

ORIGINAL ARTICLE

Membrane Stabilizing Activity and Protein Denaturation: A Possible Mechanism of Action for the Anti-Inflammatory Activity of *Phyllanthus amarus*

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Abstract:

Background: Previous studies confirm the anti-inflammatory potential of *Phyllanthus amarus* herb. **Aim and objective:** Primary aim of the present study was to investigate the possible anti-inflammatory mechanism of *Phyllanthus amarus* extract using in-vitro models. **Material & method:** Aqueous extract of *Phyllanthus amarus* was evaluated in vitro models for haemolysis of erythrocytes and % inhibition in heat induced protein denaturation at three doses 50 mg/kg, 100 mg/kg & 200 mg/kg. **Results:** The aqueous extract of *Phyllanthus amarus* significantly inhibited the haemolysis of erythrocytes and heat induced protein denaturation as compared to control. **Conclusion:** It can be postulated from the observed results that the anti-inflammatory activity of *Phyllanthus amarus* could be due to its membrane stabilizing action and inhibition of protein denaturation.

Key Words: *Phyllanthus amarus*, anti-inflammatory.

Introduction:

The species of *Phyllanthus* has long been used in folk medicine for liver protection, intestinal infections, antihepatitis B, diabetes, astringent, diuretic, cathartic, jaundice and dropsy [1, 2]. *Phyllanthus amarus* Linn (PN) (Family:

Euphorbiaceae) is a perennial annual herb, growing as a weed throughout India, commonly known as Jamgli amla, Jaramla, or Bhuiamla. The reported medicinal properties of PN are antitumor, hypolipidaemic, antiviral, etc. Previous work reports the anti-inflammatory effect of *P. amarus* extracts in rats. In the carrageenan-induced inflammation, *Phyllanthus amarus* extracts significantly inhibited the paw edema [3]. While in the another study extracts of *Phyllanthus amarus* significantly inhibited carrageenan, bradykinin, serotonin and prostaglandin E1-induced paw edema, but failed to inhibit the histamine-induced paw edema [4]. Further various doses of the aqueous extract of *Phyllanthus amarus* were investigated for analgesic and anti-inflammatory activities using both thermal and chemical models of pain assessment in rats [5]. These various studies have established the anti-inflammatory and analgesic activities of *Phyllanthus amarus* [3, 5]. Therefore, with the aim to investigate the possible anti-inflammatory mechanism of PN, present study of was carried using in-vitro models.

Materials and Methods:

Plant material:

P. amarus was obtained from different places in Karad, Western Maharashtra, India. The plant

was identified and authenticated by Department of Botany, Yashwantrao Chavan College of Sciences Karad.

Preparation of the PN extract:

The dried plant was boiled with water. The extract was concentrated and dried on water bath (yield= 10%). In all experiments three doses (50 mg/kg, 100 mg/kg & 200 mg/kg) of PN were tested in vitro models for evaluation of membrane stabilizing and protein denaturation activity.

Drugs:

Acetyl salicylic acid (ASA) of Research Lab, India and Indomethacin of Jagsonpal Pharmaceuticals Mumbai.

Animals :

Male Wistar rats (150-200g) were used in the study was maintained at ambient temperature of 25-30°C with food and water *ad libitum*. All experiments were approved by the institutional ethical committee and were carried out according to the animal ethics committee guidelines.

Effect on haemolysis:

The method of Abe *et al.* was used in the current study [6].

Erythrocyte suspension:

Whole blood was collected via retro orbital vein puncture technique from rats under ether anesthesia. Heparin was used to prevent clotting. The blood was washed three times with 0.9% saline. The volume of saline was measured and reconstituted as a 40% v/v. suspension with isotonic buffer solution of pH 7.4.

Heat-induced haemolysis:

Portions (5ml) of the isotonic buffer containing 50, 100 and 200 µg/ml of solution of *P. amarus* were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30µl) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance (optical density) of the supernatant was measured at 540 nm using Shimadzu UV-1800 spectrophotometer. Acetyl salicylic acid 200µg/ml was used as a reference standard.

Hypotonic solution-induced haemolysis:

The isotonic buffer solution was used which composed of 154 mM NaCl in 10 mM sodium phosphate buffer at pH 7.4. The experiments were carried out in duplicate pairs. Stock erythrocyte suspension 30 µl was mixed with 5 ml of the hypotonic solution containing the *P. amarus* at concentrations of 50, 100 and 200 µg/ml, while the control sample was mixed with drug free solution. The mixtures were incubated for 10 minutes at room temperature, and centrifuged for 3 min at 1300 g and the absorbance (optical density) of the supernatant was measured at 540 nm. ASA 200 µg/ml. was used as a reference standard.

