Anti-venom activities of methanol extract of *Marsilea quadrifolia* Linn (Marsileaceae) against *Russell's Viper* venom

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**ABSTRACT**

The methanol extract of *Marsilea quadrifolia* Linn is explored aimed for the first time for anti-venom activity. The *Marsilea quadrifolia* Linn extract extensively antagonized *Russell's viper* venom-induced lethality, haemorrhage, necrotizing, defibrinogenating and paw edema activities were extensively neutralized by plant extract. The above explanation confirmed that methanol extract of *Marsilea quadrifolia* Linn acquire persuasive snake venom neutralizing capability and could theoretically be used for therapeutic rationale in case of snakebite envenomation.

**INTRODUCTION**

Snakebite envenoming is a worldwide general medical issue of such size and multifaceted nature that it merits definitely more consideration from national and local wellbeing specialists than it has been surrendered up to this point. This ecological and occupational influences primarily horticultural specialists and their kids in the absolute most devastating provincial networks of creating nations. In India, on a normal 2, 50,000 snake bite on are recorded in a year (Warrell DA, 1996). There are 52 toxic types of snakes accessible in India, of which greater part of the bites and mortality are embraced to species like Daboia russelli (*Russell's viper*). The Daboia russelli (*Russell's viper*) represents a dangerous general medical issue in inexhaustible tropical and subtropical nations of the south-west area of India. Snake venom is a profoundly perplexing mixed drink of proteins, peptides, non-protein poisons, sugars, lipids, amines and other molecules. (Arid SD, 2002). The chemical composition of venom fluctuates at all ordered dimensions. Further, the arrangement can change impressively between snakes in various topographical areas and people inside those populaces. The piece is additionally subject to change dependent on eating regimen, age, season and condition. The extensively contrasting appearances of snake bite could be perceived to multifaceted nature of venom somewhat. To find the medication to anticipate affecting economy so these cures may fall inside the methods for the extraordinary loads in India whose monetary condition is low. The expanding guarantee for homegrown meds unavoidably prompted the worry of acquiring and keeping up their quality and immaculateness dependent on universally perceived guidelines. A numeral of audits has been distributed on plants screened for cure action in India and different nations. The present study focuses on plant extricate, which have a notable spot in conventional medication. Taking into clarification all the above components, an exertion has been made to evaluate the efficacy of chose as *Marselia quadrifolia* Linn as the wellsprings of antitoxin for snake envenomation.
MATERIALS AND METHODS

Snake Venom: The lyophilized snake venom crystals Russell’s viper was obtained from Calcutta Snake Park, Calcutta, India and was preserved at 4°C. It was dissolved in Phosphate buffer solution (PBS) of pH 7.2 for further use.

Plant Collection

Fresh aerial parts of M. quadrifolia Linn were collected from the field of anthi and athani region of erode district of Tamilnadu, India and authenticated by DR.P. Jayaraman PhD, Director, Plant anatomy research, Chennai, Tamilnadu, India and a voucher specimen no PARC/2014/2119. A voucher specimen (No: JKKMCP/0102/14) has been deposited in the Department of Pharmacognosy, J.K.K Sampoornai Ammal College of Pharmacy, Komarapalayam, Tamilnadu, India.

Extraction

Coarsely powdered shade dried aerial parts of the plant M. quadrifolia Linn Soxhlet extractor was used for the preparation of the extracts. The successive solvent system was used for the extraction. The dried powdered material of aerial parts of M. quadrifolia Linn. stood loaded in a thimble of Soxhlet extractor and extracted with petroleum ether, ethyl acetate, acetone, methanol with aggregate order of their polarity. A minimum of 60 cycles of siphoning was completed for successive solvent, and the process was continuous for 72 hrs until the solvent in the extractor siphon tube became colourless. Extracts were concentrated at reduced pressure in a rotary vacuum evaporator and refrigerated till more use.

