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Inhibition of Viper Venom-Induced Toxicity by *Moringa oleifera* Leaves Extracts

Asha B. Thomas[1], Snehal Karanjkhele[1], Nitin Salvi[2], Lata Kothapalli[1], Aaftab Shaikh[1], Seema Kamble[1]

[1]Department of Pharmaceutical Chemistry, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, India, [2]Department of Quality Control, Premium Serums and Vaccines Pvt. Ltd., Pune, Maharashtra, India

Corresponding Author:
Dr. Asha B. Thomas,
Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune - 411 018, Maharashtra, India.
Mobile: +91-9881236220.
E-mail: asha.thomas@dypvp.edu.in

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ABSTRACT

Snakebite, a medical emergency, is a socially significant but neglected issue in India. Antivenom serum therapy that is currently used against venomous snakebites is expensive and showed severe side effects. Traditional claims reported the use of *Moringa oleifera* to treat snakebite in rural India. To scientifically validate these claims, in the present investigation, the antivenom potential of the extracts of *M. oleifera* was investigated. The extracts effectively neutralized Russell's viper venom-induced lethality ($LD_{50:}$ 10.9 µg) at 10 µg/mice and 300 µg/mice (ED_{50}), respectively. Both extracts neutralized venom-induced hemolysis (50–100 µg). Inhibitory concentrations against phospholipase A_2 activity exhibited by aqueous and methanolic extracts were found to be 0.06 mg and 0.07 mg, respectively. In the procoagulant activity inhibition studies, the ED values in neutralizing effect of saw-scaled viper venom were found to be 1 µg for both the extracts. The presence of phenolics, flavonoids, and saponins in both the extracts was confirmed through phytochemical and high-performance thin-layer chromatography investigations which supported the obtained activity. This study indicated the potential of *M. oleifera* extracts to neutralize toxins present in *Daboia russelii* and *Echis carinatus* venom. Further bioactivity-guided fractionation of extracts to identify bioactives involved in neutralization of snake venom is necessary to establish its therapeutic potential as a venom antidote.

Keywords: *Moringa oleifera,* viper venoms, phospholipase, hemolytic, procoagulant, high-performance thin-layer chromatography

INTRODUCTION

Snakebite is a medical emergency and a socially significant issue in India, yet it is one of the most neglected public health issues, especially in poor rural communities.[1] The global burden of snakebite is estimated to be about 1.2–5.5 million snakebites, with 94,000 deaths occurring annually.[2,3] The national representative snakebite mortality survey carried out in India, indicated that about 35,000–50,000 deaths are reported every year, which signified that the death rate associated with snakebites is a serious epidemiology.[4,5] Due to the high mortality in India, the World Health Organization (WHO) has called for a global effort against "envenoming."

Snake venom is a complex mixture of enzymes, carbohydrates, proteins, and other small molecules. There are several enzymes present in venom, of which phospholipase is reported to be the most important in developing effective antidotes.[6,7] Phospholipases are predominantly present in Russell's viper (*Daboia russelii*) and saw-scaled viper (*Echis carinatus*) which are the most common poisonous snakes found in India. These multifunctional enzymes degraded membrane phospholipids, releasing arachidonic acid, the precursor molecule in mediating inflammation by the cyclooxygenase or lipoxygenase pathways. Since 1895, the only specific treatment available for snakebites is the animal derived polyclonal anti-snake venom (ASV) therapy. However, large seasonal and biochemical variations in the venom composition of snakes found in different regions, indicated that ASV developed against the four major venoms, that is, Cobra, Krait, Russell's viper, and saw-scaled viper is not always effective in the treatment of snakebite victims.[8] In addition to being an expensive therapy, several side effects such as anaphylactic shocks, pyrogen reactions, and serum sickness are also reported in ASV-treated victims.[9] Moreover,

because of the limited manufactures, the quantity of ASV produced is not sufficient to meet the growing demand. Due to these limitations of ASV therapy, there is a growing interest in exploring snake venom antidotes derived from plant sources. As per the WHO estimates, about 80% of the population, especially in rural areas, depend on herbal medicines to meet their primary healthcare needs due to their low cost and availability.[10] According to an ethnobotanical survey carried out by Samy *et al*. (2008), there are about 54 million indigenous people of different ethnic groups living in India using medicinal plants for the treatment of different ailments, including snakebites.[11]

