Research Article

In vitro anti-inflammatory and anti-venom activities of aerial parts of *Marsilea quadrifolia* Linn.

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Abstract

Objective: To evaluate the *in vitro* anti-inflammatory and anti-venom effect of ethanolic extract of *Marsilea quadrifolia* against the Human red blood cell (HRBC) membrane stabilization method, Inhibition of protein denaturation method and Neutralization of anti-coagulation activity. **Materials & Methods:** The ethanolic extract at different concentrations was incubated with human HRBC suspension and egg albumin in controlled experimental situation and subjected to determination of absorbance and membrane stabilization and viscosity to assess the anti-inflammatory property. Diclofenac sodium was used as the standard drug and with various concentrations was incubated with citrated plasma and determine clotting time to assess anti-venom activity. **Results:** The results exhibited a concentration reliant inhibition of HRBC membrane stabilization, protein (albumin) denaturation and neutralization of anticoagulant by the *Marsilea quadrifolia*. The effect of standard drug was found to be little more when compared the test extract. **Conclusion:** It can be concluded that aerial parts of *Marsilea quadrifolia* possessed marked *in vitro* anti-inflammatory and anti-venom effect.

***Keywords*:** Naja Kaouthia, Diclofenac sodium, *Marsilea quadrifolia,* anti-inflammatory, anti-venom

Introduction

Inflammation is the reaction of tissue to injury. It is a protective response which sets the storage for healing and reconstitution of normal function in the damaged tissues. This process involves functional alteration of micro vessels, leading to the accumulation of fluid and leucocytes in extravascular tissues and local pain. The inflammatory reaction is mediated by endogenously mobilized active substances, named "Chemical mediators of inflammation". Chemical mediators orginate either from plasma eg. Complement proteins, Kinins, Clotting system derived proteins or Cells eg. Eicosanoids, Cytokines, Chemokines, Histamine and Serotonin. Eicosanoids can mediate virtually every step of inflammation. Arachidonic acid, the precursor of pro – inflammatory eicosanoids is released from the membrane phospholipids in the course of inflammatory activation and is metabolized to prostaglandins and leucotrienes through the mediation of phospholipase A_2(Heller et al., 1998).

Phospholipase A_2 (PLA_2) constitute a family of structurally related proteins that hydrolyze phospholipids at the sn_2 position in a calcium – dependent manner, releasing fatty acids and lysophospholipids (Dennis, 1994). PLA_2 are commonly found in mammalian tissues as well as insect and snake venom. Venom from the both snakes and insects is largely composed of melittin, which is a stimulant of PLA_2. Due to increased presence and activity of PLA_2resulting from a snake or insect bite. arachidonic acid is released from the phospholipid membrane suspiciously. So inflammation and pain occur at the site. The enzyme PLA_2 from snake venom displays various pharmacological activities such as anticoagulant, inhibit the platelet aggregation, induce inflammation and myotoxicity (Gowda et al., 1994, Betzol et al., 1999). Extracts from plants have been used among traditional healers, especially in tropical areas where there are plentiful sources as therapy for snakebite for a long time.

Marsilea quadrifolia Linn (Family: Marsilea, English: Four leaf clover, European water clover, Tamil : Niraakkirai,

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Aarai. Kannada: Neer, hurali). It has been reported to have many medicinal properties (anti-inflammatory, anti-venom and applied to abscesses, depurative, refrigerant and diuretic etc) (Vent et al., 1985) .The aerial parts have been found to possess antidiabetic activity (Zahan et al 2011), antibacterial, antioxidant and cytotoxic activity (Ripa et al., 2009), anticonvulsant (Sahua et al., 2012), anticholinesterase potential (Bhadra et al., 2012), neurodegenerative disorders (Ashwinietal.,2012), psychopharmacological action (Reddy et al., 2012). Traditionally *Marsilea quadrifolia* Linn has been reported to have many medicinal properties but no more scientifically validated data of anti-inflammatory and anti-venom activities is available. So such studies would prove our traditional knowledge scientifically and also help in re-enforcing the country's claims.

Materials and methods

Plant collection and Authentication

The aerial parts of *Marsilea quadrifolia* were collected from the local area of Chinniampalayam in the month of November, erode district , Tamilnadu. The aerial parts of *Marsilea quadrifolia* were collected during December, 2015 from anthiyur and athani region of erode district of Tamilnadu, India (Figure1).The plant material was authenticated by DR.P. Jayaraman PhD, Director, Plant anatomy research, Chennai, Tamilnadu, India and a voucher specimen no PARC/2015/2119 was deposited at the museum, SSM college of Pharmacy, Erode (638312) Tamilnadu.

