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Neutralization of *Naja naja* venom induced lethality, edema and myonecrosis by ethanolic root extract of *Coix lacryma-jobi*

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ABSTRACT

Coix lacryma-jobi, commonly known as job's tear, is a tall grain-bearing tropical plant of the family *Poaceae*. The ethanolic root extract (ERE) of the plant was investigated for the first time for anti-venom activity against Indian cobra *Naja naja* venom.

In-vitro studies were conducted to determine neutralization of phospholipase A_2 (PLA_2) activity of the *Naja naja* venom by the ERE. ERE showed significant inhibition of PLA_2 activity, which was further confirmed from effective neutralization of human red blood cells (HRBC) lysis induced by the venom. In addition, venom-induced proteolysis, fibrinogenolysis, DNase activity were also neutralized by the ERE, which contained carbohydrates, glycolides, resins and tannins.

Oral administration of ERE at doses levels 100, 200 and 400 mg/kg effectively inhibited *Naja naja* venom-induced lethality in mice. Myotoxicity induced by *Naja naja* venom, measured by creatine kinase activity in rats was significantly neutralized by the ERE at a dose of 200 mg/kg.

Stigmasterol, as one of the component isolated from the ERE, was found to have venom phospholipase A_2 inhibition potential, which was confirmed by molecular docking studies with PLA_2. In summary, these studies indicate the ability of ERE of *Coix lacryma-jobi* to effectively neutralize the toxic effects of the venom is, in part, contributed by the inhibition of PLA_2 activity among other venom-derived factors.

1. Introduction

Focal community studies in Africa and Asia indicated 4–162 snake bite deaths per 100,000 people per year [1]. Recently published study [2] estimated annual snake bite mortality in India was around 46000. The snakes commonly associated with human mortality in India are cobra (*Naja naja*), krait (*Bungarus caeruleus*) Russell's viper (*Daboia russelli)* and saw scaled viper (*Echis carinatus*) [3]. More recent study reports that, most of the victims of snake bite are the people working in agricultural fields in the rural areas and administration of antivenom could be done nearly 4–5 h after the snake bite on an average [4]. In ancient Indian books, there are many plants recommended for use in snakebite therapy. Some of these are popularly used by snake handlers and by practitioners of traditional medicine in India for treating snakebite patients, but without any scientific validation. Therefore, this type of treatment remains questionable and needs thorough scientific investigation and validation [5].

Root extract of *Coix lacryma-jobi* (family–Poaceae) is commonly used for treatment of snake bites by the traditional healers of south India, in particular coastal Karnataka. However, there is no literature available regarding mechanistic underpinning of its benefits *in-vivo* and *in-vitro*. In an effort to bring this traditional medicine to the main frame, we have initiated studies that explore the anti-venom property of *Coix lacryma-jobi* ERE on the venom of *Naja naja*. Phospholipase A_2 (PLA_2) is a ubiquitous enzyme that specifically catalyzes hydrolysis of membrane phospholipids to release lysophospholipids and free fatty acid, namely arachidonic acid, which provides substrate for eicosanoids biosynthesis that modulate acute and chronic inflammation. Thus the compounds inhibiting PLA_2 have been implicated as potential therapeutic agents for the treatment of inflammation.

Rathnam et al., in 2016, firstly reported the molecular docking study of Njavara compounds, against Russell's viper venom PLA_2. The

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antivenom activity of bioactive compounds in *Njavara Bran* viz. tricin and its flavanolignans was proven by molecular docking studies to evaluate their potency to act as PLA_2 inhibitors. Hence in the current study, to understand the mechanism of these compounds computer aided drug discovery approach such as molecular docking was used, which provides insight into the interaction of ligands with their cognate protein target [6] of Russell's viper venom and bovine pancreatic PLA_2 as target molecules has been performed [7]. Our studies with the ERE suggested effective neutralization of *Naja naja* venom with respect to effective resolution of myonecrosis and associated pathologies dependent on the proteolytic, fibrinolytic, and nuclease activities inherent in the venom. Most imprtatnly, the ability of ERE to inhibit PLA_2 activity corroborated with the anti-hemolytic effects that was further confirmed by studies involving molecular docking of stigmasterol, a key constituent in ERE, into the active pocket of PLA_2. In conclusion, our studies provide data to support new adjunct therapies for treating snakebite victims in combination with the currently available biologics that could effectively decrease morbidity and mortality.

2. Materials and methods

2.1. Preparation of extract

Roots samples of *Coix lacryma-jobi* were collected from plants growing wild in Udupi district of Karnataka, South India. These were authenticated by Dr. Rajendra D. Shinde, Associate Professor and curator, Department of Botany, Blatter Herbarium, St. Xavier's College, Mumbai-1, and voucher specimen was deposited (No: NI-5396) at Blatter Herbarium for future use as a reference material. ERE was prepared as follows: Fresh root of *Coix lacryma-jobi* was washed and shade dried. 1 kg of dried root was ground into fine powder and subjected to extraction with ethanol (2 ml/g of dry powder) as described [8]. After 7 days, the ERE was filtered. The solvent from the total extract was distilled and the concentrate was evaporated in a water bath to a syrupy consistency, and then finally evaporated to dryness and stored in a desiccator until further use [8].

2.2. Snake venom

The lyophilized *Naja naja* venom was obtained from Irula Snake Catchers Industrial Co-operative Society (ISCICS), Kanchipuram, Tamil Nadu, India and was preserved at 4 °C. The venom was dissolved in saline and fresh dilutions of the required concentrations were prepared using saline on the day of use. All procedures of procuring the venom was as per the Wild Life Act of India.

2.3. Snake venom antiserum

Polyvalent antivenom (as standard drug) was procured from Vins Bioproducts Ltd., Hyderabad, India (Snake Venom Antiserum I.P). The polyvalent anti-venom consists of horse immunoglobulin produced against the mixtures of Cobra-Krait-Russel's viper and Saw-scale viper venom, because these three four species are commonly found near human habitation and most reported snake bites in India are from any of these three four species. Very often the victim fails to identify the snake which bit him, hence to avoid risk, polyvalent anti-venom is administered. Each ml of the polyvalent antivenom used in the study could neutralize 0.6 mg of Cobra, 0.45 mg of Common Krait, 0.6 mg of Russell's viper and 0.45 mg of Saw-scaled viper venoms (dry weight).

