Research Paper

Evaluation of Antiinflammatory Activity of Methanol Extract of *Phyllanthus amarus* in Experimental Animal Models

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The effects of methanol extract of *Phyllanthus amarus* on different phases of inflammation were examined. Investigations were performed using different phlogistic agents-induced paw edema, carrageenan-induced air-pouch inflammation and cotton pellet granuloma in rats. Methanol extract of *Phyllanthus amarus* significantly inhibited carrageenan, bradykinin, serotonin and prostaglandin E1-induced paw edema, but failed to inhibit the histamine-induced paw edema. Maximum inhibition was observed in prostaglandin E1-induced paw edema. In carrageenan air-pouch model, methanol extract of *Phyllanthus amarus* significantly reduced the volume of exudate and migration of neutrophils and monocytes. The extract significantly decreased formation of granuloma tissue in chronic inflammation model. The present study revealed that methanol extract of *Phyllanthus amarus* inhibits all the phases of inflammation.

Inflammation is a local response of living mammalian tissues to injury. It is a body defense reaction in order to eliminate or limit the spread of injurious agent. There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Edema formation, leukocyte infiltration and granuloma formation represent such components of inflammation[1].

Phyllanthus amarus (*P. amarus*, family *Euphorbiaceae*) is traditionally used for the treatment of hepatitis, diabetes and dropsy[2,3]. *P. amarus* was extensively investigated against viral hepatitis[4-7]. Recently Kiemer *et al*. have reported antiinflammatory potential of this traditionally employed herbal medicine[8]. They have reported that standardized extract of *P. amarus* inhibited iNOS, COX-2 and TNF-α, in RAW 264.7 cell lines and human whole blood[8]. Further, in carrageenan-induced inflammation, methanol extract of *Phyllanthus amarus* (MEPA) significantly inhibited the paw edema[9]. However effect of *P. amarus* on different phases of inflammation has not been studied. In the present study, we have evaluated the effect of MEPA on acute inflammation using different inflammatory mediators-induced paw edema, subacute inflammation (leukocyte infiltration and exudation) using carrageenan air-pouch model and chronic inflammation by cotton pellet-induced granuloma formation in rats.

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MATERIALS AND METHODS

The plant *Phyllanthus amarus* was collected in the month of July from Western Ghat forests near Jamboti, Belgaum Dist., Karnataka, India. The plant material was taxonomically identified by the Department of Botany, Karnataka University, Dharwad, India. Bradykinin, histamine, and serotonin were obtained from Sigma Chemical Co. (St. Louis, MO, USA): Prostaglandin E_1 was obtained from Cayman Chemical Co. (Michigan, USA), Carrageenan was obtained from Himedia Chemical Co. (Mumbai, India). Indomethacin was obtained as a gift sample from Micro Labs (Mumbai, India).

Male Wistar rats weighing 150-200 g were procured from National Institute of Nutrition, Hyderabad, India. They were acclimatized to laboratory conditions for a week prior to the initiation of the experiment. They were fed on standard rat feed and given free access to water. Twelve hours before the start of the experiment, rats were deprived of food, but given free access to water. The experiment was carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and

Institutional Animal Ethical Committee approved all the procedures.

Preparation of methanol extract:
The whole plant was shade dried and subjected to size reduction to get a coarse powder. The powdered material was subjected to successive extraction in a Soxhlet apparatus, using methanol (75%) as solvent at 50°. The extract was then evaporated on a rotary evaporator.

Phlogistic agents-induced paw edema:
The rats were divided into different groups of six each. Acute inflammation was induced by intraplantar administration of 0.1 ml of carrageenan (1%) or chemical mediators *viz*; histamine (0.1%), serotonin (0.1%), bradykinin (0.002%) and prostaglandin E_1 (0.0001%). Rats were treated with either vehicle or MEPA (250 mg/kg, p.o.), 1 h before administration of phlogistic agents. The paw volume was measured prior to injection of phlogistic agent (0 h) and then at predetermined interval for each agent. For carrageenan, bradykinin and prostaglandin E_1, the interval was 3 h, whereas, for serotonin and histamine they were 1 h and 2 h, respectively. Paw volume was measured using digital Plethysmometer (UGO BASIL, Italy). Change in the paw volume was measured and antiinflammatory activity was calculated by using the formula: % Inhibition of inflammation = 1-(Vt/Vc)×100, where, Vt represents the change in the paw volume in MEPA treated group and Vc represents the change in the paw volume in the corresponding vehicle treated control group.