(a) (b)

Figure 1. (a) *Marselia quadrifolia* leaves (b) Aerial parts of *Marselia quadrifolia*

Drug and chemicals

Diclofenac sodium (Capital Pharma), Naja kaouthia snake venom (Calcutta snake park, Kolkata). Mayer's reagent, hydrochloric acid, Wagner's reagent, ferric chloride, magnesium, sulphuric acid, acetic anhydride, bromine water, gelatin solution, Peptone, beef extract, agar, sodium chloride, Egg Albumin, Phosphate buffer saline (PBS) 6.4, Alsever solution, Human blood/Human plasma, Isolsaline, Hyposaline and Empty disc. All other chemicals were of analytical grade obtained commercially.

Preparation of extract

Fresh aerial parts of *Marsilea quadrifolia* were collected, cut into small pieces and dried under shade morning time for 10 days. The dried part were passed through sieve (coare 10/40).this powder was used for the preparation of solvent extraction and 500g of powder was extracted by maceration technique. The dry extract was kept in a vacuum desiccator until use.

In vitro Anti-inflammatory activity

Human red blood cell (HRBC) membrane stabilization method (Shinde et al., 1999)

The blood was collected from healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2% dextrose,0.8% sodium citrate, 0.5% citric acid and 0.42% Nacl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline and a 10% suspension was made. Various concentrations of extract were prepared (50, 100, 200 and 400 µg/ml) using distilled water and to each concentration 1ml of phosphate buffer, 2ml hyposaline and 0.5 ml of HRBC suspension were added. It was incubated at 37^0c for 30 min and centrifuged at 3,000rpm for 20 min. and the haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560nm. Diclofenac (100 and 200µg/ml) was used as reference standard and a control was prepared by omitting the extracts. The percentage of HRBC membrane stabilization or protection was calculated by using the following formula,

% inhibition = $\mathbf{A_1 - A_2 / A_1}$

A1 = Absorbance of control

A2 = Absorbance of test sample / standard sample

Inhibition of protein denaturation method (Sangita Chandra et al., 2012)

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of APEMQ so that final concentrations become 50, 100, 200, 400 µg/ml. Similar volume of double-distilled water served as control. Then the mixtures were incubated at 37^0 C in a BOD incubator (Labline Technologies) for 15 min and then heated at 70^0C for 5 min. After cooling, their absorbance was measured at 660 nm (SHIMADZU, UV 1800) by using vehicle as blank and their viscosity was determined by using Ostwald

viscometer. Diclofenac sodium at the final concentration of (100 and 200µg/ml) was used as reference drug and treated similarly for determination of absorbance and viscosity. The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition = **100 x (Vt / Vc - 1)**

Where, Vt = absorbance of test sample, Vc = absorbance of control.

***In vitro* anti-venom activity**

Neutralization of anti-coagulation activity (Karlsson, 1980)

The neutralization of anticoagulation activity of APEMQ induced by *Naja Kaouthia* venom was done by taking constant amount of venom which was mixed with various concentrations (50-400µg) of APEMQ. The mixtures were incubated for 30 min at 37° C and then 100µl of mixture was added to 300µl of citrated plasma, the plasma was recalcified with the addition of 100µl of 0.25M $CaCl_2$ and the clotting times was recorded by gentle tilting at every 15s till coagulation occurred.

Statistical analysis

All results will be expressed as mean ± SEM, Statistical difference in mean will be analyzed using one-way ANOVA (analysis of variance) followed by post hoc test (Dunnett's't' test). P< 0.05*, will be considered as statistically significant.

Results and discussion

Extraction and Preliminary phytochemical screening

The extraction of aerial parts of *Marsilea quadrifolia* was carried out using ethanol by cold maceration method. The ethanol extract of plant material obtained was dark green in colour and semisolid in its consistency.

The solvent extraction was subjected to preliminary phytochemical tests to determine the presence of chemical constituents the result of this examination is presented as following in Table 1.

Table 1. Preliminary phytochemical screening of ethanolic extract of aerial parts of *Marsilea quadrifolia* Linn.

