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Protective Effect of Oxalis Corniculate Extract against Toxic Enzymes of *Najanaja* and *Daboiarusselli* Venom

Kiran K S[1],KarthikN Awathade, Kumar J R[1*]Kanthesh BM[2]

[1]Division of Biochemistry, School of Life Sciences, JSS medical college campus, JSS Academy of Higher Education & Research, Mysuru-570015, Karnataka, India.
[2]Division of Molecular Biology, School of Life Sciences, JSS medical college campus, JSS Academy of Higher Education & Research, Mysuru-570015, Karnataka, India

*Corresponding author:
Dr. J R Kumar, Assistant professor, Division of Biochemistry, Department of Water & Health- School of Life sciences, JSS Academy of Higher Education & Research, JSS Medical College of Campus, Mysore 570015, kumarjr2013@gmail.com

Abstract
Snake envenomation is an imperative public health problem in many rural areas of tropical and sub-tropical Asian countries that affects thousands of people worldwide. Although anti-snake venom is the only treatment available, it is associated with several side effects. Plants have been extensively studied to obtain an alternative treatment. In Ayurveda medicine, *Oxalis corniculata* is usually used to treat Datura, Arsenic, and snakebite poisoning. The present study aims to provide a first-hand scientific explanation for the use of this plant against snake envenomation. The extracts of shade-dried *O. corniculata* (whole plant) were tested for in vitro anti-toxic effects against *Najanaja* and *Daboiarusselli*venoms. The different solvent extracts rendered a significant inhibitory effect on whole venom Hyaluronidase activity, Acetylcholinesteraseactivity, Phospholipase A_2 induced hemolytic activity, and casenolytic activity. The present study using Methanolic extract of *Oxalis corniculata* has exhibited a potential to inhibit the Haemolytic activity by 88% and 95 %, PLA_2 activity by 84% and 75%, Hyaluronidase activity by 85.6 % and 65%, Acetylcholinesterase activity by 73% and 84% and caseinolytic activity by 77% and 95% of *Najanaja* and *Daboiarusselli*venom respectively. Obtained results suggest the presence of active inhibitory components that acts against toxic properties of *Najanaja* and *Daboiarusselli* venom. Further pharmacological and in vivo studies would provide evidence that these active components may lead to a potential treatment against venom-induced toxicities.
Keywords: Oxalis corniculata, Phospholipase A_2,Hyaluronidase, Acetylcholinesterase.

Abbreviations:
O.C - *Oxalis corniculata*
PLA_2 - Phospholipase A_2
AChE - Acetylcholinesterase

1. Introduction
Snakebite is a significant public health problem, causing considerable high morbidity and mortality around the world, particularly in the equator. It is also of biomedical importance with a social and economic impact on developing regions around the world. Snakebite is now recognized as a highly neglected tropical disease (NTD) by the World Health Organization (WHO). The WHO estimates that 1.8-2.7 million venomous snakebites occur worldwide each year, resulting in an estimated 100,000 to 125,000 annual deaths, but this may be underreported [1,2]. India is among the countries, most dramatically affected by snakebite

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and accounts for almost half the total number of annual deaths in the world and it is about 1.2 million snakebite deaths [3] (representing an average of 58,000 per year).

India has about 270 species of snakes, of which 60 are considered venomous and medically relevant and with various levels of toxicity. Cobra, Krait, Russell's viper, and saw-scaled viper are the four major venomous biting species. The former two belong to the Elapidae and the latter two belong to the Viperidae family [4].

Snake venom is not composed of a single compound, but it's a complex mixture of protein probably consisting of fifty to sixty components. Each of its components possesses its activity. Further its activity and composition vary among the species and region. The protein components of snake venoms include cytotoxins, cardiotoxins, nerve growth factors, lectins, enzyme inhibitors, and various enzymes, such as phospholipase A_2 (PLA_2), metalloproteases, serine proteases, phosphor-diesterases, cholinesterases, aminotransferases, L-amino acid oxidases, catalases, ATPases, hyaluronidases, etc. [5].

To date, the best available therapy for the treatment of snakebite cases is immediate administration of polyvalent anti-venom. However, this therapy has many limitations like delayed reaction (serum sickness) and Acute reaction (anaphylactic or pyrogenic). Moreover, it has also been reported that anti-venom does not provide complete protection against hemorrhage, necrosis, and nephrotoxicity induced during envenomation [6]. Therefore, finding ways to neutralize the multiple toxicities generated post envenomation is a major challenge to the clinicians, and hence search for an alternative to anti-venom therapy for treating snakebite cases is inevitable.

India has a rich heritage of medicinal plants. Perusals of literature show that plants have been used as a source of medicine by the local healers and traditional practitioners for the treatment of various diseases including snakebite. Ethno-botanical investigation of *Oxalis corniculata* suggests that it was used against snakebite [7,8] and also reported in the traditional Ayurvedic treatment forDatura, Mercury, and Arsenic poisoning. Hence we propose furtherinvestigation of anti-venom activity of crude extracts and further bioassay-guided screening may lead to the discovery of a therapeutic candidate against snake bite.

