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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.

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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 anthiyur 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 Sampoorai 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;

$T = C \times V/M$

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),* JKKMRFCP*/IAEC/2015/01.*

Anti-venom activity

Determination of median lethal dose (MLD) of venom (LD_{50})

The median lethal dose (LD_{50}) 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 LD_{50} 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 assessed by tail vein administration of LD_{50} 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 LD_{50} dose of venom. The number of mortality in each group was counted after 48 hours. F 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 & 4 µg/20gMLD of *R.Viper* venom. Group 4: -400mg/kg of MEMQ & 4 µg/20gMLD of *R.Viper* Group 5:- Polyvalent antivenom &4 µg/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/20gMHD 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 extract of *M. quadrifolia* Linn (200 and 400 mg/kg). The standard reference groups of mice were administered anti-venom after administration of MND of venom. Group 1: Control animal will receive Phosphate buffer pH 7.2 Group 2: Venom control (2 µg/20g) *R.Viper* venom. Group 3: 200mg/kg of MEMQ &2µg/20g MND of *R.Viper* venom. Group 4: 400mg/kg of MEMQ & 2µg/20g MND of *R.Viper* venom. Group 5: Polyvalent antivenom&2µg/20g MND of *R.Viper* venom

Inhibition of venom defibrinogenating action

The Minimum Defibrinogenating Dose (MDD), i.e. the minimum concentration of *R.viper* venom which when injected intravenously (i.v.) into mice causes incoagulable blood 1 hrs later, was determined (Theakston R D G, 1983). The ability of test drug to inhibit the defibrinogenating action of venom was examined by i.v. Administration of MDD of venom into groups of mice (n=10), followed by immediately oral administration of different doses of methanol extract of *M. quadrifolia*Linn (200 and 400 mg/kg). The standard reference groups of mice were administered anti-venom after administration of MDD of venom. The condition of the blood (clotted/ non-clotted) was observed after 1 hr. Group 1: Control animal will receive Phosphate buffer pH 7.2Group 2: Venom control (2µg/20g) *R.Viper* venom. Group 3: 200mg/kg of MEMQ&2µg/20g MDD of *R.Viper* venomGroup 4: 400mg/kg of MEMQ & 2µg/20g MDD of *R.Viper* venom. Group 5: Polyvalent antivenom & 2µg/20gm MDD of *R.Viper* venom

Inhibition of venom-induced paw oedema

The Minimum Edema-forming Dose (MED) *R.Viper* venom was determined by the method. (Lomonte B *et al.,*1993; Camey Kyoko U *et al.,* 2002). MED was defined as the minimum amount of venom which, when injected subcutaneously into mice, footpad results in 30% edema within 6 hrs of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring calliper. The anti-edema 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* Group 6: Polyvalent anti-venom & 1µg/20gMED of *R.Viper* venom.

The percentage of inhibition was calculated by the following formula,

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

Where, VD= Volume of paw odema at 0 min; VT= Maximum Volume of paw odema 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< 0.001was significant.