Traditional herbal medicines prepared from medicinal plants, employed either alone or in combination are used as an antidote for snakebite by tribal and rural populations in many parts of the world, especially in tropical and subtropical countries including India. Various phytoconstituents such as phenolics, flavonoids, tannins, alkaloids, triterpenoids, steroids, and amides are reported to be active against snake envenomation.[12] However, in most cases, the efficacy of these traditional treatment regimens is unproven. Thus, the systematic and scientific study of herbal antidotes against snake venom is of great importance in the management of snakebite. *Moringa oleifera* (family – Moringaceae) is a "miracle tree" as it is an unbelievable source of all nutrients, macro- and micro-elements, amino acids, dietary fibers, and minerals. Goyal *et al.* have reported that the leaves of *M. oleifera* contain several phytoconstituents such as nitrile glycosides, mustard oil glycosides, glucosinolates, and phenolics including flavonoids.[13] It is widely cultivated throughout India and is used extensively as a food source and nutritive herb. *M. oleifera,* commonly called as drumstick tree, is reported to possess a variety of pharmacological activities. A study demonstrated that aqueous fraction of *M. oleifera* significantly enhanced proliferation, viability, and migration of human dermal fibroblast cells showing promising wound healing activity. Literature suggested that it exhibited detoxification properties; thereby, it can be used for the treatment of snakebite and scorpion bite.[13,14] Literature reports also suggested that various parts of *M. oleifera* are used to treat snakebite in several rural regions of India since ancient times.[15-17] However, there are no reports on any scientific study to support these traditional claims. Hence, in the present study, attempts have been made to evaluate the snake venom neutralization potential of different extracts of *M. oleifera* against viper venoms (Russell's viper and saw-scaled viper) [Figure 1].

MATERIALS AND METHODS

Plant collection and authentication

The plant species of *M. oleifera* (Moringaceae) was collected from local regions of Pune, Maharashtra, India, identified, and authenticated by Botanical Survey of India, Pune, India (Voucher specimen No.SSK-1). The leaflet of the plant was used in the present study.

Plant extraction

The leaflets of the plant were collected and shade dried. The dried powdered plant material (50 g) was successively macerated at room temperature with distilled water and methanol (500 ml each) for 24 h and 48 h, respectively. The aqueous (MO-A) and methanolic extracts (MO-M) were vacuum dried (Lab line) under pressure at 40ºC to obtain dry extracts, kept in stock vials and stored in refrigerator (0–4°C) until further use. For *in vitro* and *in vivo* studies, known quantity of extracts was diluted in phosphate buffer saline solution (PBS pH 7.4). The clear supernatant was used for further studies.

Venom and chemicals

Lyophilized snake venoms of *D. russelii* and *Echis carinatus* were procured from Haffkine Biopharmaceutical Cooperation Ltd., Pune, India, and were stored at 4ºC. It was diluted in 0.9% w/v of NaCl to obtain a concentration of 1% w/v (10 mg/ml), then centrifuged at 2000 rpm for 10 min. The supernatant was used as venom and stored at 4ºC until further

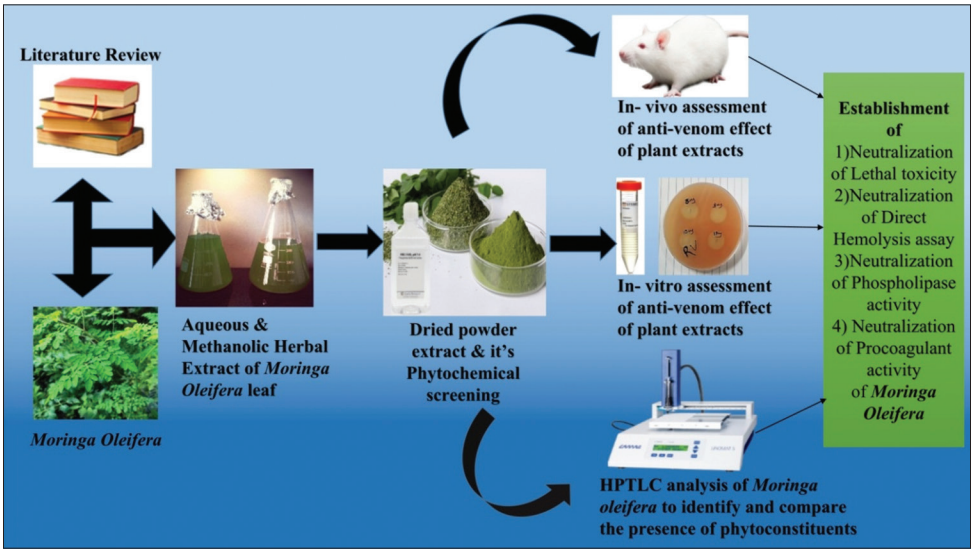

Figure 1: Flowchart depicting inhibitory effect of *Moringa oleifera* extracts on toxicity induced by viper venoms

use. All reagents and solvents used in the study were purchased from Qualigens Fine Chemicals, Mumbai, India, and HiMedia, Mumbai, India.