2.4. Selection of animals

Healthy adult albino Wistar rats, weighing about 180–220 g between 2 and 3 months of age and Swiss albino mice, weighing about 18–22 g between 2 and 3 months of age obtained from K.S. Hegde Medical Academy (KSHEMA), Deralakatte, Mangalore were used for the study. The study was approved by the Institutional Ethics Committee for Animal Experimentation, KSHEMA, Deralakatte, Mangalore (KSHEMA/AEC/38/2011). The rats and mice were housed individually in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle; 25 °C and 45–55% relative humidity). They were given standard pellet diet and water *ad libitum* throughout the course of the study.

2.5. In-vivo studies

2.5.1. Acute toxicity studies

The preliminary pharmacological studies were conducted to assess the acute pharmacological effects and LD_{50} of ERE of *Coix lacryma-jobi*. The acute toxicity study was carried out in female albino rats by "up and down" method as described earlier. The animals were fasted overnight and ERE of *C. lacryma-jobi* suspended in 0.6% (Na-CMC) was administered with a concentration starting at 2000 mg/kg. Then the animals were observed continuously for 3 h for general behavioural, neurological, autonomic responses and then every 30 min for next 3 h and finally at 24 h that represented a humane end point, when they were sacrificed and considered as dead [9].

2.5.2. Selection of doses

For the evaluation of anti-venom activity of the ERE, three dose levels were chosen in such a way that, middle dose was one tenth of the maximum dose during acute toxicity studies, and a low dose was 50% of the one tenth dose, and a high dose was twice that of one tenth dose (100 mg/kg, 200 mg/kg, 400 mg/kg). For the anti-venom study of root extract, ERE was suspended in 0.06% Na-CMC (Sodium-carboxy methyl cellulose).

2.5.3. Evaluation of LD_{50} of the venom and neutralization of lethality

The median lethal dose (LD_{50}) of *Naja naja* venom was determined according to the previously developed method [10]. Briefly, the lethality of *Naja naja* venom was assessed by i.p administration of different concentrations of the venom dissolved in 0.2 ml of sterile saline to groups (n = 6) of Swiss albino mice (18–22 g). The LD_{50} was calculated with the confidence limit at 50% probability by probit analysis of deaths occurring within 24 h after the venom administration. The neutralization potency of *C. lacryma-jobi* ERE was assessed by modified method of Theakston and Reid [10]. Twice the LD_{50} (2 LD_{50}) doses of venom was injected intra-peritoneally into different groups of mice (n = 6) immediately followed by the administration of 100, 200 and 400 mg/kg doses of the ERE by oral route. Control group received the same amount of venom without ERE (vehicle only). The standard reference group i.e. polyvalent anti-venom was administered after the administration of $2LD_{50}$ dose of venom. After 24 h, mortality was recorded and results were analyzed by probit analysis.

2.5.4. Anti-inflammatory activity against venom-induced inflammation

The minimum oedematic dose (MED) of venom is defined as the least amount of venom which, when injected in to male albino rats, produces inflammation (oedema) in the paw [11].

To assess the anti-inflammatory activity, albino rats (180–220 g) were given sub-plantar injection of *Naja naja* venom (0.1 ml) followed by 100, 200 and 400 mg/kg doses of ERE by oral route [12].

As a control, sub-plantar injection of *Naja naja* venom was given and vehicle was administered orally. The oedematogenic response was evaluated by the use of plethysmograph.

2.5.5. Neutralization of venom-induced myotoxicity

Minimum myotoxic dose (MMD) of the *Naja naja* venom was determined in rats by intramuscular injection of different concentrations of venom in the right gastrocnemius muscle. Three hours after injection, rats were bled via retro orbital plexus and the plasma creatine kinase (CK) activity was determined by means of a kinetic assay using

CK-Nac kit [13]. The MMD was defined as the amount of venom that induced an increment in plasma CK activity corresponding to 4 times the value in the control group injected with PBS alone. Neutralization assay was carried out by injecting MMD of venom followed by oral administration of different dose of ERE and ERE free vehicle served as control.

2.6. In-vitro studies

2.6.1. Neutralization of venom-induced HRBC lysis

Anti-phospholipase activity of the ERE of *Coix lacryma-jobi* L. was assessed through inhibition of *in vitro* human red blood cells (HRBC) lysis using modified method of Balu and Alagesaboopathy [14]. In this study, *Naja naja* venom was used to induce hemolysis. Graded concentrations of ERE were mixed with venom and incubated at 37° C for an hour. This mixture was added to the tubes containing 1 ml of 1% suspension of HRBC, while the control tubes were mixed with ERE free saline. The mixtures were incubated at 37 °C for 30 min and centrifuged at 112 × *g* for 3 min. The absorbance of the supernatant was measured at 540 nm using spectrophotometer. The percentage inhibition of hemolysis was calculated according to the equation {(Control − test)/ Control} × 100.

2.6.2. Neutralization of venom phospholipase A_2 by the ERE

Phospholipase A_2 assay was performed by indirect haemolytic assay by a modified method of Gutieârrez et al. [15]. Agarose gel plates containing 0.8% agarose, 1.2% washed erythrocytes, 1% lecithin and 0.1 mM $CaCl_2$ were used for the study. The venom was incubated with 100, 200 and 300 µg of ERE for 30 min at 37 °C and then added to the wells. The plates were then incubated for 20 h at 37 °C, after which they were examined for the haemolytic halos. The venom along with drug free solution served as control.

2.6.3. Assessment of fibrinogenolytic activity by SDS-PAGE

Fibrinogenolytic activity was assayed according to previously described method [16,17]. Bovine plasma fibrinogen (60 µg) was incubated for 2 h with venom sample (25 µg) in 5 mM Tris HCl buffer (pH 7.4) containing 10 mM NaCl. The reaction was terminated by adding 20 µl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and samples were analyzed on 12% SDS-PAGE as previously described. For venom neutralization studies, venom was first incubated with ethanolic root extract (venom to ERE ratio of 1:1 & 1:2) for 30 min at 37 °C and the above procedure was carried out.

2.6.4. Inhibition of venom nucleases (DNase) activity

DNA degradation study was carried out by method of Rudbeck and Dissing [18] with minor modifications. 0.5 µg onion DNA was mixed with 10 µg of venom and incubated at 37 °C for 2 h. The mixture was then subjected to agarose gel electrophoresis and observed under UV light for the changes in DNA profile. For venom neutralization studies, venom was first incubated with 25 and 50 µg of ERE for an hour. The DNA was added to the tubes containing ERE and venom at the end of an hour and incubation was continued for another 2 h. These mixtures were then analyzed by agarose gel electrophoresis as mentioned above.