RESULTS

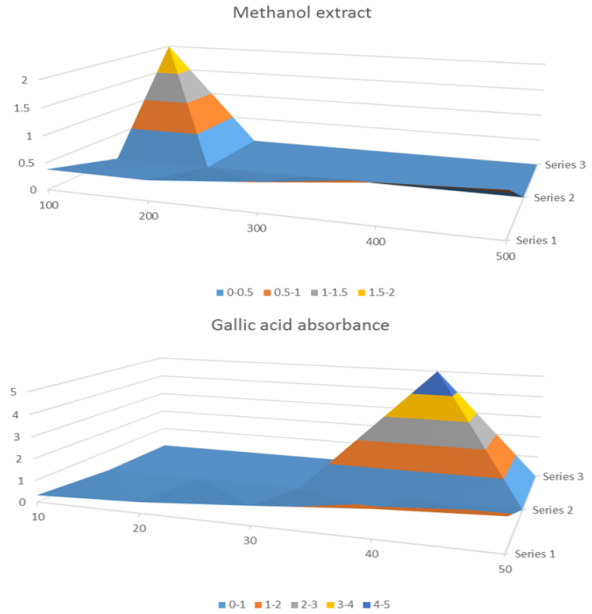

Figure 1: Total phenolic content methanol extract of M.quadrifolia Linn

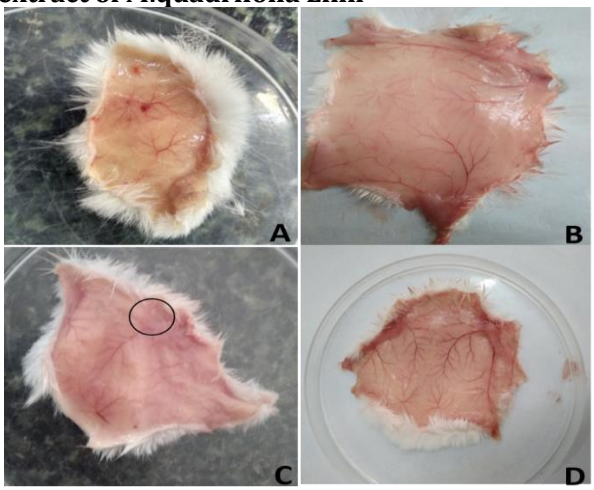

Figure 2: Hemorrhagic activity neutralization of Russell's viper venom by methanol Extract; A: Venom control; B: Anti venom; C: MEMQ 200mg/kg; D: MEME 400mg/kg

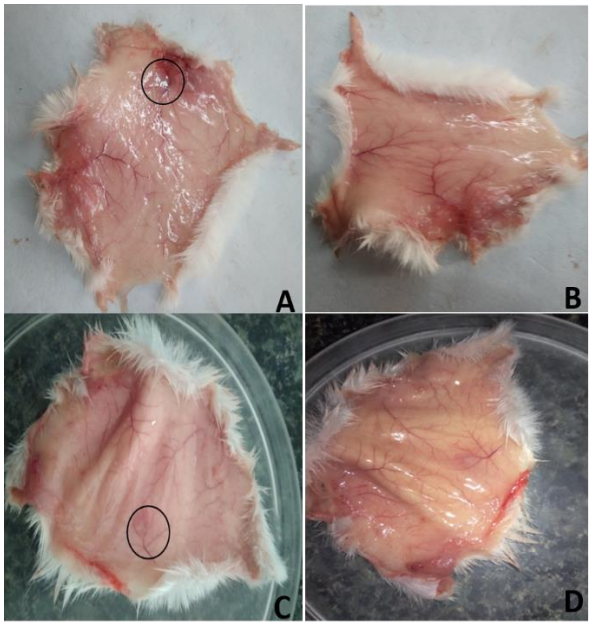

Figure 3: Necrotizing activity neutralization of Russell's viper venom by methanol Extract A: Venom control; B: Antivenom; C: MEMQ 200mg/kg; D: MEME 400mg/kg

Phytochemical Screening of *M.qudrifolia.* 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 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. viper* 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. viper* 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

Table 1: Median Lethal Dose (MLD) of Russell's viper Venom

Venom	Dose (µg/20g)	No. of deaths/No. of mice used	% Lethality
Russell's viper	10	10/10	100
	8	10/10	100
	6	09/10	90
	4	05/10	50

Table 2: Neutralization of Russell's viper venom-induced lethality by methanol extract

Group	Treatment	Venom Dose (µg/20g)	No. of deaths/No. of mice used	% Neutralized
1.	Phosphate buffer	-	0/10	-
2.	Venom control	*Russell's viper*	5/10	-
3.	MEMQ 200 mg/kg	(4 µg/20g)	4/10	60
4.	MEMQ 400 mg/kg		3/10	70
5.	Anti-venom		0/10	100

Table 3: Hemorrhagic activity neutralization of Russell's viper venom by methanol Extract

