Antioxidant and Anti-inflammatory activities of *Mitragyna parvifolia* leaves extract

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Abstract

Ethanolic leaves extract of *Mitragyna parvifolia* was screened for anti-inflammatory activity using Carrageenan-induced paw edema at various doses (100, 250 and 500 mg/kg) and the results were compared with standard drug Diclofenac sodium (50 mg/kg). It showed significant activity at 250 and 500 mg/kg ($p < 0.01$). The extract was also screened for antioxidant and free radical scavenging effects at various concentrations (100, 300 and 500 µg/ml) by reducing power assay, superoxide radical and DPPH free radical scavenging method. All these antioxidant activities were concentration dependent which were compared with standard antioxidants such as BHA and ascorbic acid. The highest antioxidant activity of *M. parvifolia* leaves extract was observed at a concentration of 500µg/ml.

Key words: *Mitragyna parvifolia*, Anti-inflammatory, Antioxidant, Reducing power

Introduction

Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli [1]. Although it is a defense mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases [2].

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl, hydrogen peroxide radical and peroxynitrite participate in the process of inflammation in various tissues [3]. In addition to their role in acute inflammation, ROS may also contribute to several chronic cutaneous inflammatory diseases such as psoriasis, atopic dermatitis, and contact dermatitis [4].

Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers [3]. Several antioxidative compounds have also been reported to show anti-inflammatory action either on arachidonic acid-induced ear edema or on TPA-induced ear edema [5]. Currently used anti-inflammatory drugs are associated with some severe side effects. Therefore,

naturally originated agents with very little side-effects are required to substitute chemical therapeutics.

The genus Mitragyna (family: *Rubiaceae*) consists of several plants used in local folkare medicine to treat fever, colic & muscular pain. It has been also used for the expulsion of worms [6]. *Mitragyna parvifolia* (Roxb.) Korth is commonly known as Kaim [7]. The plant grows throughout India, in deciduous and evergreen forests. Some of the chemical constituents reported in the plant are pyroligneous acid, methyl acetate, ketones and aldehydes. The plant is credited with innumerable medicinal properties and is widely used by tribal people and other ayurvedic practitioners. The bark and root are used to treat fever, colic, muscular pain, burning sensation, poisoning, gynecological disorders, cough and edema. The fruit juice augments the quantities of breast milk in lactating mothers and also work as lactodepurant. Wounds and ulcers are dressed with its leaves to alleviate pain, swelling and for better healing [6-9]. Though the plant has great potential for antioxidant and anti-inflammatory activity, nobody has not been yet documented these activities on the leaves of this plant. In continuation of our earlier reports on the fruit of this plant [10] we hereby reported the anti-inflammatory and antioxidant activities of leaves extract of *M. parvifolia*.

Materials and Methods

Chemicals used in this study were 2, 2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, butylated hydroxy anisole (BHA), ascorbic acid, phosphoric acid, nitro blue tetrazolium, phenazine methosulfate. All reagents used for the study were of analytical grade. All the standard drugs (Diclofenac sodium) were obtained from various chemical units - E.Merck India Ltd. and S. D. Fine Chem. Ltd. (India).

The leaves of *M. parvifolia* (MP) were collected from local areas during the November month of 2008. The plant got identified and authenticated by Department of Botany, Kurukshetra University, Kurukshetra, Haryana, (India) and a voucher specimen of the sample (Sr. No. KUK/IPS/2008/MP-105) has deposited in the Herbarium collection of Department. The leaves were cleaned and dried in the shade, then powdered to 40 mesh and stored in an airtight container.

Wistar albino rats weighing 150-200 gm were obtained from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, (India). The animals were housed in Animal house, Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra (Haryana) in polycarbonate cages, in a room maintained under controlled room temperature 22 ± 20 C, relative humidity 60 -70% and provided with food and water *ad libitum*. All the experimental procedures and protocols used in the study were reviewed by the Institutional Animal Ethics Committee (Register Number: 562/02/a/CPCSEA) and were in accordance with the guidelines of the CPCSEA, Ministry of Forests and Environment, Government of India. The animals were deprived of food for 24 h before experimentation but allowed free access to water throughout.

Preparation of Extract

The extract was prepared by cold maceration process. The dried leaves powder (750 g) divided in three parts and was treated each three times with fresh ethanol (1000 ml) separately for 48 h. The ethanolic extracts thus obtained were combined, filtered and

distilled on a water bath. The last traces of the solvent were evaporated under reduced pressure using rotatory evaporator (Heidolph Laborota 4011 digital). The ethanolic extract (yield = 2.06 % w/w) was used for pharmacological studies by suspending a weighed amount of the extract in normal saline (95 ml): tween 80 (5 ml) ratio.