2.7. Molecular docking studies

All computational studies were performed using Small Molecule Drug Discovery Suit for Windows developed by Schrödinger, running on a Intel Pentium4 HT desktop.

2.7.1. Protein minimization

Glide high throughput docking program provides performance benchmarks for docking and scoring capabilities. Glide docking can be significantly enhanced by sampling methods and scoring functions, hereafter collectively referred to as “extra-precision” (XP) Glide [19]. The major potential contributors to protein-ligand binding affinity are (1) Displacement of waters molecules by the ligand from “hydro-phobic regions” of the protein active site, (2) Protein-ligand hydrogen-bonding interactions, as well as other strong electrostatic interactions such as salt bridges [20]. Glide function considers both and determines the binding affinity of ligand with target protein [21].

XP Glide scoring function is based on enforcement of physical chemical principles to a much greater degree than is employed in many other scoring functions, appropriate protein and ligand minimization is particularly critical [22]. In the present study, PLA_2 (PDB ID: 1A3F) was downloaded from Protein Data Bank (www.rcsb.org). The downloaded structure was pre-processed for docking studies by assigning bond orders, adding hydrogens, creating zero order bonds to metals, creating disulphate bonds, filling missing side chains and deleting water molecules beyond 5 Å, followed by the refinement by assigning sample water orientations and energy minimization using force field OPLS_2005 employing protein minimization wizard provided in Schrödinger Maestro application.

2.7.2. Ligand minimization

The three dimensional conformer of Stigmasterol (PubChem ID: CID 5280794) was downloaded from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/compound/) and subjected for ligand minimization using Ligprip application provided in Schrödinger Maestro. The ligand minimization was performed by assigning force field OPLS_2005 and stereo isomers were calculated retaining specific chiralities [23].

2.7.3. Active site identification

The active site on PLA_2 was identified by employing sitemap application. Here, top ranked potential substrate binding sites were identified considering site points at reported site using restrictive definition of hydrophobicity [24].

2.7.4. Docking

The molecular docking was performed using Glide application in two stages receptor grid generation and ligand docking. Receptor grid generation has directed the binding site for stigmasterol molecule on the surface of PLA_2. Ligand docking was performed using XP glide, having ligand sampling as flexible and Epik state penalties were added for docking score [22]. The XP glide score was computationally calculated using formula

$$\text{XP Glide Score} = E_{coul} + E_{vdW} + E_{bind} + E_{penalty}$$

2.8. Isolation of chemical constituents from ERE

A portion of the ERE was subjected to straight phase column chromatography using silica gel (60–120 mesh) developed with different solvents (petroleum ether 60–80, chloroform and acetone) in order of increasing polarity. Eluents were collected, concentrated and analyzed by thin-layer chromatography (TLC) to check for purity. Eluents with identical TLC profile were combined and evaporated to dryness. Pure compounds were then subjected to ^{1}H NMR, IR and GC–MS for identification.

2.8.1. Compound 1

Octacosane – white wax, eluted with Petroleum ether had melting point of 40 °C. Molecular formula: $C_{28}H_{58}$. Molecular weight: 394. IR (KBr) V_{max} cm^{-1}: 2919 (C—H Stretch in CH3), 2851 (C—H stretch in CH2), 1461 (C—H deformation in CH3), 723 (C—H rocking vibration). ^{1}HNMR ($CDCl_3$): δ 0.82–δ 1.08 (6H, 2 CH_3): δ 1.049–δ1.5027 (52H, 26 *CH_2). Mass spectra: GC–MS (*m*/*z*): 394 (M+, $C_{28}H_{58}$), the other peaks appeared at 69, 71 (100%), 85, 99, 113, 127, 141, 239, 253, 282 and 351.

2.8.2. Compound 2

Stigmasterol – colorless crystals eluted with chloroform: ethyl acetate had melting point of 168 °C. Molecular formula: $C_{29}H_{48}O$. Molecular weight: 412. IR (KBr) V_{max} cm^{-1}: 3433 (br, OH stretch), 2929 (C−H stretch in CH_3), 2854 (C−H stretch in CH_2), 1634 (C=C stretch), 1462 (C−H deformation in gem dimethyl), 1042 (C−O stretch of secondary alcohol), 607 (rocking vibration of CH2). ^{1}HNMR ($CDCl_3$): δ 0.76 (s, 3H, H-19), δ 0.79 (s, 3H, H-18), δ 0.94 (s, 3H, H-21), δ 0.83 (t, 3H, H-24), δ 0.87 (d, 3H, H-26), δ 1.07 (d, 3H, H-27), δ 2.17 (d, 1H, OH), δ 4.69 (m, 1H, H-3), δ 5.35 (s, 1H, vinylic proton), δ 5.15 (d, 2H, allylic proton), δ 1.13 to δ 1.25 (m, 16H, 8 × CH2), δ 1.38 to δ 1.68 (m, 9H, methine protons). Mass spectra: GC–MS (*m/z*): 412 (M+, $C_{29}H_{48}O$), the other peaks appeared at 394, 351, 300, 271, 255,213, 159, 145, 133, 105, 83, 55 (100%).

2.9. Statistical analysis

The median lethal dose (LD_{50}) of the venom was expressed as µg/mouse and was calculated by probit analysis. The other data were expressed as mean ± SE., analysed by one way ANOVA followed by Dunnett's test. P value ≤ 0.05 was considered as statistically significant.

3. Results

3.1. In-vivo studies

3.1.1. Acute toxicity of ERE

The percentage yield of the ethanolic root extract was found to be 8%, which was found to be safe even at a dose 2000 mg/kg in rats and mice. No signs of toxicity were observed.

3.1.2. Evaluation of LD_{50} of the venom and neutralization of lethality

The LD_{50} of *Naja naja* venom was found to be 8 µg/20 gm mice (i.p.). The ERE at doses 100, 200 and 400 mg/kg body weight were found to effectively neutralize the lethality induced by $2LD_{50}$ of *Naja naja* venom (Table 1). Complete protection against venom induced lethality was observed at 200 and 400 mg/kg of ERE (Table 1).

