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Bioactive Potential of Aqueous Extract of *Calotropis gigantea* against *Echis carinatus* Venom

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ABSTRACT

Background: Many plants are used to cure snakebites in Indian traditional medicine and some of them have been examined for their ability to neutralize snake venom. The main aim of the present study is the screening of phytochemicals, *in vitro* assessment of venom toxicity and neutralization assays and its antimicrobial assay.

Methods: The phytochemical screening and thin-layer chromatography was used to determine and separate the chemical constituents present in the *Calotropis gigantea.* The direct hemolysis using sheep RBCs was studied for *Echis carinatus* venoms. The proteolytic, neutralizing and antimicrobial activity of the extract was also studied.

Result: The phytochemical investigation of root extract of *Calotropis gigantean* and the TLC was performed. The Protein concentration of the lyophilized snake venoms was found to be 0.692 mg/ml. *Echis carinatus* venoms showed 89% hemolysis. *Echis carinatus* venom produced a reduced zone of hydrolysis with the extract. The absence of clot formation showed the neutralizing ability of plant extracts in gelatin liquefaction assay. Thus the scientific confirmation of neutralization potential of *Calotropis gigantea* plant extract against Saw scaled viper (*Echis carinatus)* venom was performed.

Key words: Antibacterial, Antivenom, Hemolysis, Neutralisation, Phytochemicals.

INTRODUCTION

Every civilization retains its distinctive characteristics in all of its innovations and traditions, just like other traditional cultures do. Such performed and practiced facts are passed down from one generation to the next. This also includes the understanding of drugs and cures. The terrible reality is that we have little interest in modernizing our healthcare system. More study is required in this particular area.

Calotropis spp. is one of the plants widely worshipped by people and these plants are conserved as a genetic resource and used as food, fertilizer, fodder and in every other way (Kumari *et al*., 2020). *Calotropis procera* and *Calotropis gigantea* are commonly utilized not only in Indian but also in Unani, Arabic and Sudanese medicinal system (Parihar *et al*., 2016). The folk medicinal plant *Calotropis gigantea*, a member of the Apocynaceae family *C. gigantea*, is a large shrub or small tree native to Bangladesh, India, Malaysia, Southern China and Indonesia. These large shrubs have also been extensively cultivated in tropical areas around the world and typically grow to eight to 15 feet tall (Dutta *et al*., 2021). An eco-friendly nanotechnology method and *Calotropis gigantea* extract could offer promising antibacterial action against skin infections (Govindasamy *et al*., 2021). Asthma, swollen rheumatism, diarrhoea, dysentery, syphilis, ulcer, leprosy and other common diseases are all traditionally treated with it either alone or in combination with other medicinal plants (Timilsina *et al*., 2020). Barks, leaves, flowers, roots, fruits and seeds of medicinal plants can be used to make various products (Ahmad, 2020).

One of the important properties of *Calotropis gigantean* is its anti-venom property. The venom neutralization ability and prophylactic effect of *Calotropis gigantea* methanolic root extract (CGMR) against Daboia russelii envenomation using *in vitro*, in silico and *in vivo* methods were analyze. Further the extract showed significant venom neutralization ability and protection effects against venom insult. The high venom neutralization ability may be due to PLA2 inhibitors present in the extract (Vikram, 2021). Snake bites may lead to cause different pathophysiological alteration in human body such as edema, inflammation, haemorrhage, necrosis, alterations in blood coagulation process and finally leading to death. The presence of alkaloid, flavonoids, saponins, steroids, triterpenoids and tannins are responsible for the anti-snake venom activity of *Calotropis gigantea*.

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The alcoholic extract of *Calotropis gigantea* was reported for its activity to neutralize noxious effects of the venom *(Vipera russelli)* like necrotizing activity, lethality, edema and hemorrhagic activity (Chacko *et al*., 2012).