S. No.	Constituents	Tests	Ethanolic extract
1	Carbohydrate	Molish's test	–
		Fehling's test	+
		Barfoed's test	+
2	Glycosides	Modified borntrager's test	+
3	Saponin	Foam test	+
4	Phenolic compounds	$Fecl_3$ test	-
		Gelatine test	-
		Lead acetate test	-
5	Sterols &Terpenoids	Libermann-burchard test	+
		Salkowiski test	-
6	Alkaloids	Wagner's test	+
		Mayer's test	-
		Hager's test	-
7	Flavonoids	Alkaline reagent test	+
		Con.H_2so_4 test	-
		Shinoda's test	-
8	Fixed oil & fats	Spot test	-
		Saponification test	-

Where, + = Presence, – = Absence

Human red blood cell (HRBC) membrane stabilization method

The results of *in vitro* anti-inflammatory activity of APEMQ on human red blood cell were given in table 2 and figure 2.

In vitro anti-inflammatory activity of APEMQ was performed by using human red blood cell membrane stabilization method. APEMQ showed significant anti-inflammatory activity in a concentration dependent manner. APEMQ at concentration of 50,100,200,400 µg/ml showed (28.16%, 40.16%, 66.17% and 80.88 %, P< 0.05) inhibition of HRBC in hypotonic solution respectively. All the results were compared with standard Diclofenac at 100, 200, 400 µg/ml which showed (78.80% and 92.40 %, P< 0.05) inhibition of HRBC in hypotonic solution respectively.

Table 2. *In-vitro* anti-inflammatory activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on human red blood cell membrane

S. No.	Group	Concentration (µg/ml)	Optical density at 560 nm	% Inhibition of Haemolysis
1	Control	50	0.9495 ± 0.03	--
2	Plant	50	0.6821 ± 0.01	28.16
	extract	100	0.5671 ± 0.05	40.26
		200	0.3212 ± 0.03	66.17
		400	0.1815 ± 0.07*	80.88
3	Diclofenac	100	0.2012 ± 0.06*	78.80
		200	0.0721 ± 0.08*	92.40

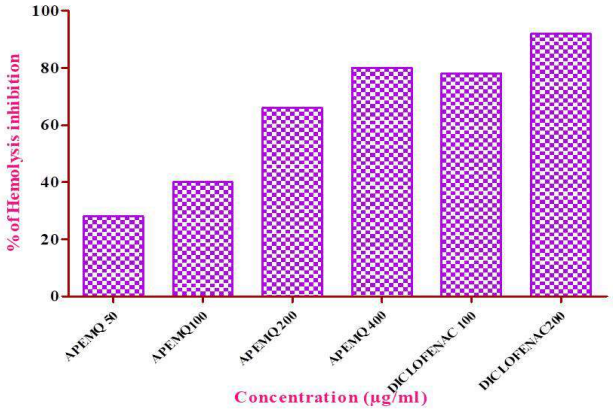

Figure 2. *In-vitro* anti-inflammatory activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on human red blood cell membrane

Inhibition of Protein (Albumin) denaturation

In the present investigation, the *in vitro* anti-inflammatory effect of APEMQ was evaluated of denaturation of egg albumin. The results are summarized in table 3 and figure 3.

In vitro anti-inflammatory activity of APEMQ was performed by inhibition of protein (albumin) denaturation method. APEMQ showed significant anti-inflammatory activity in a concentration dependent manner. APEMQ at concentration of 50,100,200,400 µg/ml showed 24.82%, 42.25%, 57.03% and 72.27%, P< 0.05 inhibition of protein denaturation respectively. All the results were compared with standard Diclofenac at 100, 200, 400 µg/ml which showed 74.26% and 89.18%, P< 0.05 inhibition of protein denaturation respectively.

Neutralization of anticoagulant activity

The Neutralization of anti-coagulant activity of APEMQ was performed using the human blood. To test the anticoagulant activity of the APEMQ was used 200 µg of venom. Standard dose

Table 3. *In-vitro* anti-inflammatory activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on inhibition of protein denaturation

S. No	Groups	Concentration (µg/ml)	% inhibition of denaturation	Viscosity (cp)
1	Control	--	--	1.39
2	Plant extract	50	24 .82	0.34
		100	42.25	0.49
		200	57.03	0.56
		400	72 .27*	0.82
3	Diclofenac	100	74.26*	0.83
		200	89.18*	1.19