Carrageenan air-pouch model:
The rats were divided into three groups (n=6). Air-pouch was produced according to the method described by Salvemini *et al.*[10]. Briefly, rats were anesthetized and air cavities were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back (0 d). An additional 10 ml of air was injected into the cavity every 3 d (3rd and 6th d) to keep the space open. On the 7th d, 2 ml of 1% solution of carrageenan dissolved in saline was injected directly into the pouch to induce an inflammatory response. The rats were orally pre-treated with either vehicle or MEPA or indomethacin 2 h prior to the injection of carrageenan into the air-pouch. The second dose of treatment was repeated after 24 h of the first treatment. Forty-eight hours after carrageenan injection, the rats were anesthetized with ether and the pouch was carefully opened by a small incision. The volume of exudate was collected and measured. An aliquot of the exudate was used for quantification of leukocyte concentration using a hemocytometer and differential cell count was performed using a manual cell counter after staining with Wright's stain. The results were expressed as the total number of neutrophils and monocytes.

Cotton pellet-induced granuloma:
The rats were divided into three groups (n=6). After shaving the fur, the rats were anesthetized with ether and 20 mg of sterile cotton pellets were surgically inserted in the groin region. The MEPA (250 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o.) or vehicle was administered for 7 consecutive days from the day of the cotton pellet implantation. The animals were anesthetized on the 8th d and the cotton pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at 37° for 24 h and dried at 60° for constant weight. Increment in the dry weight of the pellets was taken as measure of granuloma formation[11].

Statistical analyses:
All data were expressed as mean±SEM. The statistical analyses were performed using Student's 't' test. $p<0.05$ was considered as significant.

RESULTS

MEPA significantly ($p<0.05$) inhibited carrageenan, bradykinin, serotonin and prostaglandin E_1-induced paw edema, but failed to inhibit histamine-induced paw edema when compared to vehicle treated control group (Table 1). Maximum inhibition (47%) was found in prostaglandin E_1-induced paw edema, whereas, it was 37% in carrageenan induced paw edema. In case of bradykinin and serotonin-induced paw edema, the MEPA showed 20.74% and 31.32% inhibition, respectively relative to vehicle treated control group.

In the carrageenan air-pouch model MEPA and indomethacin significantly ($p<0.05$) reduced carrageenan-induced exudate volume and infiltration of neutrophils and monocytes into the air-pouch compared to vehicle treated control group (Table 2).

In the cotton pellet-induced granuloma model MEPA and indomethacin showed decreased formation of granuloma tissue by 22.89% and 43.93%, respectively when compared with vehicle treated control group (Table 3).

DISCUSSION

Inflammation has different phases; the first phase is caused by an increase in vascular permeability, the

TABLE 1: EFFECT OF MEPA ON PAW EDEMA INDUCED BY DIFFERENT PHLOGISTIC AGENTS

Treatment	Change in paw volume (ml)	% Inhibition of inflammation
Carrageenan (0.1ml of 1% solution) + Vehicle	2.79±0.13	-
Carrageenan (0.1ml of 1% solution) + MEPA	1.75±2.20*	37.27
Serotonin (0.1 ml of 0.1% solution) + Vehicle	3.48±0.16	-
Serotonin (0.1 ml of 0.1% solution) + MEPA	2.39±0.13*	31.32
Histamine (0.1ml of 0.1% solution) + Vehicle	1.74±0.25	-
Histamine (0.1ml of 0.1% solution) + MEPA	1.4±0.45	19.54
Bradykinin (0.1ml of 0.002% solution) + Vehicle	2.41±0.82	-
Bradykinin (0.1ml of 0.002% solution) + MEPA	1.91±0.12*	20.74
Prostaglandin E_1 (0.1ml of 0.0001% solution) + Vehicle	2.23±0.18	-
Prostaglandin E_1 (0.1ml of 0.0001% solution) + MEPA	1.16±0.02*	47.98

Values are mean±SEM, n=6; *p<0.05 when compared with vehicle treated control group. MEPA is the methanol extract of *P. amarus*