Preliminary Qualitative Chemical Examination of Extract

The aerial parts of M. quadrifolia Linn were exposed to systematic phytochemical screening by sequentially extracting with organic solvents, and the extract was subjected for phytochemical investigation by qualitative chemical identification tests. (Kokate C K, 1999; Khandelwal K R, 2000)

Total Phenolic Content

The total phenolic content was determined using the spectrophotometric method (Singleton et al., 1974; Shukla et al., 2016) with some little modification. Extracts were diluted with methanol to form a concentration of 1000µg ml⁻¹. The reaction mixture was organised by mixing of 1 ml of extract, 10 ml of 10% Folin-Ciocalteu’s reagent dissolved in water, and 8 ml of 7.5% sodium carbonate was added after 8 minutes. Further, the total volume is made up to mark by adding distilled water in a volumetric flask (20 ml). The whole reaction mixtures were incubated for about 45 min in the dark and at room temperature of about 25°C±2. The similar procedure was followed with gallic acid standard dilutions range of (25-700 µg ml⁻¹) and also with blank where methanol is used in place of the extract. After incubation, the absorbance was measured at 765 nm with UV-VIS spectrophotometer. Calibration curve of gallic acid was used for calculations. The total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE) / gram of dry mass by the following equation;

\[ T = \frac{C \times V}{M} \]

Wherever,

- \( T \) = Total phenolic content mg/gm of plant extract in GAE,
- \( C \) = Concentration of Gallic acid from the calibration curve,
- \( V \) = Volume of the extract in ml,
- \( M \) = Weight of the plant extract in gm.

Acute toxicity studies

The objective of the study is to categorize a dose causing major adverse effects and an estimation of the minimum dose producing lethality, according to regulatory guidelines (OECD 425). Female Swiss Albino mice weighing 25-30 gm, aged 56 to 70 days were chosen for the study. Mice were divided into two groups (n=10/group). Group I served as control and treated with Saline. The Group II received methanol extract of M. quadrifolia Linn (MEMQ) orally ranging from 175 to 2000 mg/kg body weight by using oral feeding needle sleeved on to disposable syringe. They were kept in individual polypropylene cages providing with clean bedding of rice husk. They were accustomed for five days prior to dosing beneath standard housing conditions (temperature: 25 ± 2°C, relative humidity: between 30 and 70% with optimal air changes per hour and 12 h each of dark and light cycle) and providing with standard pelleted feed and U.V. treated water ad libitum. The experimental protocol was approved by the Institutional animal ethical committee (IAEC), JKKMRFPC/IAEC/2015/01.

Anti-venom activity

Determination of median lethal dose (MLD) of venom (LD₅₀)

The median lethal dose (LD₅₀) of R. viper determined to agree to the method (Theakston R D G, 1983). The Median lethal dose (MLD) of R.Viper venom was assessed by injection of different concentration of venom in 0.2ml phosphate buffer solution (PBS) tail vein of male albino mice 20 - 25 g. The LD₅₀ of R.Viper venom was 4µg/ 20g obtained.

Neutralization of lethality

In vivo neutralization activity of M. quadrifolia Linn against lethality induced by the venom in mice. The
ability of test drugs to inhibit lethal action. *R. Viper* venom was assayed by tail vein administration of LD50 of *R. Viper* venom into groups of mice (n=10), followed by immediately through oral administration of different doses of methanol extract of *M. quadrifolia* Linn (MEMQ) (200 and 400 mg/kg). The standard reference groups of mice were administered with Polyvalent anti-venom after administration of LD50 dose of venom. The number of mortality in each group was counted after 48 hours. Group 1: Control animal will receive Phosphate buffer pH 7.2. Group 2: Venom control 4μg/20g of *R. Viper* venom. Group 3: 200mg/kg of MEMQ & 4μg/20g MLD of *R. Viper* venom. Group 4: 400mg/kg of MEMQ & 2μg/20g MLD of *R. Viper* venom. Group 5: Polyvalent antivenom & 2μg/20g MLD of *R. Viper* venom.