Oxalis corniculata Linn. commonly called creeping wood sorrel, is a well-known most versatile medicinal plant in India and has a wide spectrum of biological activities, belongs to the Oxalidaceae family [9,10]. In Ayurvedicmedicine, it is used in the treatment of liver and digestive problems. In Nepal, it is used for stomachaches; the leaves for ritual source. In Zaireanpharmacopeia, it is used as antivenom [11,12,13].It also has shown promising effects like Wound-healing, Cardio relaxant, Nematocidal, Anti-cancer, Anti-microbial, Anti-fungal, Anti-amoebic, Anti-implantation, Abortifacient, Allelopathic, Antioxidant activity, Steroidogenic activities [14 to 28]. The leaves are used as an antidote against poisoning by the seeds of Datura, arsenic, and mercury [29,30]. The leaf juice is applied to insect bites, burns, and skin eruptions.The present study investigates the neutralizing activity of *O.C* plant extracts on *Daboiarusselli*and *Najanaja*venom-induced enzymatic toxicities, by in vitro methods.

2. MATERIAL AND METHODS

Snake Venom

Lyophilized snake venom (*Najanaja* and *Daboiarusselli*) was purchased from Irula snake catcher's Industrial Cooperative Society Limited, Chennai (India).The crude venom was stored in airtight containers at 4ºC until used. Venom was dissolved in 10 mM PBS and centrifuged at 2500 rpm for 10 min and the supernatant was used for the study. Hyaluronic acid was purchased from Sigma Aldrich (USA). All other reagents and solvents were of high-quality analytical grade and were purchased from SD Fine chemicals, Sisco Research Laboratory Pvt Ltd, and Hi-Media.

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Plants and extraction

Plant material of *Oxalis corniculata* (*O.C*) was collected from MagadiTaluk, Ramanagara District, Karnataka, India, and it was authenticated by Dr. M.N Naganandini (Assistant Professor, Department of Biology, JSS College of Pharmacy, JSS AHER, Mysuru, Karnataka, India) A voucher specimen was deposited in the Herbarium of the same department under the number: JSSCMP. PCOG -116. The whole plant was washed in purified water and shade dried for 20 days.

The powdered plant material was serially extracted successively from nonpolar to polar using petroleum ether, hexane, chloroform, ethyl acetate, and methanol by soaking for 48 hours. At the end of the respective extraction, the plant extracts were filtered using Whatman No.01 filter paper. The filtrate was then concentrated under reduced pressure in a vacuum at 40 °C for 25 min using a vacuum evaporator.

Phytochemical screening

Each extract obtained from soaking powdered plant material was used for the identification of plant metabolites like alkaloids, flavonoids, terpenoids, saponins, tannins, and phenolics present [31,32].

Anti-venom assays

Preparation of Red blood cells

Blood samples from healthy male/female (non-smoker and non-alcoholic) volunteers were collected into heparinized Vacuette® tubes through venepuncture after taking informed consent. The tubes were gently swirled and centrifuged at 1500 g for 10 min at 4°C. The plasma and buffy coat were separated. The resulting erythrocytes were washed thrice with 10 volumes of PBS and centrifuged at 1500 g for 5 min. The buffy coat was carefully removed after each centrifugation and the erythrocyte suspension stock of 10% v/v was prepared in PBS, stored at 4°C, and used within 6 hours.

Indirect Haemolytic Activity

Hemolytic inhibition was determined as per the method of Boman and Kaletta [33], with slight modifications. Briefly, 100 µl of human erythrocyte suspension (in 10 mM PBS, pH 7.4) was added to the reaction mixture of inhibitor and venom (*Najanaja* and *Daboiarusselli*) pre-incubated for 2 h at 37°C. The reaction was stopped by adding 100 µl of ice-cold PBS and centrifuged at 2500 rpm (10 min at 4°C). The amount of hemoglobin released was estimated spectrophotometrically at 490 nm using a microtitre plate reader.

Phospholipase (PLA_2) inhibition

PLA_2 enzyme activity was measured using 4-nitro-3- octanoyloxy-benzoic acid (NOBA) as substrate, according to the procedure of Holzer and Mackessy [34]. The standard assay mixture contained 220 µL of buffer (10 mM Tris-HCl, 5 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 µL of 1.5 mM NOBA in acetonitrile, and 20 µl of appropriately diluted sPLA2 (about 0.2 µM) in the final volume of 260 µL. The enzyme activity (expressed as the initial velocity of the reaction) was calculated based on the increase in absorbance at 450 nm after 15 min. All assays were conducted in triplicate using Dynexmultiwell plate reader.