Preliminary phytochemical screening

Preliminary phytochemical screening of the extracts was performed to identify the presence of various phytoconstituents using standard procedures.

The presence of alkaloids was confirmed by Dragendorff's test (orange-red precipitate indicates presence of alkaloids); tannins by the ferric chloride test (appearance of a dark green color confirms the presence of tannins); Shinoda test for flavonoids (formation of pink color indicates presence of tannins); and Liebermann's test for glycosides (change in color from blue to green indicates the presence of glycosides); foam test to identify saponins (presence of saponins confirmed by foam formation); Molisch's test to identify carbohydrates (appearance of a purple or blue ring indicates the presence of carbohydrate); and sulfuric acid test for steroids (formation of red color in organic layer confirms the presence of steroids).

In vivo assessment of *Decapterus russelli* venom toxicity and antivenom effect of plant extracts

The pre-clinical testing of the extracts of *M. oleifera* for antivenom potential was evaluated as per the WHO guidelines for the production, control, and regulation of snake antivenom immunoglobulins, Annex 5.

Animals

The experimental protocol was approved by the Institutional Animal Ethics Committee (DYPIPSR/IAEC/18-19/P-27), Pune, India. Swiss albino mice of either sex (Crystal Biological Solutions, Pune, India), body weight between 25 and 30 g, were used in the study. Animals were housed in polypropylene cages with autoclaved clean rice husk as bedding material. They were maintained under favorable conditions at 22 ± 3ºC, relative humidity of 30–70%, and 12 h light/dark cycle including air change of minimum 15 changes/h. All animals were acclimatized to laboratory conditions before start of experimentation. The animals were fasted overnight before the study but were provided water *ad libitum*.

Neutralization of lethal toxicity

The median lethal dose (LD_{50}) of *D. russelli* venom was determined according to modified method developed by Theakston and Reid.[18] Various doses of venom (5–15 µg) in 0.3 ml of physiological saline were injected into tail vein of mice ($n = 5$). The LD_{50} was calculated by the analysis of deaths occurring within 24 h of venom injection (confidence limit at 50% probability). The anti-lethal potentials of extracts were determined against 2-fold LD_{50} of venom. Various amounts of plant extracts (µl) were mixed with 2-fold LD_{50} of venom, incubated at 37°C for 30 min. Then, 0.3 ml of these mixtures was injected intravenously into mice. Control mice received same amount of venom without antivenom (plant extracts). Median effective dose (ED_{50}) was calculated and expressed as µg antivenom/mouse.[19]

In vivo assessment of *D. russelli* venom toxicity and antivenom effect of plant extracts

Neutralization of direct hemolysis assay

The hemolytic action of venom and *M. oleifera* extract mixtures was studied *in vitro* as previously described James *et al*. (2013) using mice red blood corpuscles (RBC).[20] Negative control (physiological saline [5 ml] and RBC [0.5 ml]); positive control ([100% hemolysis] and DW [5 ml] with RBC [0.5 ml]); and samples (venom/extracts [5 ml] and RBC) were employed. Tubes were transferred to thermostat maintained at 37°C for 1 h, then centrifuged at 2000 rpm for 20 min. The supernatant was decanted into separate tubes and optical density was measured using spectrophotometer (Shimadzu 1800) at 540 nm against water. The percentage of hemolysis was calculated using the formula:

$$\% \text{ hemolysis} = \frac{\text{OD Experimental sample} - \text{OD negative control}}{\text{OD positive contro}} \times 100$$

Neutralization of phospholipase activity (indirect hemolysis assay)