TABLE 2: EFFECT OF MEPA ON LEUKOCYTE INFILTRATION AND EXUDATE VOLUME IN CARRAGEENAN-INDUCED AIR-POUCH INFLAMMATION

Treatment	Dose mg/kg	Exudate volume (ml)	Neutrophils (x10^6 cells)	Monocytes (x10^6 cells)
Control	0	3.33±0.20	244.50±7.58	88.10±8.04
MEPA	250	1.2±0.07*#	109.50±3.34*#	52.35±2.82*#
Indomethacin	10	0.69±0.08*	72.22±1.55*	40.35±4.43*

Values are mean±SEM, (n=6); *p<0.05 when compared with vehicle treated control group, #p<0.05 when compared with indomethacin treated group. MEPA is the methanol extract of *P. amarus*

TABLE 3: EFFECT OF MEPA ON COTTON PELLET-INDUCED GRANULOMA

Treatment	Dose mg/kg	Weight of cotton pellet (mg)	% Inhibition
Control	0	150.7±4.44	-
MEPA	250	116.2±1.96*#	22.89
Indomethacin	10	84.5±5.63*	43.93

Values are mean±SEM, n=6; *p<0.05 when compared with vehicle treated control group, #p<0.05 when compared with indomethacin treated group. MEPA is the methanol extract of *P. amarus*

second one by infiltration of leukocytes and the third one by granuloma formation. In the present study, we have examined the effects of MEPA on these phases of inflammation. In the preliminary experiment, the different doses of extract (100, 250, 500 mg/kg, p.o.) were tested in carrageenan-induced acute inflammation in rats (results not shown). We observed that MEPA at the doses of 250 and 500 mg/kg significantly inhibited carrageenan-induced paw edema. Based on these observations and the previous report[9], we selected 250 mg/kg, p.o. dose for further studies.

Histamine, serotonin, bradykinin and prostaglandins are established mediators of acute phase of inflammation causing increase in vascular permeability and vasodilatation[12]. In the present study, MEPA significantly inhibited the paw edema induced by prostaglandin E_1 (47.98%), serotonin (31.32%) and bradykinin (20.7%) but not by histamine (19.5%). Further, except prostaglandin E_1-induced edema, the magnitudes of these inhibitions were less than that observed with carrageenan-induced edema (37.27%). It has been reported that these inflammatory mediators are released endogenously and contribute to the various phases of paw edema[12]. These observations suggest that probably, MEPA shows antiinflammatory effect not only by blocking the effects of serotonin, bradykinin and prostaglandin E_1 on vascular membrane but also by inhibiting the release of these mediators. Failure of MEPA to inhibit histamine-induced paw edema suggests that MEPA may not have antihistaminic activity. Since maximum antiinflammatory effect was observed in prostaglandin E_1-induced paw edema, our results favor the notion that MEPA may contain active constituent that block prostaglandin E_1 effects.

Exudate formation and leukocyte infiltration are important components of inflammation. The air-pouch model in rodents has been extensively used to induce subacute inflammation and assess the effects of experimental drugs including NSAIDs and corticosteroids on exudate formation and leukocyte infiltration[10]. In the current study, MEPA significantly reduced the neutrophil and monocyte infiltration and volume of exudate in carrageenan-induced air-pouch inflammation. These results indicate that MEPA may alter the action of endogenous factors that are involved in neutrophil and monocyte migration to the site of inflammation. However, MEPA was less potent than indomethacin.

In our last experiment, MEPA showed significant antiinflammatory activity in cotton pellet-induced granuloma formation and thus found effective in chronic

inflammatory condition. Cytokines, such as IL-1 and TNF, as well as growth factors influence proliferation of smooth muscle cells and fibroblasts and production of granuloma[1,13]. *P. amarus* has been reported to inhibit the induction of cytokines, iNOS and COX-2 in, *in vitro* and *in vivo* studies[8]. These effects of *P. amarus* and inhibition of leukocyte migration observed in the present study may contribute to its inhibitory effect on chronic inflammation.

In conclusion, MEPA showed inhibitory effect on different phlogistic agents-induced paw edema, carrageenan-induced leukocyte infiltration and exudate formation and cotton pellet-induced granuloma formation, thus exhibiting antiinflammatory effect against acute, subacute and chronic phases of inflammation.

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