3.1.3. Anti-inflammatory activity against venom-induced inflammation

MOD of the *Naja naja* venom was found to be 4 µg when injected to the paw of the rats. The study showed that ERE at dose level 400 mg/kg significantly inhibited the oedema induced by *Naja naja* venom at all time intervals. ERE at dose level of 200 mg/kg, significantly inhibited venom-induced oedema at 180 min and 240 min (Table 2).

Table 1
Effect of ERE of *Coixlacryma-jobi* on lethality induced by $2LD_{50}$ of *Naja naja* venom in mice (*in-vivo*).

Group	Dose	Mortality (after 24 h)[no. of death/no. of mice used]	% survival after 24 h	[a]Corrected%	Probit
Naja naja venom(16 µg)					
1	Control	6/6	–	4.16	3.25
2	Std (polyvalent antivenom)	0/6	100	95.83	6.75
3	100 mg/kg root extract	1/6	66.66	66.66	5.44
4	200 mg/kg root extract	0/6	100	95.83	6.75
5	400 mg/kg root extract	0/6	100	95.83	6.75

[a] Corrected formula: for the 0% dead: 100(0.25/n); for the 100% dead: 100[(*n* − 0.25)/n]. When n is the no of animals in the group.

Table 2
The effect of different concentration of ERE of *Coixlacryma-jobi* on *Naja naja* venom-induced oedema in rats.

Time interval	Control	ERE 100 mg/kg	ERE200 mg/kg	ERE 400 mg/kg
60 min	1.10 ± 0.04	1.00 ± 0.03	0.98 ± 0.03	0.92 ± 0.02**
120 min	1.68 ± 0.09	1.58 ± 0.05	1.44 ± 0.09	1.20 ± 0.04**
180 min	1.80 ± 0.03	1.70 ± 0.03	1.62 ± 0.05*	1.46 ± 0.04**
240 min	1.50 ± 0.03	1.32 ± 0.03	1.30 ± 0.04**	1.20 ± 0.05**

The values are expressed as Mean ± SE, n = 6 rats in one group. *P ≤ 0.05 significant, **P ≤ 0.01 highly significant, when compared with control group.

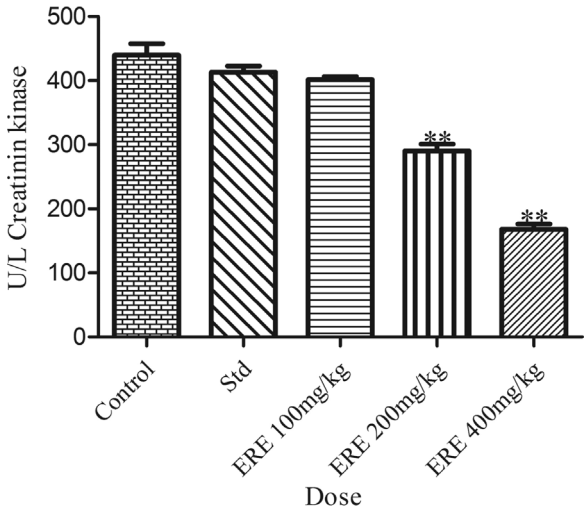

Fig. 1. Effect of *Coix lacryma-jobi* ERE on *Naja naja* venom induced myotoxicity in rat. *Naja naja* venom at a dose of three µg was given to gastrocnemius muscle. Three hours after injection, rats were bled via retro orbital plexus and the plasma creatine kinase (CK) activity was determined by means of a kinetic assay using CK-Nac kit. The Values are expressed as Mean ± SEM, n = 6 rats in one group. *P ≤ 0.05 significant, **P ≤ 0.01 highly significant, when compared with control group. U/L: units per liter; Std: standard drug; RE: ethanolic root extract; mg/kg: milligram per kilogram.

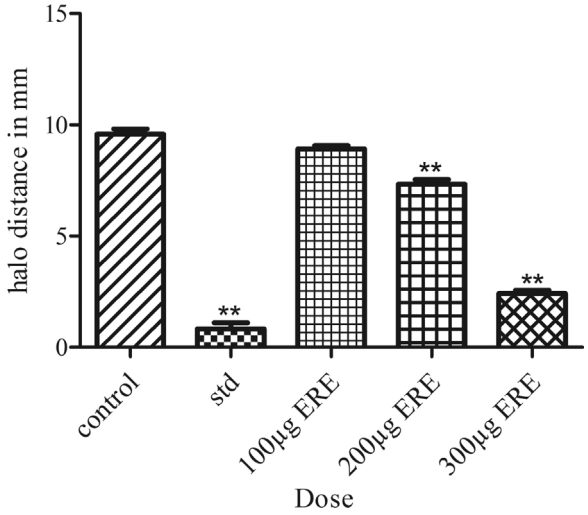

Fig. 2. Effect of ERE of *Coix lacryma-jobi* on *Naja naja* venom phospholipase A_2 activity. The venom induced indirect haemolysis was assessed by 10 µg of *Naja naja* venom produced a haemolytic halo of 10 mm. The ERE at dose level of 200 & 300 µg was used in the assay. The Values are expressed as Mean ± SEM, n = 6 samples in one group.*P ≤ 0.05 significant, **P ≤ 0.01 highly significant, when compared with control group. U/L: units per liter; Std: standard drug; ERE: ethanolic root extract; µg: microgram.