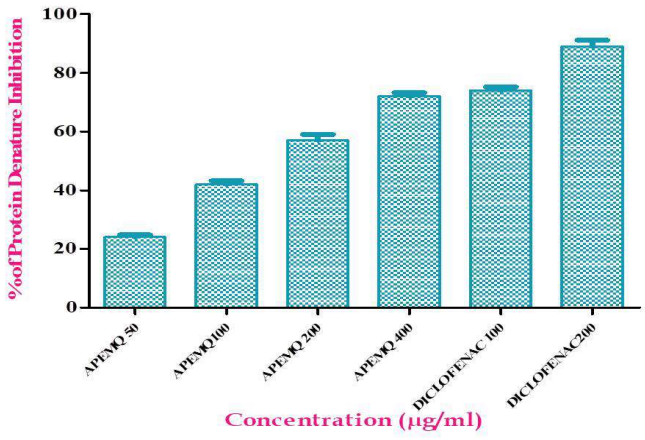

Figure 3. *In-vitro* anti-inflammatory activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on inhibition of protein denaturation. Values are mean ± SD., n=4, *P< 0.05 vs. control, Dunnett's't' test.

Table 4. *In-vitro* anti-venom activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on neutralization of anti coagulant activity

Groups	Dose of venom 200µg varying dose of plant extract in µg/ml	Formation of clots (seconds)	% inhibition of anti coagulation
Control	PBS + $CaCl_2$	28±1.20	-
Venom	200	-	-
Venom +	50	109±0.73	22
extract + blood	100	80±0.44	32
	200	45±0.54	59
	300	34±0.65	75
	400	28.67±0.87*	85

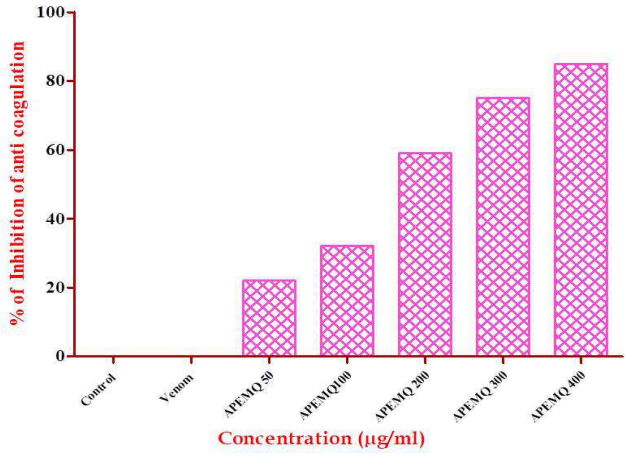

Figure 4. *In-vitro* anti-venom activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on neutralization of anti coagulant activity

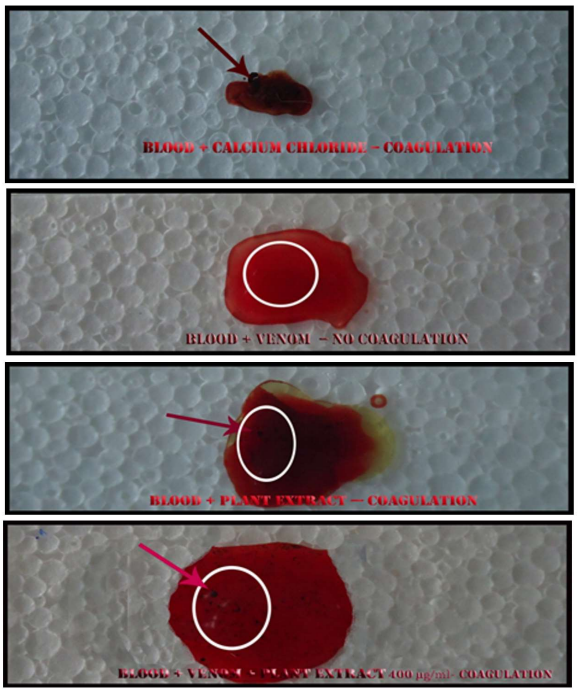

Figure 5. *In-vitro* anti-venom activity of aerial parts of *Marsilea quadrifolia* (APEMQ) on neutralization of anti coagulant activity

of 200µg was kept constant and varying doses of APEMQ were used. Normal coagulation produced (blood+PBS+CaCl2) at time period of 28±1.20 sec. APEMQ at a dose of 400µg showed a high significant reduction in the neutralization of anti-coagulant by 85 % ($P<0.05$) as compared to the *N. kaouthia* venom were given in table 4 and figure 4.

Conflict of Interest

The authors have no conflict of interest

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