Hyaluronidase inhibition

Hyaluronidase assay of crude venom was determined turbidometrically by the method of Pukrittayakamee et al. [35]The assay mixture contained buffer Tris–HCl (pH 8.0), 25 µg of hyaluronic acid (0.5 mg/mL in buffer), and enzymes in a final volume of 1.0 mL. The

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mixture was incubated for 15 minutes at 37°C and the reaction was quenched by the addition of 2 mL of 2.5% (w/v) cetyl- trimethylammonium bromide in 2% NaOH (w/v). The absorbance was read at 400 nm (within ten minutes) against a control solution containing 1 ml of the same buffer and 2 mL of 2.5% (w/v) cetyl-trimethylammonium bromide in 2% NaOH (w/v). Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube in which no enzyme wasaddedas100%. One unit was defined as the amount of enzyme that provoked 50% turbidity reduction. Specific activity was defined as turbidity reducing units per milligram of the enzyme. For the inhibition studies, venom was preincubated with extracts for 30 minutes at 37°C.

Acetylcholinesterase

AChE activity was assayed following Ellman et al. method [36]. The reaction mixture comprised 3.0 ml of the phosphate buffer (pH 8.0), 10 µL of DTNB (10 mmole/L) and 20 µL of acetylethiocholine iodide (158.5 mmol/L). A total of 50 µL of 0.1% crude venom and 3 mL of buffer solution was incubated at room temperature for five minutes. Then, 10 µL of DTNB (a strong oxidizing agent) and 20 µL of substrate acetylethiocholine iodide were added to reach a final concentration of 1 mmole/L. The increase in absorbance at 412 nm was measured on a double beam spectrophotometer against a control mixture prepared at the same time. However, in the latter case, 50 µL of the enzyme was replaced with 50 µL of buffer solution. For the inhibition studies, venom was pre-incubated with extracts for 30 minutes at 37°C.

Caseinolytic activity.

The procedure of Satake et al. [37]was followed. The venom sample (20µg), from each venom (*Najanaja* and *Daboiarusselli*), was incubated with 1 ml of the casein substrate (2% fat-free casein in 0.2 M Tris- HCl pH 8.5) for 2 h at 37³C. The reaction was terminated by adding 1.5 ml of 0.44 M trichloroacetic acid. After 30 min, the protein precipitate was separated by centrifugation. To 1 ml of the supernatant, 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of Folin-Ciocalteu's reagent were added. The blue color developed was read after 20 min at 660 nm.

3. RESULTS

Phytochemical screening.

The preliminary phytochemical screening has shown that the presence of alkaloids, flavonoids, saponins, carbohydrates, proteins, phenols, and tannins in the selected solvent extracts of the plant. From the table-1, it was evident that all solvent extracts were found to contain selected phytochemicals. However, when compared with other solvent extracts, methanolic extract was found to contain most of the plant metabolites. The summary of the results is presented in Table 1.

Tests	Petroleum ether	Hexane	Chloroform	Ethyl acetate	Methanol
Alkaloids	-	-	-	++	++
Terpenoids	-	-	-	-	-
Flavonoids	-	-	++	++	++
Phytosterols	-	-	-	-	-
Proteins	-	-	-	-	++
Carbohydrates	-	-	-	-	++
Glycosides	-	-	-	-	-
Phenolics/tannins	-	-	-	-	++

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Resins	-	-	-	-	-
Saponins	-	-	-	-	++

(+) indicates present, (−) indicates absent

Table 1:Phytochemical analysis of Oxalis corniculata

Indirect Hemolytic Activity.

The anti-hemolytic activity of the different solvents extracts of *O.C* through the inhibition of RBC lysis induced by *Najanaja* and *Daboiarusselli* venom and the results were shown in figure-1. *Najanaja* and *Daboiarusselli* venom-induced hemolysis in a dose-dependent manner and minimum indirect hemolytic concentration of *Najanaja* and *Daboiarusselli* venom was established at 4µg and 6µg respectively. However, methanolic extracts of *O.C* inhibited *Najanaja* and *Daboiarusselli* venom-induced hemolytic activity by 88 % and about 95% inhibition at 1:100 w/w and more than 50% inhibition observed at 1:40 w/w and 1:60 w/w concentration respectively for *Najanaja* and *Daboiarusselli* venom. Other solvents extracts of plants such as chloroform, ethyl acetate, Petroleum ether, and hexane extracts did not show significant inhibition of the hemolytic activity of venom even at 1:100 w/w results are shown in figure 1a and 1b.