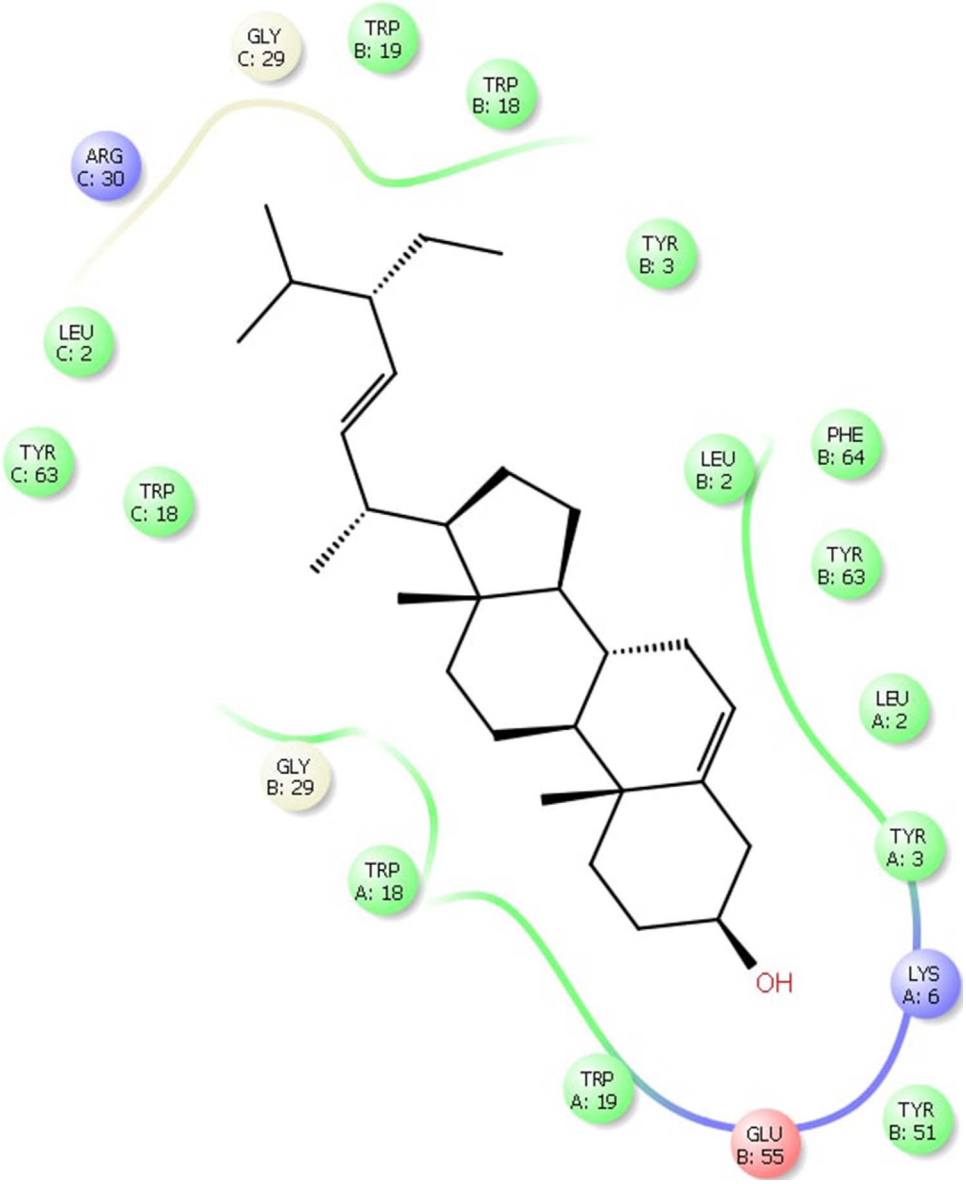

Fig. 3. Top ranking pose of stigmasterol in the active pocket of PLA_2 predicted using Glide docking method. The molecular docking was performed using Glide application in two stages-receptor grid generation and ligand docking. Receptor grid generation indicated the binding site for stigmasterol molecule on the surface of PLA_2. Ligand docking was performed using XP glide, having ligand sampling as flexible and Epik state penalties were added for docking score.

3.1.4. Neutralization of venom-induced myotoxicity

Myotoxicity caused by *Naja naja* venom was assessed by serum creatin kinase (CK) level. *Naja naja* venom at dose of three µg produced four fold increase in plasma CK level after 3 h of intramuscular injection compared to control. There was significant dose dependent inhibition in serum CK activity by the ERE at 200 mg/kg and 400 mg/kg body weight in *Naja naja* venom treated rats. Inhibition of CK activity at 400 mg/kg ERE was significantly higher than 200 mg/kg (Fig. 1).

3.2. In-vitro studies

3.2.1. Neutralization of venom phospholipase A_2 activity

The venom induced indirect haemolysis was assessed and its neutralization by the ERE was carried out. 10 µg of *Naja naja* venom produced a haemolytic halo of 10 mm. The ERE at dose level of 200 & 300 µg, significantly neutralized this haemolytic effect of the venom (Fig. 2).

3.2.2. Neutralization of venom-induced HRBC lysis

Venom at dose of 50 µg added to 1 ml of 1% HRBC suspension produced haemolysis comparable with hyposaline-induced haemolysis. It is evident from the results obtained for HRBC lysis studies that the ERE shows dose dependent protection against the venom induced haemolysis up to a dose of 1000 µg (Fig. 5).

3.2.3. Neutralization of fibrinogenolytic activity

In the present study, it was observed that, crude lyophilized venom of *Naja naja* completely degraded α chain and partial degradation of β and γ-chain of fibrinogen was observed. In neutralization study, it was observed that, ERE at 1:1 ratio partially protected α chain, whereas β and γ-chain remained unaffected. At 1:2 venom to ERE ratio, all the 3 chains appeared more prominently, indicating protection from proteolytic degradation when compared to 1:1 venom to ERE ratio group (Fig. 6).

3.2.4. Inhibition of venom nucleases (DNase) activity

In the present study, it was observed that, 10 µg of *Naja naja* venom completely degrades 0.5 µg of DNA. In the neutralization study, an incremental protection against the DNA degradation was observed from ERE dose at 25 µg. Highest protection was observed at 100 µg of ERE. From the present study, it was evident that ERE of *Coix lacryma-jobi* provides protection against *Naja naja* venom induced DNA degradation