Acute toxicity test

Acute toxicity tests were performed according to OECD – 423 guidelines (acute toxic class method) [11]. Swiss mice (n = 6) of either sex selected by random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water only. The ethanolic extract of *M. parvifolia* suspended in normal saline:tween 80 (95:5) was administered orally at a dose of 5 mg/kg initially and mortality was observed for 3 days. If mortality was observed in 5/6 or 6/6 animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in less than four mice, out of six animals then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher doses such as 100, 300 and 1500 mg/kg.

All studies were carried out by using five groups of ten animals each for both anti-inflammatory and anti oxidant activity.
Group I received normal saline : tween 80 (p.o),
Group II received Diclofenac sodium (50 mg/kg i.p.),
Group III received MP extract (100 mg/kg p.o.),
Group IV received MP extract (250 mg/kg p.o.) and
Group V received MP extract (500 mg/kg p.o.).

Anti-inflammatory activity *(Carrageenan-induced paw edema)*

The anti-inflammatory activity of ethanolic extract of *M. parvifolia* using carrageenan-induced paw edema was studied according to Winter et al [12]. Thirty minutes after administration of test and standard drugs, 0.1 ml of 1% w/v of carrageenan suspension in normal saline was injected to all animals in the left hind paw (plantar region). The paw volume, up to the tibiotarsal articulation, was measured using a plethysmometer (model 7140, Ugo Basile, Italy). The measures were determined at 0 h (before carrageenan injection) and 30, 60, 90 and 120 minutes after drug treatment. The anti-inflammatory effect of ethanolic extract was calculated by the following equation:

$$\text{Anti-inflammatory activity (\%)} = (1-V_t/V_c) \times 100$$

Where V_t represents the paw volume in drug treated animals and V_c represents the paw volume of control group of animals.

Antioxidant assay

DPPH free radical scavenging activity

The free-radical scavenging activity of MP extract was measured as decrease in the absorbance of methanol solution of DPPH [13]. A stock solution of DPPH (33 mg in 1 L) was prepared in methanol, which gave initial absorbance of 0.493, and 5 ml of this stock solution was added to 1 ml of extract solution at different concentrations (100, 300

and 500 μg/ml). After 30 min, absorbance was measured at 517 nm and compared with BHA and ascorbic acid taken as standards. Scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\% \text{ Anti} - \text{radical activity} = \frac{\text{Control Abs} - \text{Sample Abs}}{\text{Control Abs}} \text{ X } 100$$

Superoxide radical scavenging assay

The reaction mixture consisting of 1ml of nitro blue tetrazolium (NBT) solution (156 mM NBT in phosphate buffer, pH 7.4), 1 ml NADH solution (468 mM NADH in phosphate buffer, pH 7.4), and 1ml of sample solution of extract was mixed. The reaction was started by adding 100 ml of phenazine methosulfate (PMS) solution (60 mM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25^0 C for 5 min and the absorbance was measured at 560 nm against blank sample and compared with standards [14, 15]. Decreased absorbance of reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \text{ x}100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of MP extract or standard compounds.

Reducing power assay

The reducing power of extracts was determined as per the reported method [16]. Different concentrations of MP extract (100, 300 and 500 μg/ml) in 1 ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50^oC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical analysis

All data were represented as mean ± SEM and as percentage. Results were statistically evaluated using Dunnett's t- test. $P<0.01$ was considered significant.

Results and Discussion

Acute toxicity test

M. parvifolia leaves extract did not produce any mortality even at the dose of 1500 mg/kg, p.o. All the doses (5, 50 and 300 mg/kg, p.o.) of *M. parvifolia* were thus found to

be non-toxic. On the basis of above results, three doses (100, 250, 500 mg/kg, p.o.) of *M. parvifolia* were selected for further pharmacological studies.

Anti-inflammatory activity

The anti-inflammatory effects of the ethanolic extract of *M. parvifolia* is shown in Table 1. The alcoholic extract at 250 and 500 mg/kg p.o. showed very significant results and caused an inhibition in the carrageenan - induced rat paw edema. The maximal inhibition in edema volume was achieved at a dose of 500 mg/kg, which is significant (P<0.01) in comparison to standard drug Diclofenac sodium (50 mg/kg).