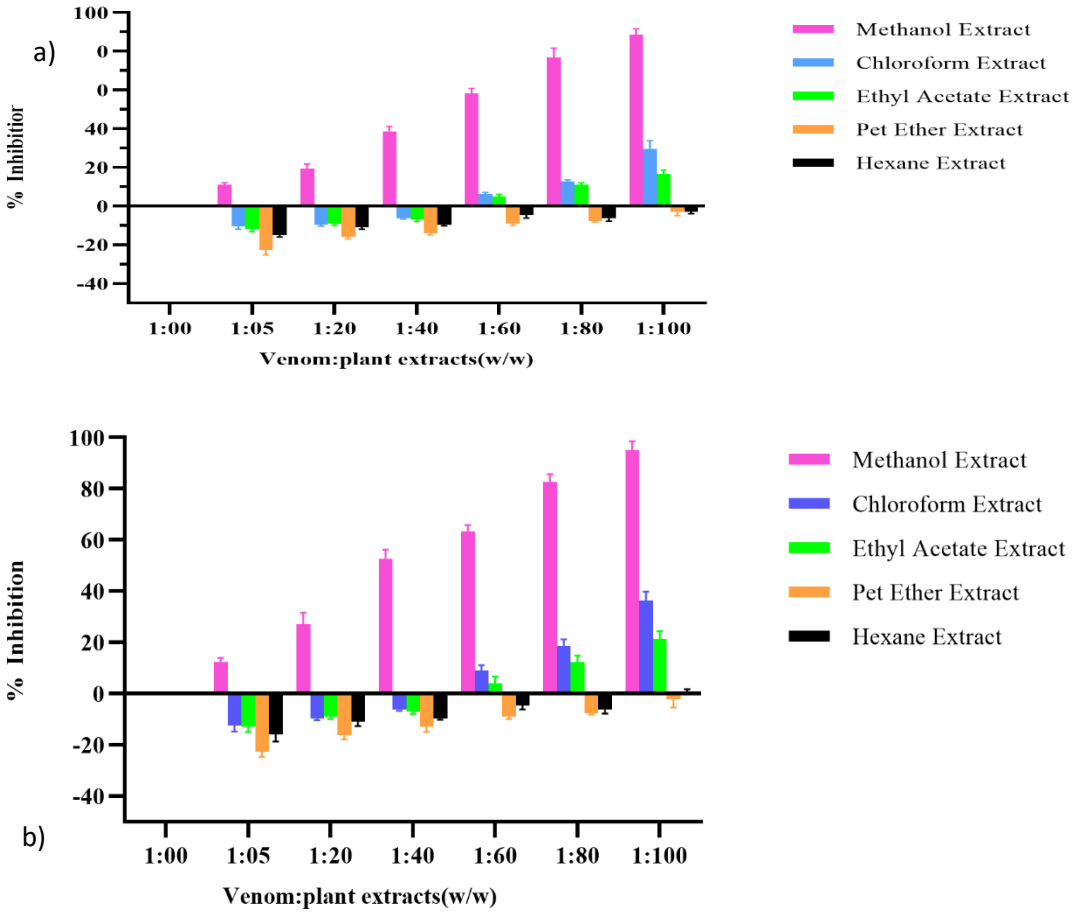

Figure-1: Effect of Methanol, chloroform, ethyl acetate, Pet ether, and hexane extracts against hemolysis induced by a) *Najanaja* b) *Daboiarusselli* venom.Results are shown as mean ± SEM

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Phospholipase (PLA_2) inhibition

Results of Phospholipase A_2 inhibition by the different solvents extracts against *Najanaja* and *Daboiarusselli* venom are depicted in Figures 2a and 2b respectively. At 1:80 w/w concentration methanolic extract inhibited PLA_2 activity by 84 % and 75% and at the same concentration chloroform extract has shown inhibition of about 44%and 45% respectively for *Najanaja* and *Daboiarusselli* venom.

