaloids	+
3	Glycosides	+
4	Triterpenoids	_
5	Flavonoids	++
6	Phenols	++
7	Tannins	+
8	Steroids and sterols	+
9	Saponins	_
10	Proteins and amino acids	++

+= Present, ++ = More present, - = Absent

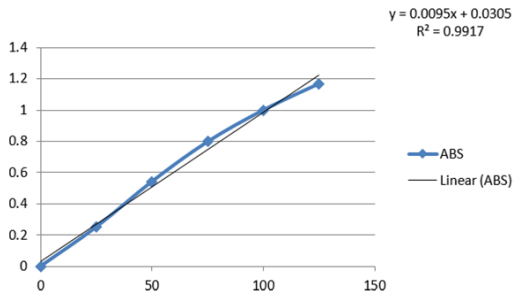

Fig. 1: Calibration Curve of Gallic Acid.

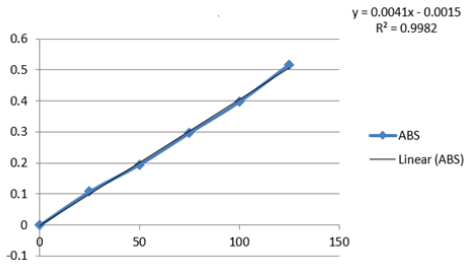

Fig. 2: Calibration curve of Quercetin.

Table: 3 DPPH Radical Scavenging Activity.

	% inhibition			DPPH
Conc	STD	Extract	Extract	IC 50 in µg/ml
50	12.905	1.869	STD	134.4747
100	30.96	12.81	Extract	240
150	56.111	28.26		
200	68.298	43.65		
250	86.66	49.77		

Table: 4 ABTS Radical Scavenging Activity.

ABTS	% inhibition			ABTS
Conc	STD	Extract	Extract	IC 50 in µg/ml
25	20.9736	17.3761	STD	44.495
50	35.0822	22.89	Extract	97.928
75	47.2373	38.0606		
100	66.0193	51.5586		
125	86.3962	64.2219		

Table 5: Acute Toxicity Study.

Response	Group 1	Group 2	Group 3	Group 4
Alertness	Normal	Normal	Normal	Normal
Grooming	Normal	Normal	Normal	Normal
Touch response	Normal	Normal	Normal	Normal
Torch response	Normal	Normal	Normal	Normal
Pain response	Normal	Normal	Normal	Normal
Tremor	Normal	Normal	Normal	Normal
Convulsion	Normal	Normal	Normal	Normal
Lighting reflex	Normal	Normal	Normal	Normal
Gripping strength	Normal	Normal	Normal	Normal
Pinna reflex	Normal	Normal	Normal	Normal
Corneal reflex	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal
Lacrimation	Normal	Normal	Normal	Normal
Diarrhoea	Normal	Normal	Normal	Normal

Table 6: Effect of Extract on Histamine Induced Rat Paw Edema In Rats.

Group	Treatment and Dose	Mean edema volume(ml) and % inhibition			
		0 h	1 h	2 h	3 h
I	Histamine 0.1% (0.1 ml)	0.066±0.003	0.120±0.002	0.217±0.003	0.3133±0.002
II	Diclofenac(20 mg/kg)	0.067±0.004	0.061±0.0015***(49.166%)	0.082±0.001***(62.24%)	0.092±0.001***(70.60%)
III	Extract (200 mg/kg)	0.063±0.004	0.101±0.005**(15.97%)	.112±0.001***(48.112%)	0.130±0.002***(58.46%)
IV	Extract(400 mg/kg)	0.064±0.002	0.076±0.002***(36.83%)	0.095±0.003***(55.89%)	0.103±0.003***(67.09%)

Values in brackets denote percentage inhibition of the edema paw volume. Values represent mean ± SEM, n=6 compared with control, statistical analysis was done by one way analysis of variation (ANOVA) followed by Dunnett's test. ***P<0.001, **P<0.01, *P<0.05 and ns- non significant.

CONCLUSION

S. oleosum (family Myrtaceae) is a well-known bioactive plant in Ayurvedic system of medicine. The present investigation was aimed at determining the anti-inflammatory and anti-oxidant activity of ethanolic bark extract of *S. oleosum*. Extract showed significant *in-vitro* antioxidant activity by terminating the actions of free radicals. The extract was studied for its anti-inflammatory activity using Histamine induced inflammation in which Diclofenac (20mg/kg) was used as the standard. Thus the study proved that the extract possesses significant (P<0.05) anti-inflammatory activity which was evident with reduction in mean paw edema volume in histamine induced inflammatory models.

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