Effect on protein denaturation:

Test solution (1ml) containing different concentrations (50 - 200 µg/ml) of plant extract and / or indomethacin (100 µg/ml) was

mixed with 1ml of egg albumin solution (1mM) and then incubated at 27 ± 1 °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. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was carried out in triplicate and the average was taken.

The percentage inhibition of haemolysis in tests (b) and (c) was calculated according to the equation: % inhibition of haemolysis = $100 \times [1 - (OD2-OD1 / OD3-OD1)]$

Where, OD1= test sample unheated or in isotonic solution;

OD2=test sample heated or in hypotonic solution; and

OD3=control sample heated or in hypotonic solution.

Data were statistically analyzed by Student's *t*-test and $p < 0.001$ vs. control were considered to be significant.

Results:

In the study of membrane stabilization activity the PN extract at concentration range of 50-200 µg/ml protected significantly the erythrocyte membrane against lysis induced by heat as well as hypotonic solution. Aspirin (200 µg/ml) also offered a significant ($p < 0.01$) protection of the RBC's against the damaging effect induced by heat and hypotonic solution. At a concentration of 200 µg/ml, the PN extract showed 56.50 ± 1.049 % inhibition of heat-induced haemolysis and 75.50 ± 1.049 % of Hypotonic solution-induced haemolysis when compared with blank (Table 1).

The inhibitory effect of different concentrations of PN on protein denaturation is shown in (Table 2). PN extract (50-200 µg/ml) showed significant inhibition of denaturation of egg albumin in concentration dependent manner. PN extract at concentration of 200 µg/ml and Indomethacin at concentration of 100 µg/ml showed significant inhibition 54.16 % and 82.83 % respectively of protein denaturation when compared with control.

Table 1: Effect of *P. amarus* on heat-induced and hypotonic solution-induced haemolysis of erythrocyte membrane

Treatment	Concentration (µg/ml)	% Inhibition of haemolysis	
		Heat-induced	Hypotonic solution-induced
Control	-	-	-
<i>P. amarus</i>	50*	26.83 ± 1.472	65.16 ± 1.722
	100*	47.33 ± 1.366	71.33 ± 1.506
	200*	56.50 ± 1.049	75.50 ± 1.049
Aspirin	20*	25.12 ± 1.72	76.23 ± 1.35

Values are mean \pm S.D., n=6; * $P < 0.001$ vs. control, Student's *t*-test.

Treatment	Concentration (µg/ml)	% Inhibition of protein denaturation
Control	-	-
<i>P. amarus</i>	50*	23.33 ± 1.633
	100*	42.83 ± 1.722
	200*	54.16 ± 2.317
Indomethacin	10*	82.83 ± 1.602

Values are mean ± S.D., n=6; *P< 0.001 vs. control, Student's t-test.

Discussion:

In the current study in-vitro results confirm the reported anti-inflammatory activity of *P. amarus*. Raphael & Kuttan have showed that methanolic extract of *Phyllanthus amarus* significantly inhibited gastric lesions, induced by intragastric administration of absolute ethanol and the anti-oxidant activity of the extracts [9]. Kierner et al, have investigated potential anti-inflammatory properties of standardized *P. amarus* extracts concerning a potential influence of *P. amarus* on endotoxin-induced nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and cytokine production in vivo and in vitro [10].

Kassuya et al have investigated the anti-allodynic and anti-oedematogenic effects of the hexanic extract, lignan-rich fraction and purified lignans from, *Phyllanthus amarus*, in the inflammatory and neuropathic models of nociception [11].

A possible explanation for the stabilizing activity of *P. amarus* could be an increase in the surface area to volume ratio of the cells

which could be brought about by an expansion of membrane or shrinkage of the cell, and an interaction with membrane proteins [4, 5]. Moreover, it has also been shown that the deformability and cell volume of erythrocytes is closely related to the intracellular content of calcium [7, 8]. Hence, it may be speculated that the cytoprotective effect on erythrocyte membrane may be due to the ability of the test extract to alter the influx of calcium into the erythrocytes. The present investigation suggests that the membrane stabilizing activity of *P. amarus* may be playing a significant role in its anti-inflammatory activity.

Denaturation of proteins is a well documented cause of inflammation and rheumatoid arthritis [9]. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation. Ability of PN extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity.

Several bioactive molecules, such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins, have been shown to be present in the extracts of *P. amarus* [1, 2]. The anti-inflammatory activity of PN extract in the present study may be due to the presence of therapeutically active lignans and flavonoids [10-13]. The anti-inflammatory and analgesic activity of extracts rich in flavonoids, lignans and their rich fractions on inflammation have previously been reported [1-2 and 10-14].

The data of our studies suggests that PN shows significant anti-inflammatory activity in both the in-vitro methods tested. Further studies involving the purification of the chemical

constituents of the plant and the investigations in the biochemical pathways may result in the development of a potent anti-inflammatory agent with a low toxicity and better therapeutic index.

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