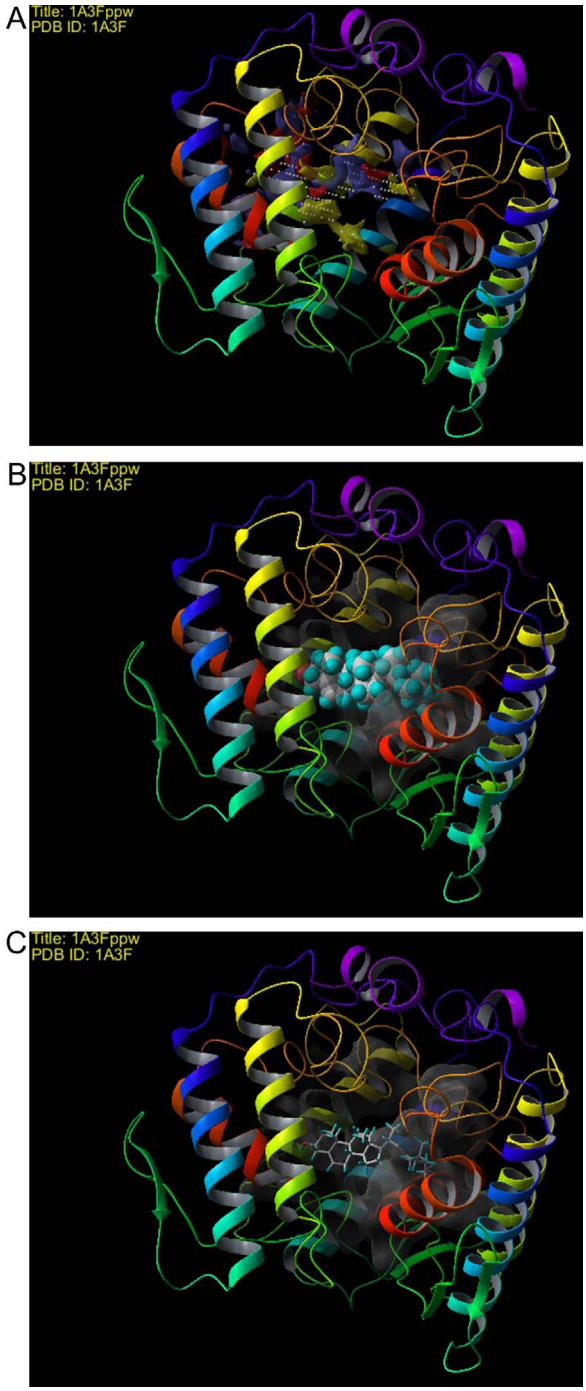

Fig. 4. Docked poses of Stigmasterol with PLA_2 in 3D space A) Illustrates the active site on PLA_2, B) Stigmasterol (in CPK pattern) has occupied the active site of PLA_2 (Ribbon pattern), C) Illustrates the interaction of Stigmasterol in docked workspace with active site amino acids.

(Fig. 7).

3.2.5. Molecular docking studies

From the docking studies it was observed that the PLA_2 activity is inhibited by the interaction of stigmasterol with PLA_2. Fig. 3 illustrates the best-scored docking pose showing the active pocket of PLA_2 in which several noncovalent interactions anchors the ligand into the protein. No hydrogen bonds were formed between the constituent active site amino acids. However, the noncovalent interactions like van der Waal's force and hydrophilic interactions of −4.6 kcal/Mol and hydrophobic enclosure reward of −2.7 kcal/Mol appeared to remarkably contribute to the glide score of −7.2 kcal/Mol. The molecular docking of stigmasterol in the active pocket of PLA_2 (Fig. 4A–C) was in agreement with above discussed results. In conclusion, molecular docking studies strongly suggested stigmasterol to be a potential candidate for further screening as an inhibitor of PLA_2.

3.3. Isolation of compounds from the ERE of Coix lachrymal-jobi

In the present work, five chemical entities were isolated from the ERE of *Coix lacryma-jobi* of which two components have been previously identified.

Compound 1 was isolated as white wax. Molecular formula of the compound was determined as $C_{28}H_{58}$. The IR spectrum revealed the presence of C−H stretching in CH_3 at 2919 cm^{-1}, C−H stretching in CH_2 at 2851 cm^{-1}, C−H deformation in CH_3 at 1461 and C−H rocking vibration at 723 cm^{-1}. ^{1}HNMR spectra showed signal of methyl group at δ 0.82–1.08 and methylene group at δ 1.049–1.5027. The mass spectra revealed the molecular ion peak at 394. The comparison of spectral data by IR, ^{1}HNMR and MS suggested that the compound 1 corresponded to octacosane (Fig. 8).

Compound 2 was isolated as colourless crystals. The molecular formula was determined as $C_{29}H_{48}O$. The IR spectra revealed presence of hydroxyl group at 3433 cm^{-1}, C−H stretching in CH_3 at 2929 cm^{-1}, C−H stretching in CH_2 at 2854 cm^{-1} and C=C stretching at 1634 cm^{-1}. ^{1}HNMR spectra showed signal of vanilic proton at δ 5.35, allylic proton at δ 5.15, secondary hydroxyl group appeared at δ 2.17. The mass spectra revealed the molecular ion peak at 412. The comparison of spectral data of IR, ^{1}HNMR and MS suggested that the compound 4 corresponded to stigmasterol (Fig. 9).

4. Discussion

Coix lacryma-jobi is a very common grass in India. The roots of this plant are extensively used to treat snake bite by the traditional healers of coastal Karnataka, India. Present study was carried out to assess the neutralization potency of the ERE of the plant against *Naja naja* venom. While our studies demonstrated effective resolution and recovery of rodents exposed to the venom, ERE of *Coix lacrymal-jobi* did not exhibit any signs of toxicity in the acute toxicity studies.

Present study revealed the effectiveness of ERE against $2LD_{50}$ dose of *Naja naja* venom at all dose tested. In a previously reported study, using a different root extract of *Andrographis paniculata,* it was reported that venom to extract ratio of 1:500 increased the survival time of the animal [25]. Similarly, *Mucuna pruriens* plant extract was tested for neutralization of *Naja naja* venom lethality, and it was reported that 0.16 mg of the extract completely neutralized lethality caused by $2LD_{50}$ (20 μg) of the *Naja naja* venom [26]. Another study carried out using *Mimosa pudica* extract showed that 0.14 mg of extract completely neutralized lethality of $2LD_{50}$ (20 μg) of *Naja naja* venom [27]. In the present study, 200 and 400 μg/Kg of ERE provided complete protection against *Naja naja* venom induced lethality.