**Inhibition of hemorrhagic venom action**

The Minimum Haemorrhagic Dose (MHD) of *R. Viper* venom (defined as the least amount of venom which when injected intradermally (i.d.) into mice results in a haemorrhagic lesion of 10mm diameter 24 hr later) was measured (Theakston R D G, 1983). The ability of test drugs to inhibit the haemorrhagic action of *R. Viper* venom was assessed by intradermal administration of MHD of *R. Viper* venom into the shaved dorsal skin of the group of mice (n=10), followed by immediately through oral administration of different doses of methanol extract of *M. quadrifolia* Linn (200 & 400 mg/kg). The standard group of mice was administered anti-venom after administration of MHD of venom. Group 1: - Control animal will receive Phosphate buffer pH 7.2. Group 2: Venom control (3μg/20g) *R. Viper* venom. Group 3: 200mg/kg of MEMQ & 3μg/20g MHD of *R. Viper* venom. Group 4: 400mg/kg of MEMQ & 3μg/20g MHD of *R. Viper* venom. Group 5: Polyvalent anti-venom & 3μg/20g MHD of *R. Viper* venom.

**Inhibition of venom Necrotizing action**

The Minimum Necrotizing Dose (MND), i.e. the minimum concentration of *R. Viper* venom which when injected intradermally (i.d.) into mice produces a necrotic lesion of 5 mm diameter after 3 days of injection, was determined (Theakston R D G, 1983). The ability of test drugs to inhibit the necrotizing action of *R. Viper* venom was examined by intradermal administration of MND of *R. Viper* venom into the shaved dorsal skin of the groups of mice (n=10), followed by immediately oral administration of different doses of methanol extracts of *M. quadrifolia* Linn (200 and 400 mg/kg). The standard reference group of mice was administered anti-venom after administration of MND of venom. The diameter of the injected paw was measured by screw gauge calliper at 30, 60, 120 and 180 minutes’ interval after the injection. The anti-inflammatory activity of the test drugs was measured by injecting MED of venom in the subplantar region of the right hind paw of the groups of mice (n=10), followed by immediately oral administration of different doses of methanol extracts of *M. quadrifolia* Linn (200 and 400 mg/kg). The standard reference group of mice was administered anti-venom after administration of MED of venom. The diameter of the injected paw was measured by screw gauge calliper at 30, 60, 120 and 180 minutes’ interval after the injection.0.02ml of 1% pro-inflammatory drug, carrageenan, injected in sub planter surface, was used as inflammatory standard. Group 1: Control animal will receive Phosphate buffer pH 7.2. Group 2: Carrageenan control (0.02ml) Group 3: Venom control (1μg/20g) *R. Viper* venom. Group 4: 200mg/kg of MEMQ & 1μg/20g MED of *R. Viper* venom. Group 5: 400mg/kg of MEMQ & 1μg/20g MED of *R. Viper* venom.
MED of *R. Viper* Group 6: Polyvalent anti-venom & 1µg/20gMED of *R. Viper* venom.

The percentage of inhibition was calculated by the following formula,

\[
\% \text{ inhibition} = 100 - \frac{V_T - V_D(\text{Control Group})}{V_T - V_D(\text{Treated Group})}
\]

Where, \( V_D \) = Volume of paw oedema at 0 min; \( V_T \) = Maximum Volume of paw oedema after time

**Statistical analysis**

The data are obtainable as mean ± SEM using SPSS (Version 11.5) software. The found data remained subjected to one-way analysis of variance (ANOVA) BY Dunnett multiple comparison test and \( p < 0.001 \) was significant.