Table 1. Anti-inflammatory activity of ethanolic extract of leaves of *M. parvifolia*

Treatment	Doses (mg/kg) p.o.	Paw edema volume(ml)			
		30 min	60 min	90 min	120 min
Control Normal Saline (95): tween 80 (5)	--	0.57±0.07	0.78±0.09	0.85±0.03	0.88±0.03
Standard (Diclofenac Sodium)	50 i.p.	0.47±0.08 (17)[a]	0.62±0.03** (20)	0.64±0.09** (24)	0.74±0.09** (15)
MP extract	100	0.55±0.08 (3)	0.72±0.01 (7)	0.75±0.06 (11)	0.85±0.06 (3)
	250	0.52±0.01 (8)	0.66±0.01 (15)	0.69±0.01** (18)	0.80±0.01** (9)
	500	0.50±0.01 (12)	0.67±0.08 (14)	0.65±0.09** (23)	0.75±0.09** (14)

All values are expressed as mean of ten rats in each group. Statistically significant ** p<0.01 compared to control. [a]Values in parenthesis represents % inhibition.

DPPH free radical scavenging activity

DPPH radical was used as a substrate to evaluate free radical scavenging activities of MP extract. It involves reaction of specific antioxidant with a stable free radical 2, 2-diphenyl-1- picryl-hydrazyl (DPPH). As a result, there is reduction of DPPH concentration by antioxidant, which decreases the optical absorbance of DPPH; this is detected by spectrophotometer at 517 nm. BHA and ascorbic acid were used as standards. The scavenging effect of MP extract on the DPPH radical was 87.6%, (Fig.1) at a concentration of 500 μg/ml. These results indicated that extract has a noticeable effect on scavenging the freeradicals.

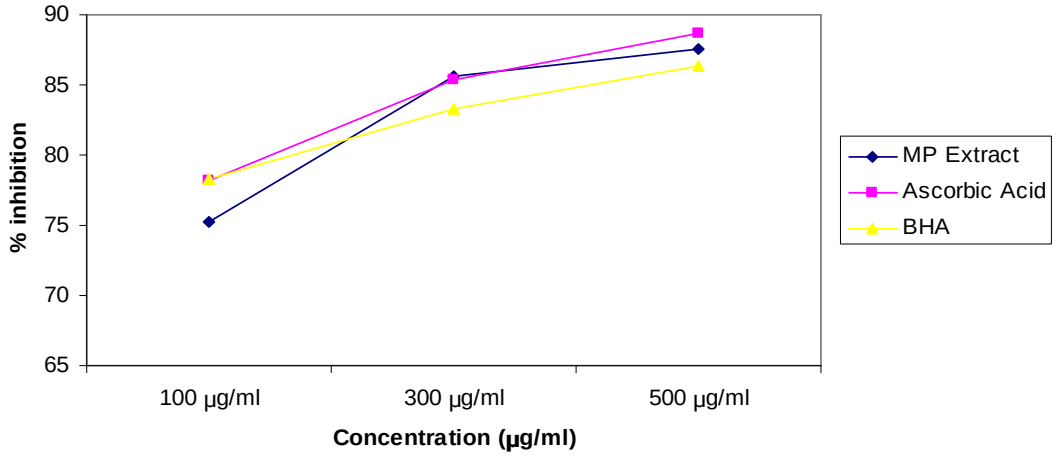

Fig.1. Anti-radical activity of MP extract, Ascorbic acid and BHA against 1,1-diphenyl-2-picrylhydrazyl free radicals

Superoxide radical scavenging assay

The superoxide anion radical scavenging activities of extracts were assayed by the PMS-NADH system. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The percentage inhibition of superoxide generation by 500 µg/ml concentration of MP extract was measured as 65.0 % (Fig. 2).