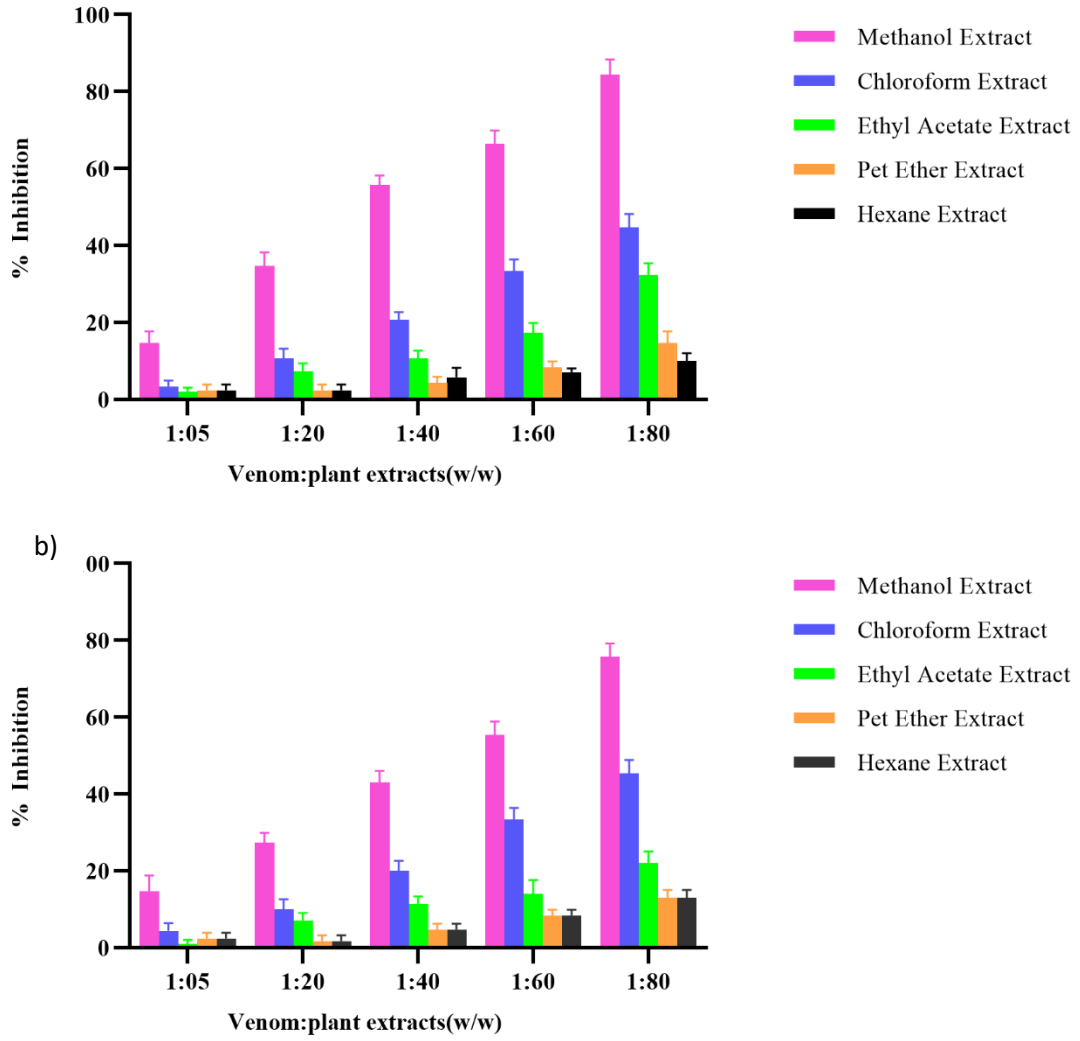

Figure-2: Effect of Methanol, chloroform, ethyl acetate, Pet ether, and hexane extracts against PLA2 Activity induced by a) *Najanaja* b) *Daboiarusselli* venom. Results are shown as mean ± SEM

Hyaluronidase inhibition

Hyaluronidase activity inhibition by different plant extracts is depicted in Figures 3a and 3b respectively for *Najanaja* and *Daboiarusselli* venom. Methanolic extract of plant offered about 85.6% and 65 % inhibition against hyaluronidase activity induced by *Najanaja* and *Daboiarusselli* venom respectively. At 1:80 w/w chloroform and ethyl acetate extracts could able to offer only about 38.33% and 55% inhibition respectively for *Najanaja* venom and 35 % each extract for *Daboiarusselli* venom. Other extracts did not show significant inhibition against hyaluronidase activity of venom