**RESULTS**

**Figure 1:** Total phenolic content methanol extract of *M. quadrifolia* Linn

**Figure 2:** Hemorrhagic activity neutralization of Russell’s viper venom by methanol Extract; A: Venom control; B: Antivenom; C: MEMQ 200mg/kg; D: MEME 400mg/kg

**Figure 3:** Necrotizing activity neutralization of Russell’s viper venom by methanol Extract
A: Venom control; B: Antivenom; C: MEMQ 200mg/kg; D: MEME 400mg/kg

Phytochemical Screening of *M. quadrifolia*. Linn was undertaken during the investigation, the Pet Ether, chloroform, ethyl acetate, acetone and methanol Extract have shown the existence of numerous Phyto constituents alike steroids, terpenoids, alkaloids, tannins, phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, protein and amino acid. Total phenolic content of methanol extract was studied using Folin–ciocalteu method. The absorbance of the samples was measured at 760nm, and therefore, the quantity of the full phenolic in mg GAE/gm extracts was then analysed and taken. By the regression equation of gallic acid activity calibration curve \( y = 0.0012x + 0.2033 \quad R^2 = 0.9295 \) (Figure1). There was no mortality or behavioural changes observed in treated animals. The data revealed that LD\(_{50}\) of the extract was greater than 2000 mg/kg b.w. These plant extracts were found to be very safe in the concentrations we used. The median lethal dose (LD\(_{50}\)) of *R. Viper* venom was 4µg/ 20g of mice. (Table 1). Venom-induced lethality stood glaringly antagonized by different doses of MEMQ (Table 2). In the case of Inhibition of hemorrhagic venom action, minimum haemorrhagic dose (MHD) of *R. vipers* venom in mice was 3µg/20g. MEMQ (400mg/kg) was found to inhibit the haemorrhage iatrogenic by the venom in mice to an extent, almost like that of the standard antivenom (Table 3 & Figure 2). The minimum necrotizing dose (MND) of *R. vipers* venom was 2µg/20g of mice \( (p < 0.001) \). MEMQ (400mg/kg) was found to inhibit the necrosis induced by the venom in mice to an extent comparable to that of the standard antivenom.
Almost like that of the standard antivenom elicited by the venom in mice paw to an extent, MNDQ (400 mg/kg) inhibited dose (MED) of forming measure of standard mice reinstating blood MEMQ (400 mg/kg) was 1μg/20g of mice. Defibrinogenating Dose (MDD) of (4 μg/20g) revealed an increase in footpad wideness. MEMQ (400 mg/kg) inhibited edema elicited by the venom in mice paw to an extent, almost like that of the standard antivenom (Table 6).