There is a polyvalent antivenom available in India that is effective against the venoms of the cobra, common krait, Russell's viper and saw-scaled viper. According to estimates, the indigenous medical books contain about 25000 potent plant-based medicines. Around 4 billion people, or 80% of the world's population, according to the World Health Organization (WHO), currently utilize herbal medicine for some part of primary healthcare (Purohit and Vyas, 2004). Traditionally, *Calotropis gigantea* has been used against snake bites. Pandey *et al.* (2011) reported the anti-venom properties of *Calotropis gigantea* against cobra venom via *in-vivo*. Many works of literature via orally and research advocated *Calotropis gigantea* holds toxic properties due to the presence of some phyto compounds like Cardenolide. The benefits of herbal medicine include its accessibility, known source, simple extraction procedures and lack of literacy requirements, ability to be developed by people of any age, low cost mechanism, affordable storage features and cost effectiveness when compared to other polyvalent animal antibodies. In our present study, we investigate the anti-venom properties *Calotropis gigantea* and qualitative investigation of toxic compounds present in it *via in vitro* aginst *Echis carinatus* venom.

MATERIALS AND METHODS

Collection of medicinal plant

The medicinal plant was collected in and around the Coimbatore and Palakkad regions. The herb was legitimately identified as coming from the Keralan Arya Vaidya Pharmacy Research Centre in Palakkad. The soxhlet apparatus was filled with 20 g of the finely ground fresh root of the *Calotropis gigantea* sample. Distilled water was used as the extraction solvent during the soxhlet extraction method (250 ml) (Azrie *et al*., 2014).

Phytochemical studies of plant extracts

The crude plant extract was subjected to phytochemical analysis for detecting the chemical compounds in it. The phytochemicals Alkaloids (Evans, 1997), Saponin (Kokate, 1999), Tannins (Mace, 1963), Anthraquinone, Flavanoids, Phenol, Steroids and Terpenoids, Salkowsky test (Cholesterol), Phytosterols (Finer, 1986), Proteins, sugars (Yemm and Wills, 1954), Reducing Sugar, glycosides were analyzed.

Separation of plant components using thin layer chromatography

About 20 gm of silica gel (Merc-105553) was used to prepare the plates. The solvent system used was Ethyl acetate, Formic acid, Glacial acetic acid and Water (100:11:11:26) Following Thin Layer chromatography bands have appeared and it was visualized by UV- chamber at a wavelength of 254 nm (Wagner and Bladt, 1996).

Quantitative determination of *Calotropis gigantea* using HPTLC analysis

HPTLC analyses were carried out for aqueous root extract of *Calotropis gigantea.* The solvents used for HPTLC analysis were obtained from MERCK. 5 µl of the test solution and 5 µl of available standard solutions such as quercetin, gallic acid, rutin, Stigmosterol, Beta-sitosterol and lupeol were loaded (Arivukkarasu and Rajashekaran, 2015).

Collection and study protein profile of snake venom

The freeze-dried venom powders of *Echis carinatus* were obtained from Irulas Snake catcher's Industrial co-operative society Chennai, Tamil Nadu and was stored at 4°C. The stock solution was prepared by dissolving 1 mg of lyophilized venom in 1 ml of physiological saline (1 mg/ml). The protein profile of venom samples was analyzed by SDS PAGE. SDS PAGE was done according to the method of Laemmli (1970) with a 4% stacking and 12% separating gel. The protein content of the venom was estimated by the method of Lowry (1951). The protein content of the test samples was extrapolated from the standard graph.

In vitro assessment of venom toxicity and neutralization assays

Direct haemolysis assay

The hemolytic action of *Echis carinatus* venoms and extract was studied *in vitro* by using RBC. Briefly, 5 ml of citrated blood was centrifuged for 10 minutes at 900 rpm. The supernatant was poured off and the pellet was washed twice with the physiological salt solution. 5 ml of physiological saline and 0.5 ml of RBC mixture served as a control. 5 ml of distilled water with 0.5 ml of washed RBC was used for 100% hemolysis. 5 ml of venom/extract and 0.5 ml of washed RBC served as the experimental sample. The tubes were put in a thermostat for 1 hr at 37°C and centrifuged at 2000 rpm for 20 mts. The supernatant fluid was poured off to separate tubes to measure the optical density using a spectrophotometer at a wavelength of 540 nm against water (Thushara James *et al*., 2013). The calculation of hemolysis was done by the formula:

$$\frac{\text{Experimental sample-Control sample}}{\text{100\% hemolysis}} \times 100$$

Indirect haemolysis assay (PLA_2 activity)

Phospholipase A_2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez *et al*., 1988. Increasing concentrations of *Echis carinatus* venoms (µg) were added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM $CaCl_2$.