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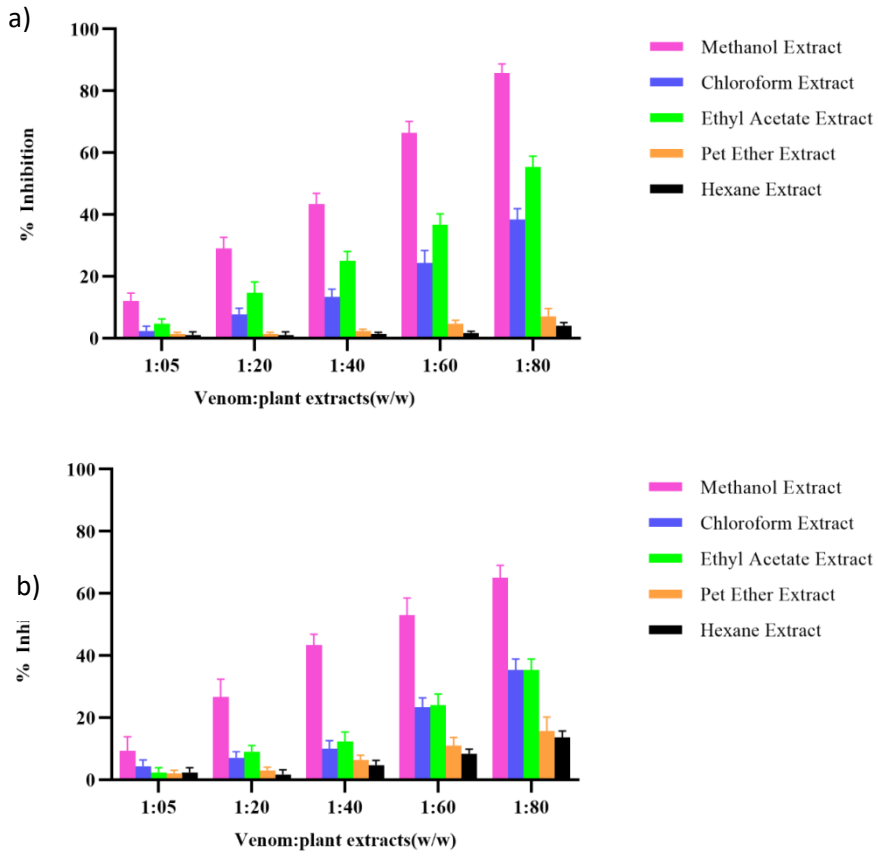
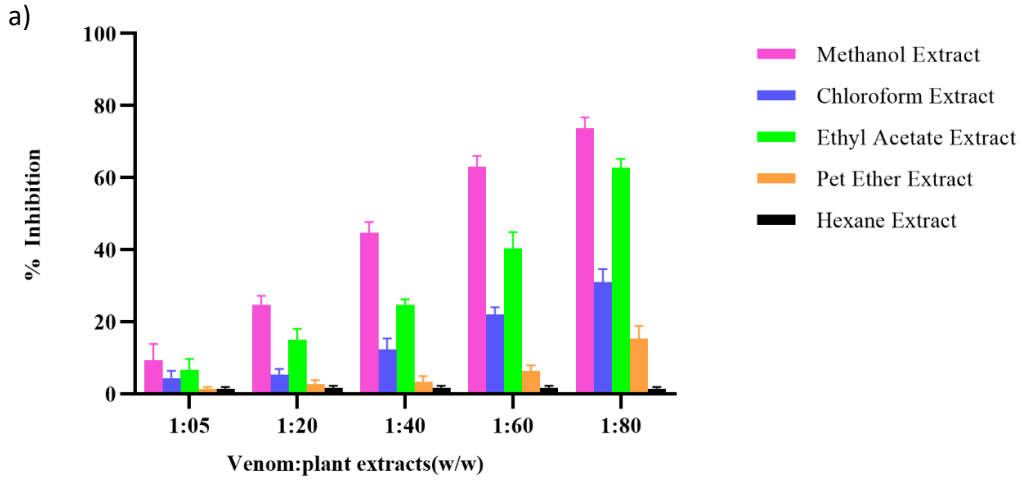


Figure-3: Effect of Methanol, chloroform, ethyl acetate, Pet ether, and hexane extracts against Hyaluronidase induced by a) *Najanaja* b) *Daboiarusselli* venom. Results are shown as mean ± SEM

Acetylcholinesterase

Acetylcholinesterase activity inhibition by methanolic extract of *O. C was* 73 % and 84% at 1: 80 w/w Concentration respectively for *Najanaja* and *Daboiarusselli*venom. Whereas, the same concentration of ethyl acetate extract inhibited AChE activityby 62% of *Najanaja*venom and 56 % of *Daboiarusselli* venom. Results are shown in Figures 4a and 4b.