In the study using *Mucuna pruriens* extract, oedema was reduced by 30% at 2.8 mg of extract pre incubated per every mg of *Naja naja* venom [26]. Another study was carried out using *Mimosa pudica* extract which showed 30% reduction in oedema when 2.8 mg of extract was pre incubated per every mg of *Naja naja* venom [27]. Methanolic extract of *Azadirachta indica* leaves were reported to neutralize 52% of *Naja naja* & 49% of *Naja kauthia* venom PLA_2 activity for venom to extract ratio of 1:50 [28]. Whereas, aqueous extract of *Mimosa pudica* was reported to have a protective action up to 86% against *N. kauthia* venom PLA_2, in which, dose of venom used was not specified [29]. A similar study with leupeol acetate from plant source, reported that it can effectively inhibit *Naja kauthiya* (5 μg) and *D. russelli* (5 μg) venom PLA_2 with ED_{50} of 60.1 and 73.3 μg respectively [30]. Yet another study carried out by Gopi et al. [31], reported that the methanolic extract of *Leucas aspera* failed to exhibit protection against *N. naja* venom PLA_2,

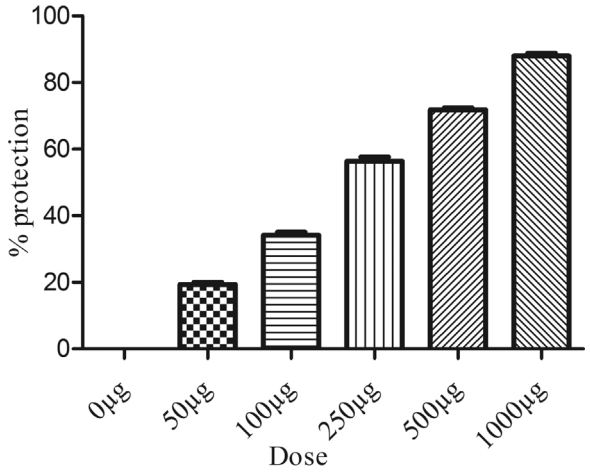

Fig. 5. Effect of *Coix lacryma-jobi* ethanolic root extract on *Naja naja* venom-induced HRBC lysis. Venom at dose of 50 µg added to 1 ml of 1% HRBC suspension produced haemolysis comparable with hyposaline-induced haemolysis.

whereas it effectively inhibited protease activity of the venom. Compared to all the above mentioned studies where the plant extracts were preincubated with the venom, our studies differed given that the ERE was administered post venom treatment, which correctly stimulates a natural scenario of snakebite rather than being more of a prophylactic. In addition, inhibition of indirect haemolysis was seen at much higher dose of 300 µg when compared to 200 µg of ERE (Fig. 2). These results observed with ERE of *Coix lachryma-jobi* was much more significant for venom to ERE ratio of 1:30 (i.e. 10 µg of venom & 300 µg of ERE inhibited PLA_2 activity by 69.36%) when compared with previously reported studies.

From the docking studies it is observed that stigmasterol targeted the PLA_2 activity given the interaction of stigmasterol with PLA_2. Molecular docking was performed to understand the pose variation of the stigmasterol in the active pocket of PLA_2 ubiquitous enzyme. PLA_2 specifically catalyzes hydrolysis of membrane phospholipids to produce lysophospholipids and free fatty acid, namely arachidonic acid, which is the precursor for eicosanoids biosynthesis that prevails post venom exposure. Most of the bioactive eicosanoids such as prostaglandins, leukotrienes and epoxy-derivatives are most likely pro-inflammatory, significantly contribute to and exacerbating inflammation that potentially overwhelms the pathways of resolution.

Condrea et al. [32] reported cobra venom to exhibit a direct haemolytic effect that is manly attributed to its ability to catalyze hydrolysis of phospholipids into lysophospholipids. Further, it was reported that venoms of *Naja* sp. contain a direct haemolytic factor, in the absence of which the phospholipase failed to show any direct haemolytic effect suggesting an activation step, which could also be a target of the ERE, which remains to be confirmed. The present study demonstrated dose-dependent inhibition of phsopholipase/haemolytic activity, which can be attributed to the active principles (such as stigmasterol) in the ERE that can inhibit PLA_2 in the venom.

It is reported that *Naja naja* venom degrades α-A chain of the fibrinogen, whereas β-B chain and γ-chain were not affected by the venom [33]. ERE of *Coix lacryma-jobi* demonstrated effective inhibition of fibrinolytic enzyme of *Naja naja* venom. In another study, *Naja nigricollis* (spitting cobra) venom showed degradation of α chain of the fibrinogen [34]. It is plausible that the ERE of *Coix lacryma-jobi* could show cross-species protection, but remains to be tested in the future.

Katkar et al. [35] reported the presence of DNase activity in the venom of *Naja naja*, which is also confirmed in the present study. Furthermore, presence of 5′ nucleotidase in *Naja naja* venom was reported by Dhananjaya et al. [36], where the in the anti-coagulant activity correlated with DNase activity. de Roodt et al. [37] reported hydrolysis of DNA by venom of *Naja siamensis.* Taken together our results indicate that active constituents in the ERE effectively inhibits nucleotidase (DNase) enzyme present in the *Naja naja* venom that contributes, in part towards the protective effects of ERE.

5. Conclusion

In the present study, it was observed that ERE of *Coix lacryma-jobi* effectively neutralized lethality induced by 16 µg ($2LD_{50}$) of *Naja naja* venom at all dose levels (100, 200 and 400 mg/kg body weight). Inflammation caused by 4 µg of venom was neutralized effectively by 400 mg/kg body weight of ERE. *N. naja* venom-induced increase in serum creatine kinase level was effectively inhibited at 200 and 400 mg/kg body weight of ERE. *In-vitro* studies conducted to assess the enzyme inhibition studies showed effective inhibition of PLA_2, nucelotidase (DNase), fibrinogenolytic enzyme activity. Furthermore, two chemical constituents isolated from the ERE were identified to be octacosan, and stigmasterol. These two constituents need to be further characterized individually and/or in combination for their anti-venom property.

From the molecular docking studies, stigmasterol appeared to be a candidate that could potentially target PLA_2 activity of *N. naja* venom. However, experimental validation is needed to complement the chemi-