Group	Treatment	Venom MHD (µg/20g)	Diameter of haemorrhagic lesion (mm)
1.	Phosphate buffer	-	----
2.	Venom control	*Russell's viper* (3 µg/20g)	9.87 ± 0.41
3.	MEMQ 200 mg/kg		8.73 ± 0.40^{d***}
4.	MEMQ 400 mg/kg		8.01 ± 0.55^{c**d***}
5.	Anti-venom		2.45 ± 0.43 ^{c***}

Table 4: Necrotizing activity neutralization of Russell's viper venom by methanol Extract

Group	Treatment	Venom MND (µg/20g)	Diameter of necrotic lesion (mm)
1.	Phosphate buffer	-	----
2.	Venom control	*Russell's viper* (2 µg/20g)	5.60 ± 0.41
3.	MEMQ 200 mg/kg		5.21 ± 0.31^{d***}
4.	MEMQ 400 mg/kg		4.28 ± 0.20^{c***d***}
5.	Anti-venom		0.67 ± 0.09^{c***}

MND. Minimum necrotic 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

Table 5: Defibrinogenating activity neutralization of Russell's viper venom by methanol Extract

Group	Treatment	Venom MDD (µg/20g)	Clotting time in (Sec)
1.	Phosphate buffer	-	51.83 ± 2.23
2.	Venom control	*Russell's viper* (2 µg/20g)	194.67 ± 5.45 ^{c***d***}
3.	MEMQ 200 mg/kg		158.33 ± 6.09 ^{c***} ^{d***}
4.	MEMQ 400 mg/kg		119.12 ± 8.41 ^{c***} ^{d***}
5.	Anti-venom		52.50 ± 3.82

MDD. Minimum Defibrinogenating 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

(Table 4 & Figure 3). The minimum defibrinogenating Dose (MDD) of *R.viper* venom remained 1µg/20g of mice. (p<0.001).

MEMQ (400mg/kg) was found to be precise real in reinstating blood coagulability in mice further as like that of the standard antivenom (Table 5). In an edema-forming measure, the minimum oedema-forming dose (MED) of *R.viper* venom was 1µg/20g of mice. (p<0.001) revealed an increase in footpad wideness. MEMQ (400mg/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,

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

Group	Treatment	Venom MED (µg/20g)	Paw volume in mm				
			0 min	30 min	60 min	120 min	180 min
1.	Carrageenan	-	4.47 ± 0.09	4.59 ± 0.09	4.76 ± 0.07	4.83 ± 0.07	4.89 ± 0.07
2.	Venom control	*Russell's viper* (1µg/20g)	4.62 ± 0.08	4.87 ± 0.07	5.03 ± 0.07	5.17 ± 0.05	5.24 ± 0.04
3.	MEMQ 200 mg/kg		4.45 ± 0.15	4.77 ± 0.13	4.88 ± 0.12	4.94 ± 0.10 ^{c***d***}	5.03 ±0.09
4.	MEMQ 400 mg/kg		4.47 ± 0.13	4.82 ± 0.11	4.79 ± 0.07	4.51 ± 0.12 ^{c***d***}	4.16 ± 0.07 $^{c*** d***}$
5.	Anti-venom		4.38 ± 0.10	4.68 ±0.10	4.63 ± 0.04^{c**}	4.01 ± 0.07 ^{c***}	3.33 ± 0.14 ^{c***}

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

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 of to anything to 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_{50} tests for the viability of antivenom dependent on testing the neutralizer against a set different of the venom ED_{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; Pithanyanukal *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. PLA_2 can prompt different biological and pathological impacts, as in the PLA_2 present in R.Viper venom. This impact is brought about by expanded dimensions of intracellular arachidonic acid that

stimulate the action of cyclooxygenase - 2. Tea polyphenols were as of late found to exhibit against – cyclooxygenase and hostile to lipoxygenase activities (Katiyur SK *et al.,*1992). Inhibition of edema inducing action to propose the inhibition of inflammatory responses, no doubt PLA_2 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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