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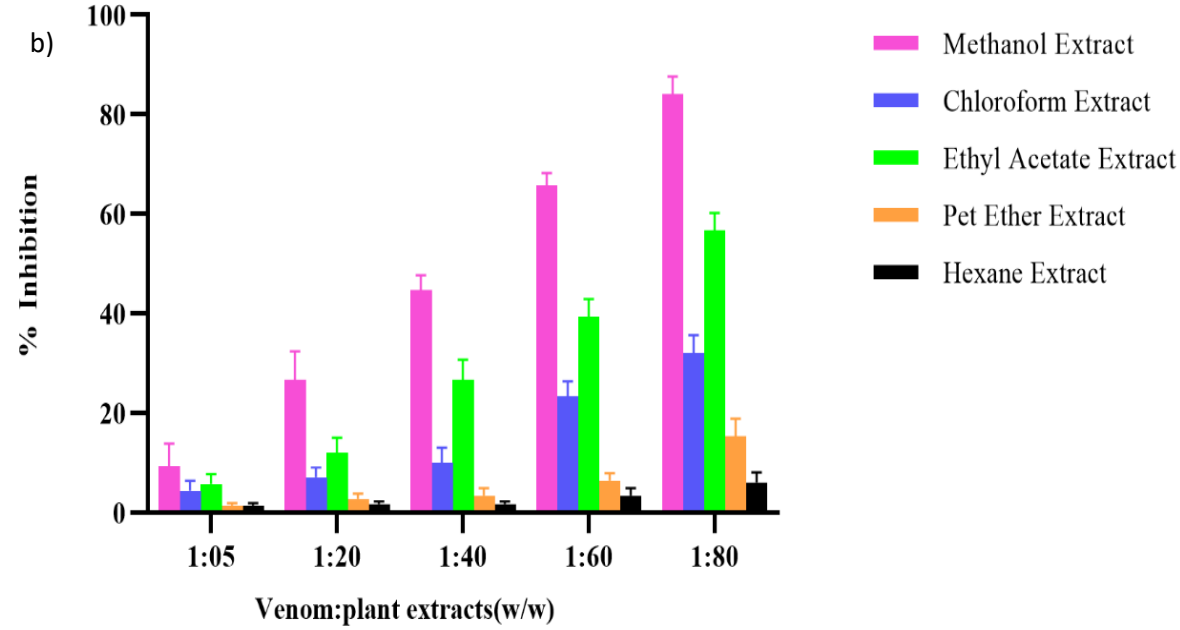
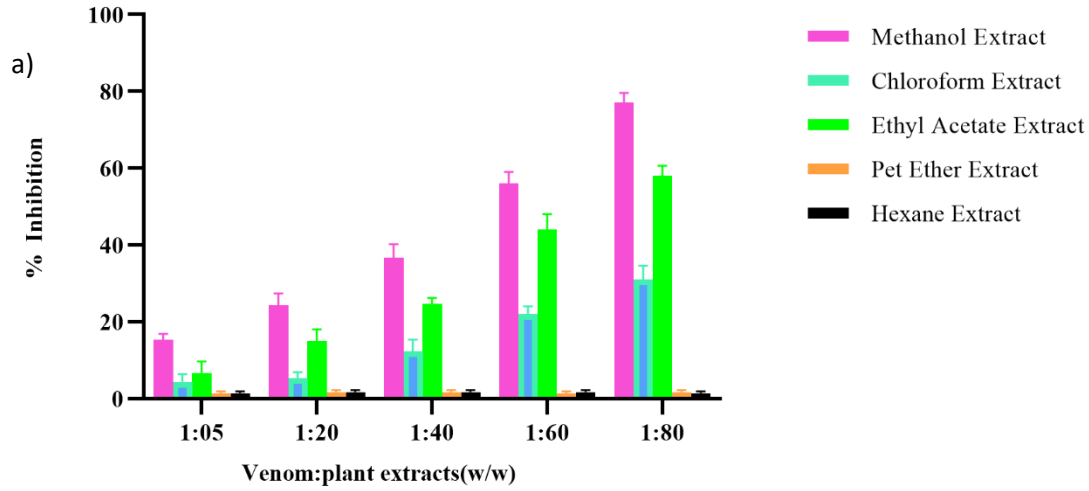


Figure-4: Effect of Methanol, chloroform, ethyl acetate, Pet ether, and hexane extracts against Acetylcholinesterase activity induced by a) *Najanaja* b)*Daboiarusselli* venom. Results are shown as mean ± SEM

Caseinolytic activity.

Protease activity of *Najanaja* and *Daboiarusselli* venom are inhibited by methanolic extracts of O.C by more than 77% and 95% at 1:80 w/w concentration respectively. The methanolic extract was able to inhibit protease activity of *Najanaja* and *Daboiarusselli* venom by more than 50% at 1:20 w/w concentration. Ethyl acetate extract inhibited *Najanaja* and *Daboiarusselli* venom protease activity by 62% and about 58% respectively at 1:80 w/w concentration. Results are shown in Figures 5a and 5b. Chloroform, petroleum ether and hexane extract couldn't able to inhibit protease activity significantly.



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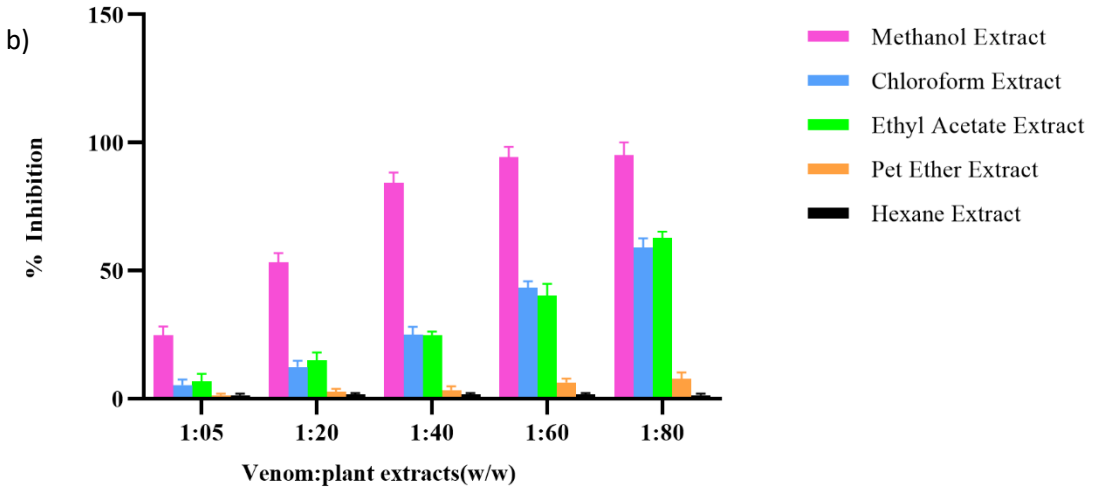