Proteolytic activity

Skim milk agar plates (1%) were prepared. Agar wells (2 wells per plate) were cut and 20 µl of *Echis carinatus* venom was added to the plate and incubated for 24 h at 37°C.

20 µl of PBS alone served as a control. Zone of hydrolysis of casein on milk agar plate was measured. Neutralization expressed as the ratio mg plant extract/mg venom able to reduce by 50% the diameter of the zone of hydrolysis when compared to the effect induced by venom alone was measured (Meenatchisundaram, 2017).

Neutralization of procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid (1983) and modified by Gene *et al.* (1989). The constant amount of venom was mixed with various dilutions of extracts and incubated for 30 minutes at 37°C. Then 0.1 ml of a mixture containing two minimum coagulant doses of venom was added to samples of 0.2 ml of citrated plasma and the clotting times were recorded. In control tubes, plasma was incubated with either venom alone or extracts alone.

Neutralization assay using gelatin liquefaction method

A constant amount of venom and gelatin was mixed with various dilutions of extracts are incubated for 30 minutes at 37°C. In control tubes, gelatin was incubated with venom alone. Neutralization was expressed or noticed by the liquefaction of gelatin when it was treated with various concentrations of plant extracts (Philpot and Deutsch, 1956).

RESULTS AND DISCUSSION

Calotropis gigantea is a non-cultivable weed frequently referred to as "Madar" in Hindi in India. It's so poisonous that snakes can't take the stench, therefore snake charmers use it to keep snakes away (Meenatchisundaram *et al.,* 2009). Our present investigation explores *Calotropis gigantea* plant extract neutralizes the effect of Saw-scaled viper venom. The soxhlet extraction of the aqueous root of *Calotropis gigantea* was prepared. The plant extract (root) was subjected to phytochemical analysis and the presence of flavonoid, phenol alkaloids and glycoside compounds were quantitatively detected (Table 1). The phytoconstituents of *Calotropis gigantea* that are derived from various parts of the plant include numerous glycosides, alkaloids, flavones, tannins and so forth, such as calotoxin, uscharin, uschridin and proceroside (Zhu-Nian Wang, 2008). Based on the literature review *Calotropis gigantea* possess a highly toxic effect even though it has many medicinal properties. The cardenolides might result in contributing toxicity to the snakebite-affected person that might result in cardiac arrest or other complications. In such a case either the extract devoid of this steroid component or its very low dose should be administered. On Phytochemical screening, dried root extract showed the presence of glycosides. Based on the observations made under the UV - chamber at a wavelength of 254 nm the spots were observed in the definite distance (Fig 1). The HPTLC of the extract was also performed where it showed the presence of antioxidant compounds when compared with Rf value of the standard and well-known free radical scavengers rutin, quercetin and gallic acid, Stigmosterol, Beta-sitosterol and lupeol in *Calotropis gigantea* various extract (Fig 2, Table 2). For identifying these free radical scavengers' rutin, quercetin and gallic acid, Stigmosterol, Beta-sitosterol and lupeol we used UV light at 254 nm. HPTLC is a fast, accurate and quantitative analytical method used in immunotechnology (Dey, Abhijit and Devendra Kumar Pandey, 2014).

The protein concentration of the lyophilized snake venoms was analyzed. Saw-scaled viper was found to be 0.692 mg/ml. The protein profile of Saw-scaled viper venom was analyzed by SDS-PAGE (Fig 3). A Group of protein bands was detected in Saw-scaled viper venom.