Phospholipase A2 (PLA2) activity was measured using indirect hemolytic assay on agarose- erythrocyte-egg yolk gel plate by method described by Gutierrez *et al.,* 1988.[21] Literature reports suggest that PLA2 present in venom can act through various mechanisms. This includes inhibition of the release, synthesis, storage, or turnover of neurotransmitters. Some PLA2 can damage skeletal muscles. PLA2 present in some venom through the formation of lysolecithin from extra erythrocyte lecithin sources can induce indirect hemolysis.[22] Increasing doses of Russell's viper venom (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 $CaCl_2$. Plates were incubated overnight at 37°C and diameters of hemolytic halos were measured. The minimum indirect hemolytic dose (MIHD) corresponds to the dose of venom producing hemolytic halo of 11 mm diameter. The efficacy of extracts in neutralizing the phospholipase activity was carried out by incubating constant amount of venom (µg) with different amounts of plant extracts (µl), incubated for 30 min at 37°C for 20 h. Neutralization of phospholipase was expressed as ratio mg antivenom/mg venom able to reduce diameter of hemolytic halo by 50% when compared to the effect induced by venom alone.[23]

Neutralization of procoagulant activity

The pro-coagulant activity was carried out according to the method described by Theakston and Reid modified by Laing *et al*., 1992.[24] Initially, minimum coagulant dose (MCD) of *E. carinatus* venom was determined as the venom concentration which induced clotting of plasma within 60 s. In the neutralization assays, constant amount of venom was mixed with various dilutions of plant extracts, incubated for 30 min at 37°C. Then, 0.1 ml of mixture was added to citrate plasma (0.3 ml) and clotting times were recorded. Neutralization was expressed as ED, defined as ratio of µL antivenom (extracts)/mg venom which increased the clotting

time 3 times when compared with clotting time of plasma incubated with 2-fold MCD of venom alone.[25]

High-performance thin-layer chromatographic (HPTLC) analysis of extracts

The HPTLC analysis of the leaf extracts of *M. oleifera* was carried out to identify and compare the presence of phytoconstituents in aqueous and methanolic extracts. The densitograms obtained were compared with commercially available marketed formulation of *M. oleifera* (Shigru Capsules, The Himalaya Drug Company, Bengaluru, India) to confirm that the extracts employed in the study are comparable to the marketed formulation.

The HPTLC analysis was carried out using aluminum plates pre-coated with silica gel 60 F254 (E. Merck, Darmstadt, Germany; Merck India, Mumbai, India). Camag Linomat V sample applicator (Muttenz, Switzerland) with Camag TLC scanner III densitometer (620 mm × 620 mm × 345 mm) operated in reflectance-absorbance mode fitted with grating type of monochromator at a scanning speed of 5–100 mm/s was used in the study. The source of radiation used was deuterium, halogen tungsten, and mercury vapor lamps emitting continuous ultraviolet spectrum between 190 and 800 nm (wavelength accuracy ± 1 nm).

Preparation of extract stock solution A and B

Accurately weighed quantity (5 mg) of aqueous and methanolic extract of *M. oleifera* (MO-A) was transferred to separate 10 ml volumetric flask respectively, 2 ml of distilled water was added, ultrasonicated for 10 min, the volume was then made up to the mark with methanol (50 µg/µl).

Preparation of stock solution of capsule formulation C

Accurately weighed quantity (8.03 mg of capsule powder which is equivalent to 5 mg of extract) of marketed formulation of *M. oleifera* was transferred to a 10 ml volumetric flask, 2 ml distilled water was added, ultrasonicated for 10 min, the volume was then made up to the mark with methanol (50 µg/µl).

Optimization of chromatographic conditions

Aliquot portions of stock solutions A, B, and C, 20 µl each, were applied on TLC plates (band size: 6 mm) and plates were developed in twin-trough glass chamber saturated with different solvent systems (toluene: ethyl acetate; toluene: methanol; ethyl acetate: methanol; and toluene: ethyl acetate: methanol) for 20 min with densitometric scanning at varied wavelengths to determine the best conditions for effective separation of the phytoconstituents present in the extracts. The solvent phase consisting of toluene: ethyl acetate: formic acid (5:4:0.5 v/v/v) gave relatively well-separated sharp bands at 294 nm. Further analysis was carried out under the optimized conditions.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The preliminary phytochemical investigations of the extracts of *M. oleifera* leaves indicated the presence of phenols, flavonoids, alkaloids, saponins, glycosides, and carbohydrates in both MO-A and MO-M extracts. However, the MO-A extract also showed positive test for tannins while the MO-M extracts exhibited positive for steroids. Makhija *et al.* (2010) have reported that phytoconstituents such as tannins, steroids, amides, flavonoids, phenols, and alkaloids are active against snake envenomation.[16] As majority of these constituents are present in the extracts, they may prove effective against snake venom toxicity.

