

ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIAL OF *PTEROSPERMUM ACERIFOLIUM*

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ABSTRACT

Leaves of *Pterospermum acerifolium* L. (Sterculiaceae) are used in India for reducing oxidative stress and inflammation. The objective of this study was to investigate the antioxidant and anti-inflammatory activities to justify the use of the plant in folkloric medicine. Antioxidant activity of different fractions were evaluated by using in-vitro antioxidant assays models like determination of total phenolics, DPPH radical scavenging assay, nitric oxide scavenging assay, hydroxy radical scavenging assay and superoxide anion scavenging assay. Anti-inflammatory activity was evaluated using carrageenan induced inflammation and thermally induced protein denaturation. Ethyl acetate fraction of *P. acerifolium* (EAF) showed highest free radical scavenging activity in all the models. EAF also produced significant anti-inflammatory activity in both in-vivo and in-vitro model. The results obtained in this study showed that the leaves of *Pterospermum acerifolium* L. have antioxidant and anti-inflammatory properties which provide a basis for the traditional use of the plant.

Keywords: Antioxidant, anti-inflammatory, *Pterospermum acerifolium*, total phenolic content.

INTRODUCTION

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. The research into plants with alleged folkloric use as anti-inflammatory agents should therefore be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs.

Pterospermum acerifolium Linn. has a wide application in traditional system of Indian medicine for example, in ayurvedic anticancer treatment flowers are mixed with sugars and applied locally [1]. Flowers and bark, charred and mixed with kamala applied for treatment of smallpox. Flowers made into paste with rice water used as application for hemicranias [2]. Stem bark of the plant was found to have antimicrobial activity [3]. Isolation of boscalin glucosides from leaves of *Pterospermum acerifolium* have been reported [4]. Hepatoprotective effect of ethanol extract of leaves of *Pterospermum acerifolium* was also reported [5]. Chronic effects of *Pterospermum acerifolium* on glycemic and lipidemic status of type 2 model diabetic rats was found beneficial [6].

There is no scientific information on the anti-inflammatory and antioxidant study of the plant. The present study aims to evaluate the antioxidant and anti-inflammatory potential of *Pterospermum acerifolium* to justify the folkloric use of this plant.

MATERIALS AND METHODS

Plant Material

Leaves of *Pterospermum acerifolium* were collected from Jatara, Tikamgarh, Madhya Pradesh (India) during the month of September 2007. The plant was identified by

botanist, College of Pharmaceutical Sciences, Berhampur, Orissa, India.

Chemicals and Reagents

All the chemicals and reagents used were of analytical grade includes DPPH (diphenyl picryl hydrazyl), nitro blue tetrazolium (NBT), NADH, phenazine methosulphate nicotinamide (PMS), ammonium molybdate, carrageenan, dextran, trypsin, egg albumin (Lancaster Research Lab, Chennai, India), α -tocopherol, trichloroacetic acid, ascorbic acid, casein (Himedia Lab, Mumbai India) and indomethacin (Advance Scientific Center, Bhopal, India).

Extraction of Plant Material

About 1.5 kg of shade dried powder of leaves of *Pterospermum acerifolium* was successively extracted with petroleum ether and methanol to obtain the petroleum ether and methanol extract. The crude methanol extract, after removal of the solvent, was dissolved in 10% sulfuric acid solution and partitioned with chloroform, ethyl acetate, n-butanol and water successively to give chloroform, ethyl acetate, n-butanol and water soluble fractions respectively.

Antioxidant activity

Determination of total phenolics

The concentrations of phenolic content in all the fractions were determined with Folin-Ciocalteu's phenol reagent (FCR) according to the method of Slinkard and Singleton [7]. 1 ml of the solution (contains 1 mg) of the fraction in methanol was added to 46 ml of distilled water and 1 ml of FCR, and mixed thoroughly. After 3 min, 3 ml of sodium carbonate (2%) were added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of phenolic compounds was calculated according to the following

equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance} = 0.001 \times \text{pyrocatechol } (\mu\text{g}) + 0.0033$$