Figure-5: Effect of Methanol, chloroform, ethyl acetate, Pet ether, and hexane extracts against Protease activity induced by a) *Najanaja* b) *Daboiarusselli* venom. Results are shown as mean ± SEM

Discussion:

The search for novel natural molecules capable of inhibiting the local damage caused by snake envenomation is a promising field of research. Plant extracts provide an alternative for the treatment of snakebites, plants have active compounds with many biological activities, and many such plants have been used traditionally to treat snakebites [38]. In some cases, plant bioactive molecule has proved with scientific validation for its anti-venom activity. The ability of plant extracts to neutralize venom has been attributed to the presence of plant "secondary metabolites" capable of binding venom proteins and inhibiting enzyme activity [39 to 42].

In our present study, preliminary phytochemical analysis of *O corniculata* revealed the presence of secondary metabolites such as flavonoids, tannins, phenols, carbohydrates, proteins, and alkaloids and earlier reports have also suggested that these metabolites will interact with toxic proteins and inhibit enzymes of snake venom particularly PLA_2[43].

Snake venoms are a complex mixture of toxins that shows the biological and biochemical potential of proteolytic, hemolytic, phospholipase activities. Hemolysis is one of the significant lethal effects of snakebite, this effect is due to the presence of phospholipase and hemolysin proteins [44] which act on phospholipids to liberate lysolecithin. Lysolecitihin in turn acts on the membrane of RBC causing hemolysis [45]. In our present study methanolic extract of *O corniculata* interacted with both *Najanaja* and *Daboiarusselli* venom proteins and stabilized the cell membranes of HRBC and protected it from lysis significantly (figure 1). However similar kinds of protection were observed in methanolic extracts of Euphorbia hirta[46], LeucasAspera [47], and Azimatetracantha ethyl acetate extracts [48].

Snake venoms are rich sources of PLA_2 enzymes and often contain a large number of isozymes. So far, several hundred snake venom PLA_2 enzymes have been purified and characterized [49]. Phospholipase A_2 is one of the most common and abundant multi-toxic protein presents in both *Najanaja* and *Daboiarusselli* venom, which brings many pharmacological effects such as edema activity, myotoxicity, neurotoxicity, and cardiotoxicity upon envenomation [50 to 53]. Methanolic extracts of *O.C* inhibited PLA_2 activity by 84% and 75% of its activity respectively for *Najanaja* and *Daboiarusselli* venom (figure 2). Similarly, the aqueous stem bark extract of *Mangifera indica* [54,55],

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*Andrographispaniculata*and *Aristolochia Indica* plant extracts [56]showed significant inhibition of PLA_2.

Hyaluronidases play a crucial role in spreading the toxic venom components from the bite site to the systemic circulation and eventually to multiple tissue targets. they degrade the connective tissues surrounding the blood vessels, capillaries, and smooth muscle resulting in loss of structural integrity [57,58]. hyaluronidase inhibition is important in the treatment of snakebite. in the present study methanolic extract of *O.C* inhibited hyaluronidase activity by 85.6 % and 65 % for *Najanaja* and *Daboiarusselli* respectively (figure 3). Similar kinds of inhibitions were observed in Mimosa pudica root extract [59] and Albizialebbeck seed methanolic extract [60]. Acetylcholinesterase has a neurotoxic effect, it is more profound in elapids, very little, or no activity is observed in viperids. However, the plant extracts were able to antagonize the activity exhibited by the venom by 84% and 75% (figure 4).

Proteases on the other hand are responsible for the blood coagulation and hemostatic plug formation and degradation of the extracellular matrix components of the victims of snakebite [61]. About 77% and 95 % of caseinolytic Inhibition was observed at 1:80 w/w concentration of methanolic extract of *O.C*(figure 5)and similarly, Vitexnegundo leaves extract[62], Pergulariadaemia (Forsk.) chiov.[63] showed protease inhibition activity, The inhibitory activities of plant extracts against snake venoms should be further investigated by in vivo studies using animal models and by pharmacological analysis in vitro. The authors believe that these plant extracts could lead to the development of new drugs against the venom-induced morbidities of *Najanaja* and *Daboiarusselli* snakes.

Conclusion

In conclusion, in this study, we for the first time present demonstrated the inhibitory potential of different solvent extracts of O Corniculata on toxic enzymes of *Najanaja* and *Viperarusselli* Venom. Further investigation on the potent solvent extract of the plant could help in unraveling the lead molecule that could be used to treat snake envenomation and its specific inhibitory mechanism.

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