In vivo assessment of *D. russelii* venom toxicity and antivenom effect of plant extracts

Neutralization of lethal toxicity

The summary of the *in vivo* and *in vitro* testing is summarized in Table 1. The LD_{50} of *D. russelli* venom was assessed using Balb/c strain mice. The mean LD_{50} in the tested mice was 10.9 µg (11 µg). The neutralization of lethality was carried out by mixing 15 µg of venom (2-fold LD_{50}) with 5 µg–300 µg concentrations of the extracts of *M. oleifera* which showed that 10 µg of MO-A and 300 µg MO-M extracts were able to completely neutralize the lethal effect (2-fold LD_{50}) of *D. russelli* venom [Table 1]. The MO-A extract showed neutralization at lower concentrations compared to MO-M extract which may be due to the improved solubility of aqueous extract, leading to increased plasma concentrations, resulting in higher potency of phytoconstituents that may be responsible for activity. Furthermore, the presence of tannins in aqueous extract may have contributed to this increased activity.

In vivo assessment of *D. russelii* venom toxicity and antivenom effect of plant extracts

Neutralization of hemolytic activity

In this assay, *a*queous and methanolic extracts of *M. oleifera* were able to neutralize the hemolysis of RBCs produced by *D. russelii* venom (34.52%) up to 22.1% and 0.35%, respectively [Table 1]. The viper venoms are able to induce hemolysis due to the presence of PLA2 and more specific cardiotoxins and cytotoxins. The MO-M extract was able to effectively protect the RBC from direct hemolysis probably due to the presence of phytoconstituents such as flavonoids and steroids present in the extract which inhibits these toxic proteins.

Neutralization of phospholipase activity

In the indirect hemolysis assay, evaluated by neutralization of phospholipase activity, *D. russelii* venom was effectively able to produce hemolytic halo in agarose-sheep erythrocytes gels. The MIHD was set at10 µg of venom (produced halo of 16 mm diameter). The aqueous and methanolic extracts of *M. oleifera* (concentration ranged from 5 to 80 µg) effectively inhibited the PLA2 activity as demonstrated by significant reduction in the diameters of haloes [Figure 2]. MO-A significantly reduced the halo diameter from 16 mm to 8 mm at the concentration of 0.06 mg while MO-M extract produced similar effect at 0.07 mg [Table 1]. Both

the extracts showed effective neutralization of PLA_2. Lindahl and Tagesson (1997) reported that quercetin selectively inhibits Group II PLA_2 activity which is present in viper venoms at lower concentrations.[26] In addition, Gil *et al.* (1994) also reported that kaempferol-3-O-galactoside inhibited human recombinant synovial PLA_2 with low IC_{50} values (12.2–17.6 μM).[27] The leaf extracts of *M. oleifera* are reported to contain quercetin-3-O-glucoside, quercetin-3-O-(6″-malonyl-glucoside), and kaempferol-3-O-(6″-malonyl-glucoside), which may have contributed significantly for the PLA_2 inhibitory activity.[28]

Neutralization of procoagulant activity

The MCD of *E. carinatus* was found to be 2 μg which resulted in clotting of human citrate plasma in 60 s [Table 1]. In neutralization assay, the ED values of aqueous and methanolic extracts were found to be 1 μg for both the extracts [Table 1]. The extracts when tested in increasing concentration were able to effectively increase the clotting time indicating its capacity to effectively neutralize the coagulation activity of *E. carinatus* venom.

HPTLC analysis

In the HPTLC analysis of extracts and marketed formulation of *M. oleifera* carried out under optimized conditions, the densitograms obtained showed comparable pattern of peaks observed at specific R_f values for both extracts. Among multiple peaks obtained, peaks at similar R_f values were observed at 0.12, 0.24, 0.30, 0.39, 0.46, and 0.49 for MO-A; 0.11, 0.13, 0.29, 0.35, 0.43, and 0.48 for MO-M, and 0.11 and 0.21 for marketed formulation on spraying with aluminum chloride. As aluminum chloride is used for the detection of flavonoids, the presence of these peaks suggests the presence of flavonoids in the extracts [Figure 3]. The densitograms recorded using phosphomolybdic acid which is a general locating reagent showed the presence of several peaks at similar R_f values for both the extracts and marketed formulation. The presence of these peaks at similar R_f values indicated that both the extracts and the marketed formulation may contain some of the reported phytoconstituents that may be effective against snake envenomation.