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured as per the process described by Blois et al [8]. Briefly, 0.1 mM solution of DPPH solution in methanol was prepared and 1ml of this solution was mixed with 3ml of sample solutions in water at different concentrations. Finally, after 30 min, the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. DPPH radical-scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = ((A_0 - A_1) / A_0 \times 100)$$

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO_2^-) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color which can be measured at 546 nm [9]. Sodium nitroprusside (10 mM, 2 ml) in phosphate buffer saline was incubated with the test compounds in different concentrations at room temperature for 30 min. After 30 min, 0.5 ml of the incubated solution was added with 1 ml of Griess reagent and the absorbance was measured at 546 nm. The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = ((A_0 - A_1) / A_0 \times 100)$$

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract.

Superoxide anion scavenging activity assay

Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT). About 1 ml of nitro blue tetrazolium (156 μM), 1 ml NADH (468 μM) in 100 mM phosphate buffer of pH 7.8 and 0.1 ml of sample solution of different concentrations were mixed. The reaction started by adding 100 μl PMS (60 μM). The reaction mixture was incubated at 25°C for 5 min and absorbance of the mixture was measured at 560 nm against blank samples [10]. The percentage inhibition was determined by comparing the results of control and test samples.

$$\% \text{ Inhibition} = ((A_0 - A_1) / A_0 \times 100)$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydroxy radical scavenging activity

Hydroxy radical scavenging activity was determined using 2-deoxyribose oxidative degradation as described previously [11]. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with thiobarbituric acid (TBA). The reaction mixture contained deoxyribose (2.8mM); FeCl_3 (100 mM); KH_2PO_4 -KOH buffer (20mM, pH 7.4); EDTA (100 mM); H_2O_2 (1.0 mM); ascorbic acid (100m M), and various concentrations of the test compounds in a final volume of 1 ml. Ferric chloride and EDTA (when added) were premixed just before addition to the reaction mixture. The reaction mixture was incubated at 37°C for 60 min. After incubation at 37°C for 1 h, 1.0 ml of 2.8% trichloroacetic acid and 1.0 ml of 1% aqueous solution of TBA were added to the sample; test tubes were heated at 100°C for 20 min to develop the color. After cooling, TBARS formation was measured spectrophotometrically at 532 nm against an appropriate blank. The hydroxyl radical-scavenging activity was determined by comparing absorbance of the control with that of test compounds.

Anti-inflammatory activity

Animals

Healthy, albino rats (Wistar Strain) of either sex, weighing 150 – 200 Gm were obtained from Animal house of CPS Mohuda. They were housed in good condition in the department's animal house and given standard mouse pellet and water *ad Libitum*. All the rats were kept at controlled light condition (light: dark, 12:12 hr) and temperature $22 \pm 1^\circ \text{C}$. (According to CPCSEA norms)

Acute toxicity study

Albino rats of either sex were used for acute toxicity study. The animals were divided into six groups containing six animals each. EAF was suspended in Tween 80 and administered orally as a single dose at different dose levels viz. 500, 750, 1000, 1250, 1500 and 2000 mg/kg of body weight (b.w.) The Mice were observed periodically for symptoms of toxicity and death within 24 h and then daily for next 14 days.

Carrageenan induced inflammation

Pedal inflammation was produced by subplantar administration of 0.1 ml of carrageenan (1% w/v) to the right paw of each rat [12]. Different groups of animals were pretreated with EAF (150 and 300 mg/kg, *p.o.*) or with 5 ml/kg of distilled water (vehicle control) or 10 mg/kg reference drug (indomethacin) at 1h before eliciting paw edema. The paw volume was measured by dipping the foot in the mercury bath of the plethysmograph up to the anatomical hairline on lateral malleolus and compared with control animals, which received only the vehicle. Measurement was done immediately before, first and third hour following carrageenan injection. The edema inhibitory activity was calculated according to the following formula-

$$\text{Edema } (\%) \text{ inhibition} = (1 - D/C) \times 100$$

where, D represents the percentage difference in increased paw volume after the administration of test drugs to the rats and C represents the percentage difference of increased volume in the control groups.