The red cell lysis which may develop following snakebite does not play a predominant role in the overall venom lethality, but should rather be considered as one of the manifestations of the digestive action of the venom (Condrea, 1978). The hemolytic study on *Echis carinatus* venoms showed 89% hemolysis. The root extract was able to neutralize the venom-induced hemolysis and the hemolysis was reduced (Fig 4). The various parts of various plants were practiced in venom neutralization. *A. paniculata, C. magna, G. superba* and *H. javanica* plant extracts possess potent snake venom neutralizing capacity and could potentially be used as an adjuvants for antivenin therapy in case of snakebite envenomation, especially against the local effects of cobra venoms were proved (Kumarapppan *et al*., 2011).

Table 1: Phytochemical screening of plant extract.

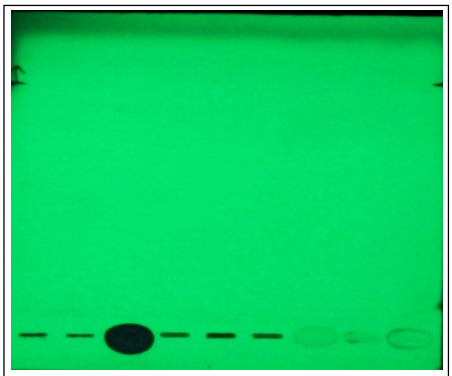
Tests	Dried root extract
Alkaloids	Positive
Glycoside	Positive
Saponins	Negative
Tannins	Negative
Anthraquinones	Negative
Flavanoids	Negative
Phenol	Negative
Steroids and terpenoids	Positive
Salkowsky	Positive
Phytosterol	Negative
Proteins	Negative
Aminoacids	Negative
Sugars	Positive
Reducing sugars	Negative

Table 2: Rf values of markers and extract of *Calotropis gigantean.*

Track number	Sample	Amount (µl)	Rf value
1	Root extract	5	0.89
2	Root extract	10	0.90
3	Root extract	20	0.86
4	Rutin	5	0.92
5	Quercetin	5	0.89
6	Gallic acid	5	0.6
7	Stigmasterol	5	0.89
8	B-Sitosterol	5	0.88
9	Lupeol	5	0.89



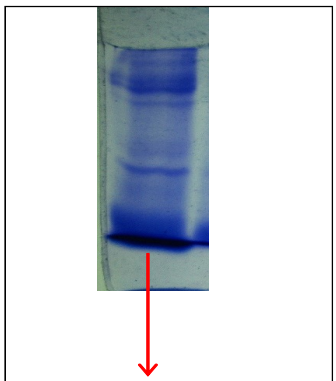
Fig 1: Separation of components using thin layer chromatography. Based on the observations made under the UV-chamber in a wavelength of 254 nm the spots were observed in the definite distance in extracts.



1 2 3 4 5 6 7 8 9

1. Root extract (5 µl), 2. Root extract (10 µl), 3. Root extract (20 µl). 4. Rutin standard, 5. Quercetin standard 6. Gallic acid standard 7. Stigmasterol standard 8. B-Sitosterol. 9. Lupeol.

Fig 2: Chromatogram of *calotropis gigantea* and three standards after development in the mobile phase.



A

A. Echis carinatus venom. (Group of protein bands was detected in Saw-scaled viper venom).

Fig 3: Protein profile of snake venom by SDS-PAGE.

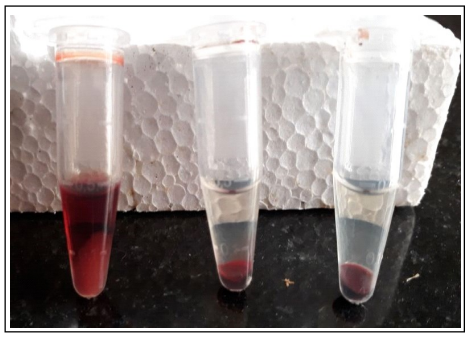


Fig 4: Direct hemolysis.

In phospholipase activity (PLA_2) 10 µg of *Echis carinatus* venom were able to produce 11 mm diameter hemolytic halo, which is considered to be 1 Unit (Fig 5). The extract was capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by snake venoms in a dose-dependent manner. In another study, they proved the inhibition of PLA2-dependent hemolysis of sheep RBC's induced by Daboia russelli venom by *Rauvolfia serpentina* extract in a dose-dependent manner (Thushara James, 2013). The medicinal plants *Thea sinensis Linn* and *Cordia verbenacea* effectively neutralized the phospholipase A2 activity induced by snake venoms (Laing *et al*., 1992).