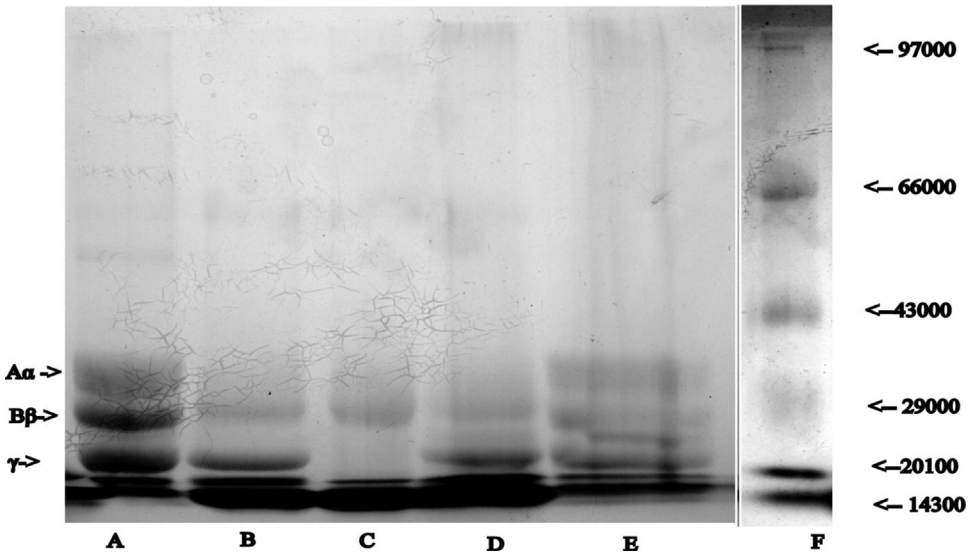

Fig. 6. Inhibition of *Naja naja* venom-induced fibrinogenolytic activity demonstrated by SDS-PAGE. Bovine plasma fibrinogen (60 µg) was incubated for 2 h with venom sample (25 µg) in 5 mM Tris HCl buffer (pH 7.4) containing 10 mM NaCl. The reaction was terminated by adding 20 µl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and samples were analyzed on 12% SDS-PAGE. Lane A- Fibrinogen, Lane B- fibrinogen + *N. naja* venom 25 µg, Lane C-*N. naja* venom, Lane D-fibrinogen + *N. naja* venom + ERE 50 µg, Lane E-fibrinogen + *N. naja* venom + ERE 100 µg, Lane F-Molecular weight marker respectively. Gel Shown is representative of n = 3.

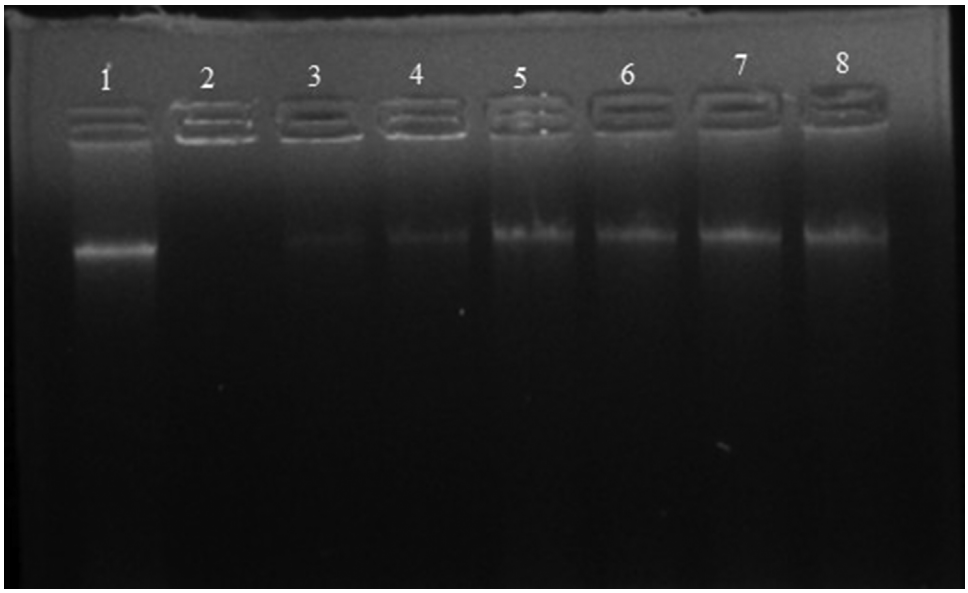

Fig. 7. Inhibition of *Naja naja* venom nucleases (DNase) activity demonstrated by agarose gel electrophoresis. 0.5 μg onion DNA was mixed with 10 μg of venom and incubated at 37 °C for 2 h. The mixture was then subjected to agarose gel electrophoresis and observed under UV light for the changes in DNA profile. For venom neutralization studies, venom was first incubated with 25 and 50 μg of ERE for an hour. The DNA was added to the tubes containing ERE and venom at the end of an hour and incubation was continued for another 2 h. These mixtures were then analyzed by agarose gel electrophoresis 1. DNA, 2. DNA + *N. naja* venom 10 μg, 3. *N. naja* venom 10 μg + ERE 25 μg + DNA, 4. *N. naja* venom 10 μg + ERE 25 μg + DNA, 5. *N. naja* venom 10 μg + ERE 50 μg + DNA, 6. *N. naja* venom 10 μg + ERE 50 μg + DNA, 7. *N. naja* venom 10 μg + ERE 100 μg + DNA and 8. *N. naja* venom 10 μg + ERE 100 μg + DNA. Gel Shown is representative of n = 3.

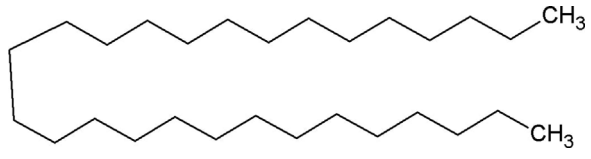

Fig. 8. Structure of octacosane.

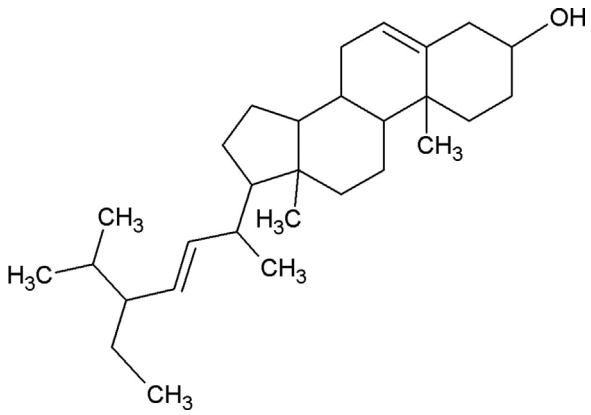

Fig. 9. Structure of stigmasterol.

informatics studies. In summary, data from the present study demonstrates the ability of ERE of *Coix lacryma-jobi* to neutralize the toxic enzymes present in the *Naja naja* venom. This can be utilized alone or in combination with anti-venom therapy so as to reduce the use of anti-venom and to down-regulate the immune reactions associated with venom exposure and anti-venom treatment.

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