Inhibition of protein denaturation

Test solution having different concentration (50-250 $\mu\text{g/ml}$) of drug was taken with 1ml (1mM) of egg albumin solution. The mixture was incubated at $27 \pm 1^\circ \text{C}$ for 15 min. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 min. After cooling the turbidity was measured spectrophotometrically at 660 nm [13]. Percentage inhibition of denaturation was calculated from control where no drug was added.

Statistical analysis

The experimental data were expressed as mean \pm SEM. The significance of difference among the various treated groups and control group were analyzed by means of one-way ANOVA followed by Dunnett's multiple comparison tests using Graphat InStat Software (San Diego, CA, USA). The level of significance was set at $p < 0.05$.

RESULTS

In this study in-vitro antioxidant activity of different fractions and anti-inflammatory activity of ethyl acetate

soluble fraction of leaves of *Pterospermum acerifolium* was evaluated by different in vivo and in vitro screening methods.

Preliminary phytochemical screening of the different fractions of *Pterospermum acerifolium* leaves revealed the presence of steroids, flavonoids, alkaloids and glycosides. In the acute toxicity assay no deaths were observed during the 72 h period at the doses tested. At these doses, the animals showed no stereotypical symptoms associated with toxicity, such as convulsion, ataxy, diarrhoea or increased diuresis thus the median lethal dose (LD50) was determined to be higher than the dose tested i.e. 2.0 g/ kg b.w.

The total phenolic content of different fractions is presented in Table 1. It was calculated as quite high in ethyl acetate fraction ($169.2 \pm 12.5 \mu\text{g mg}^{-1}$ pyrocatechol equivalent) other than n-butanol ($32.7 \pm 8.92 \mu\text{g mg}^{-1}$) and chloroform fraction ($21.2 \pm 6.13 \mu\text{g mg}^{-1}$).

The results of the free radical scavenging potentials of different fractions tested by DPPH method are depicted in Figure 1. The IC_{50} value of EAF was found as $26.2 \mu\text{g/ml}$, whereas the IC_{50} value of NF was found as $66.2 \mu\text{g/ml}$. The low IC_{50} value of ethyl acetate fraction is due presence of high polyphenolics and flavonoids in it.

Table 1: Total phenolic content and comparative scavenging activity of different fractions at highest concentration (100 $\mu\text{g/ml}$)[#]

Fractions	Total phenolic content	DPPH radical	Nitric oxide radical	Superoxide radical	Hydroxy radical
EAF	169.2 ± 12.5	79.7 ± 2.25	70.7 ± 4.04	66.1 ± 2.3	69.4 ± 1.21
NF	32.7 ± 8.9	73.8 ± 2.25	57.6 ± 3.2	54.2 ± 2.71	58.31 ± 6.7
CF	21.2 ± 6.13	45.08 ± 1.97	33.9 ± 6.3	33.7 ± 3.51	41.3 ± 4.8

[#]Values are mean \pm SEM (n=6)

Table 2: Effect of EAF of *Pterospermum acerifolium* on carrageenan induced paw edema in rats[#]

Treatment	Dose	Paw volume (ml)			Edema inhibition (%)
		Initial	1 h	3 h	
Normal saline	5 ml/kg	3.41 ± 1.25	3.98 ± 0.29	4.49 ± 0.32	-
EAF	150	3.39 ± 0.23	3.72 ± 0.25	$3.42 \pm 0.20^*$	23.83
EAF	300	3.32 ± 0.14	3.61 ± 0.17	$2.98 \pm 0.21^{**}$	33.6
Indomethacin	10	3.22 ± 0.21	3.39 ± 0.18	$2.79 \pm 0.24^{**}$	37.86

[#]Values are mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$ significant as compared to control group

Different fractions of methanol extract of *Pterospermum acerifolium* also moderately inhibited nitric oxide in dose dependent manner (Figure 2). IC_{50} value of EAF, NF was found as $32.4 \mu\text{g/ml}$ and $78.5 \mu\text{g/ml}$ respectively.

In superoxide anion scavenging assay, ethyl acetate fraction showed maximum superoxide anion scavenging activity and the results are presented in Figure 3. The IC_{50} value of EAF ($51.8 \mu\text{g/ml}$) was lower than NF ($\text{IC}_{50} = 91.4 \mu\text{g/ml}$).