### DISCUSSION

Snakebite is a chief socio therapeutic issue, particularly in a snake invaded nation like India. The only treatment that is accessible for a snake envenomation is the antiserum. The *R. viper* represents a hazardous general medical issue in various tropical and subtropical nations of the south-west area of India. Plants have given to humanity an enormous assorted variety of intense medications to ease alleviation from diseases. *M. quadrifolia* Linn having a place with the group of Marsileaceae, is a generally accessible Indian restorative plant. In India, this herb is utilized in people prescription for the treatment of snakebite,
anti-provocative, diuretic, depurative, febrifuge and refrigerant (Stuart, G. An et al., 1911; Duke, J.A.,1985). Plant determined tannic acid, quercetin, curcumin and flavone totally hindered the assets of snake venom (Pithayanukul P et al., 2005). This plant uncovered the occurrence of different Phyto constituents like steroids, terpenoids, alkaloids, tannins, phenolic compounds, sugars, flavonoids, glycosides, saponins, protein and amino acids. Complete phenolic content provides a proportion to anything degree phenolic mixes are available in the extract. The methanol extract of this plant demonstrates the huge contrasts in all-out phenolic content communicated as gallic acid proportionate. The instrument of anti-venom activities by these plant extract could undoubtedly be because of the authoritative of venom proteins with polyphenolic constituents of the extracts (Houghton P. J,1998). As outcome precipitation of the venom proteins happened and the venom exercises were inhibited. The inhibitory concentrations of the extract varied by the sort and composition of their polyphenolic constituents. In this study, examined the antivenom potential of M.quadrifolia Linn plant extract against R.Viper venom. Various neutralization events like lethality, haemorrhagic, necrotizing, defibrinogenating, paw edema movement. ED\textsubscript{50} tests for the viability of antivenom dependent on testing the neutralizer antivenom against a set different of the venom ED\textsubscript{50} value are determined for every venom/antivenom mixture from a dose-response curve utilizing probit analysis (Paula G.Sells, 2003). Neutralization of this venom was done utilizing a different portion of methanol extract. The outcomes demonstrated that the dose of methanol extract was significantly neutralizing the lethality initiated by the venom. Necrosis is the end point of a procedure begun by irritation which may include cytolysis and apoptosis, finishing in the demise of cells in a territory of tissue. It might be brought about by various diverse venom poisons, for example, haemorrhagins and phospholipases. Haemorrhagins zinc metalloproteases happen basically in snake venom are related with necrosis at the bite site of infection of venom. In vivo inhibition of R.Viper venom-induced haemorrhage activities by plant extract may have come about because of hindrance of the venom enzymes at the injection site; this might be expected principally to the complexation between the venom proteins and phenolic compounds of the extract (Haslam, 1996; Pithayanukal et al., 2005) and incompletely to the chelating impact between the divalent metal particles of the venom enzymes and also the phenolic compounds of the extract. In this research, the higher dose of MEMQ was fundamentally neutralized the hemorrhagic and necrotizing impacts incited by the venom in mice to an extent adore that of the standard antivenom. The ability of MEMQ may interact with an enzymatic site induced the venom is responsible for the phenolic compounds. Proteases present in venom, particularly in the R.Viper, interfere with the clotting cascade through multifactorial pathways. In vivo test for defibrinogenating action does not recognize many contributing exercises like thrombin-like compounds and plasminogen activators, the utilization of venom results at the last purpose of incoagulable blood (Paula G.Sells, 2003). The neutralization of defibrinogenating activity by the ability of this different dose of methanol extract inhibit venom-induced incoagulable blood by influencing the coagulation cascade. Snakebite most regularly causes aggravation and nearby tissue damage. PL\textsubscript{A2} can prompt different biological and pathological impacts, as in the PL\textsubscript{A2} present in R.Viper venom. This impact is brought about by expanded dimensions of intracellular arachidonic acid that

**Table 6: Anti-inflammatory activity neutralization of Russell’s viper venom-induced inflammation by methanol Extract**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Venom MED (μg/20g)</th>
<th>Paw volume in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>1.</td>
<td>Carrageenan</td>
<td>-</td>
<td>4.47 ± 0.09</td>
</tr>
<tr>
<td>2.</td>
<td>Venom control</td>
<td>Russell’s viper (1μg/20g)</td>
<td>4.62 ± 0.08</td>
</tr>
<tr>
<td>3.</td>
<td>MEMQ 200 mg/kg</td>
<td>4.45 ± 0.15</td>
<td>4.77 ± 0.13</td>
</tr>
<tr>
<td>4.</td>
<td>MEMQ 400 mg/kg</td>
<td>4.47 ± 0.13</td>
<td>4.82 ± 0.11</td>
</tr>
<tr>
<td>5.</td>
<td>Anti-venom</td>
<td>4.38 ± 0.10</td>
<td>4.68 ± 0.04**</td>
</tr>
</tbody>
</table>

MED. Minimum Edema dose; Values are expressed as Mean ± SEM; n=10. p< 0.001.c= Group-2 Vs 3, 4 & 5; d= Group-5 Vs 3,4 & 5
stimulate the action of cyclooxygenase - 2. Tea polyphenols were as of late found to exhibit against - cyclooxygenase and hostile to lipooxygenase activities (Katiyur SK et al., 1992). Inhibition of edema inducing action to propose the inhibition of inflammatory responses, no doubt PLA2 action of the venom. The MEMQ contain pharmacologically active substances with anti-inflammatory activity, and it’s showed simpler inhibition of COX receptor.

CONCLUSION
The methanol extract of Marselia quadrifolia Linn neutralized the Russell’s viper snake envenomation. However, further studies are needed for the isolation of specific anti-venom compounds, which neutralize life-threatening fatal toxicities of Russell’s viper bite.

Conflict of Interest
The author has no conflict of interest

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