Echis carinatus venom produced a zone of hydrolysis of 11 mm in diameter in skim milk agar when the proteolytic study was carried out (Fig 6). Neutralization assays were carried out by pre-incubating extract with a known concentration of venom and found to produce reduced zone size of 2.5 mm. After one hour the proteolytic activity was carried out and the neutralization expressed as the ratio, mg plant extract/mg venom indicated that the plant extracts were able to reduce the diameter of the zone of hydrolysis by maximum when compared to the effect induced by venom alone. Plant extract can be able to reduce the zone of hydrolysis produced by venoms.

Venom-induced clotting was neutralized by increasing the anti-venom concentration sufficiently. The minimum coagulant dose (MCD) of venom was mixed with various concentrations of extracts and dissolved in 0.1 ml of human citrated plasma. The absence of clot formation shows the neutralizing ability of plant extracts. Saw-scaled viper venom requires a high concentration (50 µl) for clotting. Venom-induced clotting was neutralized by increasing the anti-venom concentration sufficiently (Fig 7). In the gelatin liquefaction study, the minimum coagulant dose (MCD) of venom was mixed with various concentrations of extracts and dissolved in 50 µl of gelatin. The absence of clot formation shows the neutralizing ability of plant extract (Fig 8). *Calotropis gigantea* is effective in the neutralization of coagulant activity induced by snake venoms. Snake envenomations cause different pathophysiological changes such as inflammation, haemorrhage, necrosis, edema, alterations in blood coagulation system and ultimately leading to death (Pillay, 2008).

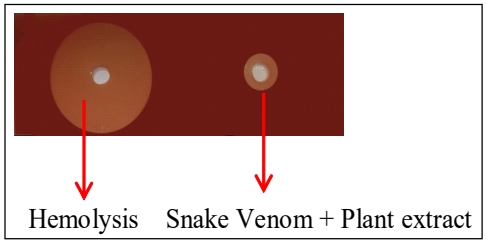


Fig 5: Indirect hemolysis.



Fig 6: Proteolytic activity.

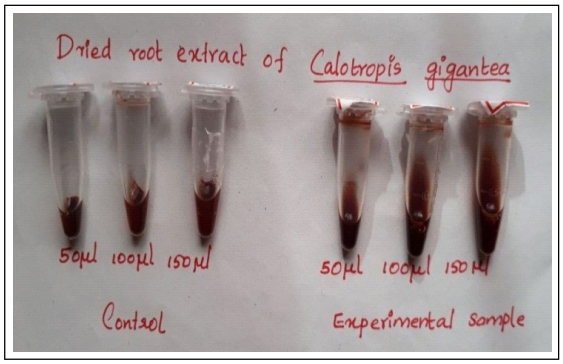


Fig 7: Procoagulant activity.



Fig 8: Gelatin liquefaction.

Hence, the presence of these anti-snake venom compounds in the aqueous extract from *Calotropis gigantea* root could have contributed to its efficient antivenom activity. The glycoside is reported to be toxic if present at appreciable levels. The secondary metabolites of the plant are responsible for conferring its biological activities. Though *Calotropis gigantea* has various medicinal applications, still the phytochemicals of this plant need to be standardized to explore its medicinal values with the help of various methods. Further research is necessary to elucidate the phytochemical and pharmacological aspects of this plant and further investigations could be done.

CONCLUSION

In the current investigation, we were able to convincingly demonstrate the scientific validity of the neutralization capability of plant extracts from *Calotropis gigantea* against the venom of Saw Scaled Vipers (*Echis carinatus*). People in India are aware of the tree, *Calotropis gigantea*, which is widely distributed there. To identify the cardenolide molecules that are found in *Calotropis gigantea*, more research is required. As more harmful compounds are eliminated from the plant's various components, *Calotropis gigantea* may be detoxified and used as a natural remedy for various snake bites. Experimental validation is required for the development of low-cost phytotherapeutic compounds.

Conflict of interest

All authors declare that they have no conflict of interest.

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