All the fractions suppressed dexyribose degradation in a concentration-dependent manner (figure 4). In this assay also ethyl acetate fraction showed lowest IC_{50} value ($28.7 \mu\text{g/ml}$) other than NF ($86.6 \mu\text{g/ml}$) and chloroform fraction ($\text{IC}_{50} > 100 \mu\text{g/ml}$).

The in vivo anti-inflammatory effects of the EAF were assayed by using carrageenan induced paw edema. The administration of sub plantar injection of carrageenan (0.1

ml, 1% w/v), produced significant edema in the rat paws, reaching maximum at 3 h (Table 2). Oral administration of EAF caused doses related inhibition of carrageenan induced inflammation. The extract with dose of 150 mg/kg induced significant ($p<0.05$) anti-inflammatory activity at 3 h after carrageenan administration.

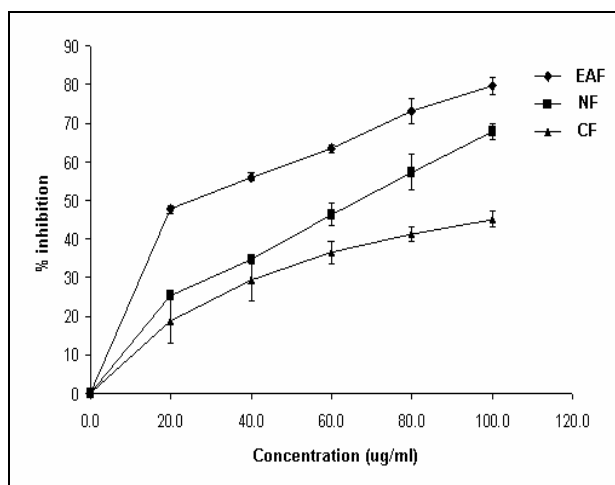


Figure 1: Radical scavenging potential of different fractions by DPPH method at different concentrations (µg/ml).

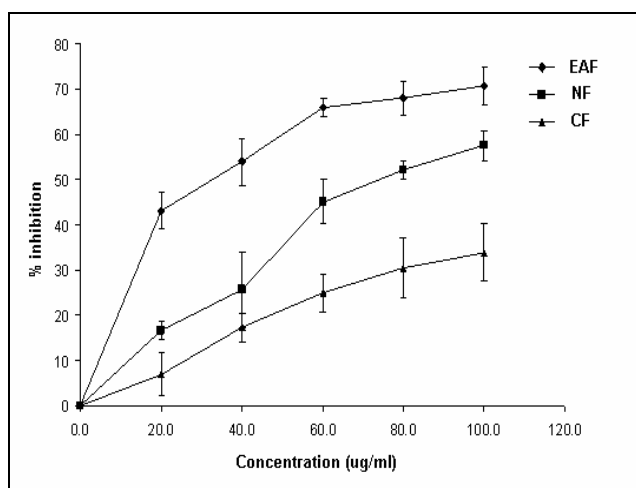


Figure 2: Nitric oxide scavenging potential of different fractions at different concentrations (µg/ml).

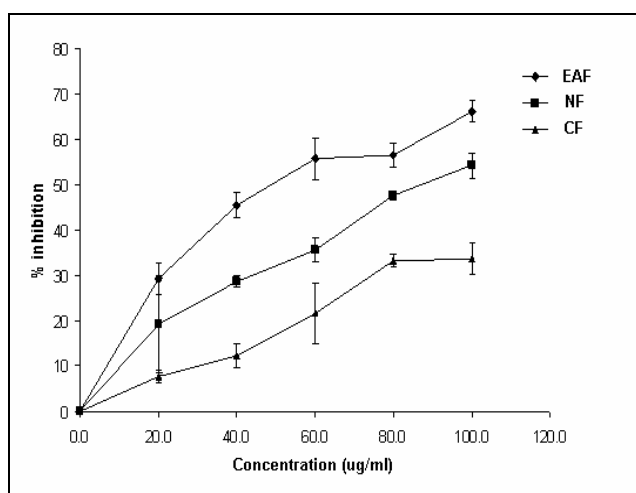


Figure 3: Scavenging potential of different fractions at different concentrations (µg/ml) on superoxide radicals generated by the PMS/NADH system.