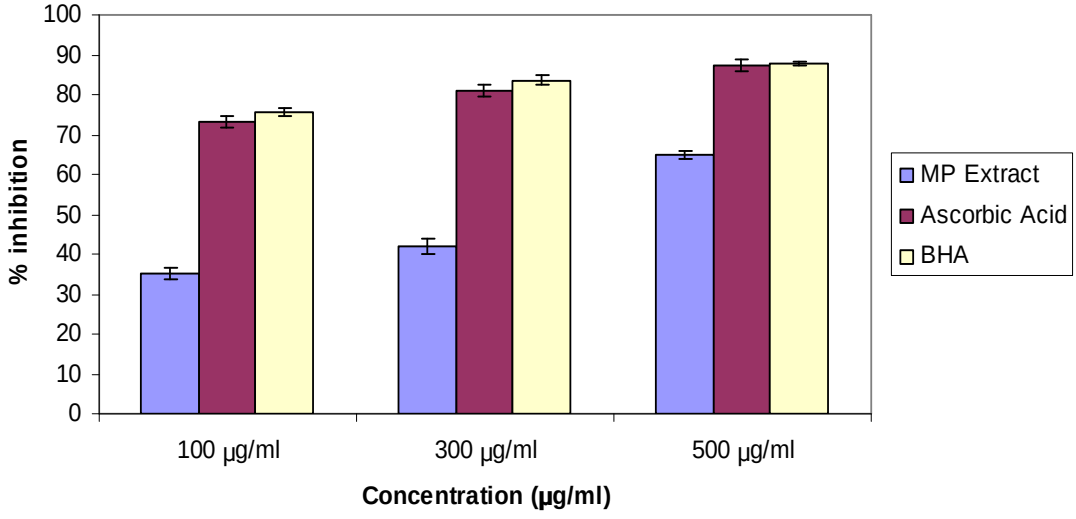

Fig. 2. Comparison of % inhibition of Superoxide radicals generation by different concentration of ascorbic acid, BHA, and MP extract using PMS-NADH-NBT Method.

Reducing power assay

For the measurements of the reducing ability, 'Fe^{3+}- Fe^{2+} transformation' in the presence of MP extract was found. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reductive capabilities of extract were compared with BHA and ascorbic acid. The reducing power of MP extract was found to increase with increasing concentrations (Fig. 3).

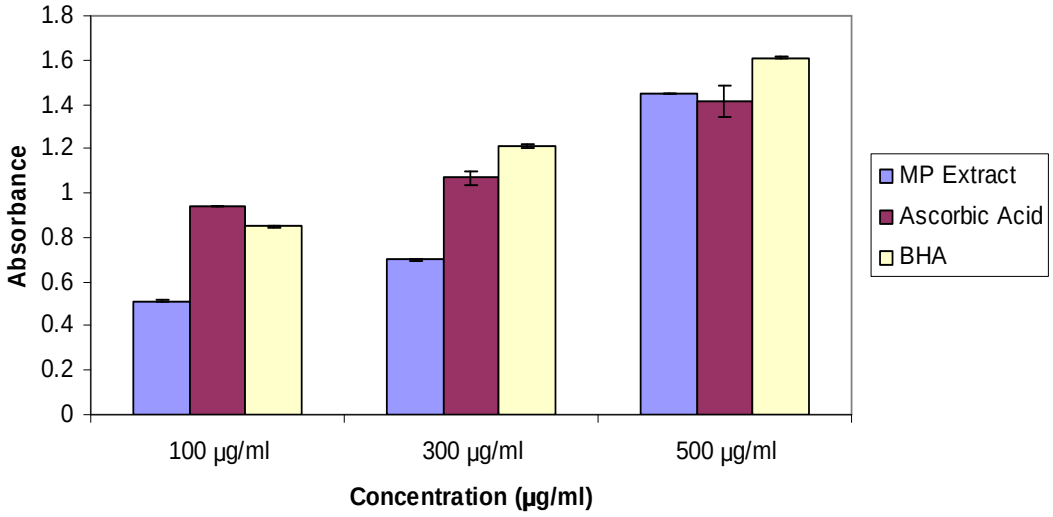

Fig. 3 Reducing power assay of MP extract, Ascorbic acid and BHA

The most widely used primary test for screening of anti-inflammatory agents is carrageenan induced edema in rat hind paw [12]. The development of edema in the paw of the rat after injection is believed to be biphasic event. The initial phase observed during first hour is attributed to release of histamine and serotonin; the second phase is due to release of prostaglandin-like substances [17]. Based on this, it may be argued that the suppression of first phase may be due to inhibition of the release of early mediators, such as histamine and serotonin, and the action in the second phase may be explained by an inhibition of cyclooxygenase [18].

The free radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH. This assay provided useful information on the reactivity of the compounds with stable free radicals, because of the odd number of electrons. Superoxide radical is a highly toxic species, which is generated by numerous biological and photochemical reactions [19]. In the PMS-NADH-NBT system, superoxide anion, derived from dissolved oxygen from the coupling reaction of PMS-NADH, reduces NBT. The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture.

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [20]. Therefore, antioxidants with free radical scavenging

activities may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals [21].

Conclusion

The ethanolic MP extract possesses good antioxidant and anti-inflammatory potential. So, the further investigation of chemical constituents responsible for the above activity is needed.

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