Table 1: *In vivo* and *in vitro* testing of extracts of *Moringa oleifera* against *Daboia russelii* and *Echis carinatus*

Extract	ED50 (mg)*	Antihemolytic activity*	PLA2 inhibition*	Prolongation of coagulation activity**
	Dose of venom (2LD50: 15 μg)	% hemolysis	Halo diameter (mm)	Clotting time
MO-A	10 μg	22.1 (100 μg)	7 mm (60 μg)	1 min 28 s (1 μg)
MO-M	300 μg	0.35 (100 μg)	7 mm (70 μg)	4 min 10 s (1 μg)

MO-A: Aqueous extract; MO-M: Methanolic extract, *activity against *Daboiarusselii*; **activity against *Echis carinatus*

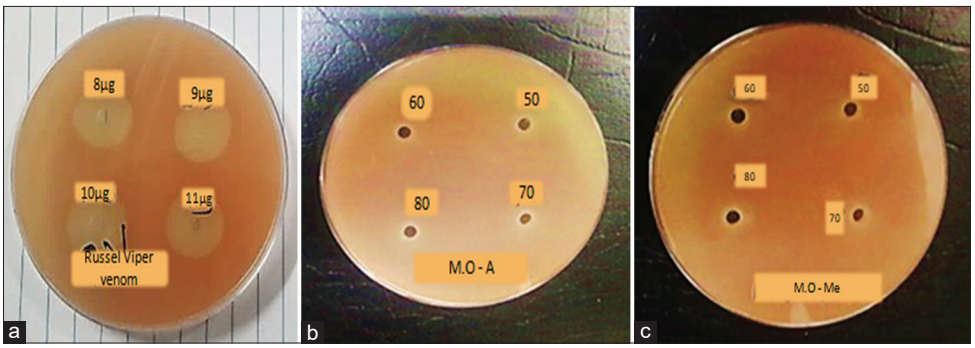

Figure 2: Reduction in *Daboia russelli* venom-induced hemolytic haloes on treatment with *Moringa oleifera* aqueous extract. (a) Haloes with different concentrations of *D. russelii* venom; (b) reduced haloes due to increasing concentration of MO-A. (c) Reduced haloes due to increasing concentration of MO-M

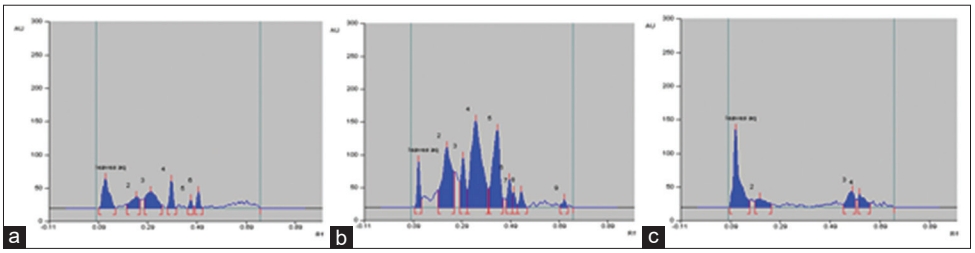

Figure 3: Densitograms of *Moringa oleifera* when stained with 1% ethanolic solution of aluminum chloride (for flavonoids): (a) MO-A, (b) MO-M, and (c) marketed formulation

CONCLUSIONS

In the present study, an attempt has been made to evaluate the leaves extracts of *M. oleifera* for the treatment of snakebite. The preliminary phytochemical investigations supported by HPTLC study indicated that both aqueous and methanolic extracts contain phytoconstituents such as phenolics, flavonoids, saponins, alkaloids, and glycosides. The *in vivo* lethal toxicity assay demonstrated that the aqueous extract at a dose of 10 μg in mice effectively neutralized the venom-induced toxicity. Furthermore, the extracts reduced the venom-induced hemolysis and showed the dose-dependent inhibitory ability toward PLA_2-dependent hemolysis. *Echis*-induced procoagulant activity was also found to be antagonized due to the treatment of same extracts. As the extracts show promising dose-dependent PLA2 inhibitory activity, it is anticipated to effectively act as an antidote through this mechanism.

In conclusion, the result from this study indicates that *M. oleifera* extracts can effectively neutralize the toxins present in *D. russelii* and *E. carinatus* venoms. Further *in vitro* and *in vivo* studies and bioactivity-guided fractionation of the extracts need to be carried out for the isolation, purification, and identification of bioactive components that are involved in neutralization of the snake venom toxicity to establish its therapeutic potential as a venom antidote.

FUNDING

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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