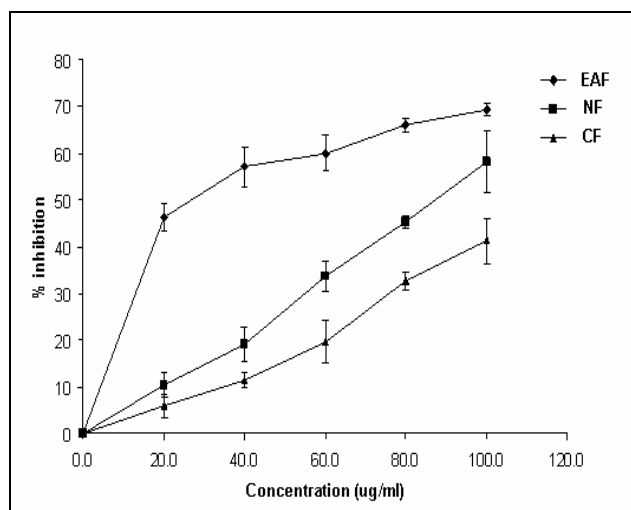


Figure 4: Hydroxy radical scavenging potential of different fractions at different concentrations (µg/ml) on deoxyribose degradation method.

The dose of 300 mg/kg abolished inflammation more significantly ($p<0.01$) at 3 h and it was comparable to the untreated control group. A standard drug indomethacin (10 mg/kg, *p.o.*) showed potent inhibition against carrageenan induced inflammation. EAF showed 23.83 and 33.6 % edema inhibition at 3 h against carrageenan induced inflammation.

The inhibitory effect on protein denaturation by EAF is shown in Table 3. EAF (50-250 µg/ml) showed significant ($p<0.05$) inhibition of denaturation of egg albumin in concentration dependent manner. EAF at concentration of 250 µg/ml and indomethacin at concentration of 100 µg/ml showed significant ($p<0.01$) inhibition (68.86 and 82.67% respectively) of protein denaturation when compared with control.

Table 3: Effect of ethyl acetate fraction of *Pterospermum acerifolium* on *in vitro* assays.[#]

Concentration (µg/ml)	Inhibition of protein denaturation (%)
50	24.07±0.62
100	29.8±0.66
150	34.59±0.23
200	54.6±0.67
250	68.76±0.85

[#]Values are mean ±SEM (n=3)

DISCUSSION

The present study established the anti-inflammatory activity of ethyl acetate fraction of methanolic extract of *Pterospermum acerifolium*. It is earlier reported that carrageenan induced inflammation is useful for the detection of orally active anti-inflammatory agents [14]. Vinegar *et al*, [15] reported that edema formation due to

carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine & serotonin. The second phase of edema is a result of liberation of prostaglandins, lysosomes, bradykinins and protease [16]. Dirosa *et al* had earlier stated that the second phase is sensitive to most clinically active anti-inflammatory drugs [14]. The EAF showed dose dependent inhibitory activity over a period of 3hrs and extract showed maximum inhibition of carrageenan induced paw edema at 3h. This indicates EAF attenuates with both early and delayed phases of carrageenan induced inflammation.

Denaturation of proteins is well documented cause of inflammation and rheumatoid arthritis [17]. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation [18].

Ability of EAF to bring down thermal denaturation of protein is possibly a contributing factor with the mechanism of action.

The presence of flavonoids and triterpenes in the leaves of *Pterospermum acerifolium* has been observed by general identification test. EAF was also found as a rich source of polyphenolic compounds. Therefore the anti-inflammatory activity of EAF of *Pterospermum acerifolium* seems to be due to the high polyphenolic compounds in it.

This study has revealed the potential for antioxidant activity and also lent scientific justification to the traditional use of plant in anti-inflammatory conditions. However, the exact structure of the bioactive compounds and mechanism(s) involved in anti-inflammatory activities of the EAF are yet to be elucidated.

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