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Research Article

EVALUATION OF ANTIVENOM ACTIVITY OF ETHANOLIC EXTRACT OF BUCHANANIA LANZAN BARK AGAINST NAJA KAOUTHIA SNAKE VENOM

Naseeb KM, Hegde Karunakar*, Syed Aamir, Deepak TK, Kalangottil Ashar

Department of Pharmacology, Srinivas College of Pharmacy, Valachil, Post- Farangipete, Mangalore, 574143. Karnataka, India

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*Corresponding Author**: Karunakar Hegde**
Tel: +91-0824-2274722 Fax: +91-824-2274725 E-mail: khegde_sh2003@yahoo.co.in

ABSTRACT

In the present study the effect of ethanolic extract of Buchanania lanzan bark (EEBL) was studied against toxicity induced by Naja kaouthia snake venom by various in vivo and in vitro studies. The EEBL was evaluated for neutralization of lethality, myotoxocity, Phospholipase A2 activity and Human RBC lysis produced by Naja kaouthia snake venom. The EEBL 200mg/kg and 400mg/kg significantly neutralized the lethality produced at different concentration of snake venom. Myotoxicity also decreased up to a significant level characterized by decline in creatine phosphokinase (CPK) level. In vitro models for assessing hemolytic activity were found to be significantly decreased in the presence of EEBL. Both direct and indirect hemolytic study was performed at various concentration of EEBL. More than 50% of hemolysis was significantly neutralized by the extract. Results showed significant neutralization of toxicity produced by N. kaouthia snake venom. However, further study is needed to determine the mechanism of action and to find out the active components responsible for its antivenom activity.

Keywords: Antivenom, Buchanania lanzan, Lethality, Myotoxicity, Naja kaouthia, Phospholipase A2.

INTRODUCTION

Snake bite remains a public health problem in many countries even though it is difficult to be precise about the actual number of cases. It is estimated that the true incidence of snake envenomation could exceed 5 million per year. It remains medical emergency encountered in the tropics and estimated 35,000 to 50,000 people die of snakebite every year in India. The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bangarus caeruleus*), Russell's viper (*Daboia russelli*) and Saw-scaled viper (*Echis carinatus*)[1]. *Naja kaouthia* is recognized phenotypically with the presence of O-shaped or monocellate hood pattern. They are widely distributed in Nepal, North East India, Bangladesh, Myanmar, Thailand and Peninsular Malaysia. According to WHO, it belongs to Category 1 of venomous snakes. The symptoms of cobra bite are general neurotoxicity leading to flaccid paralysis and death by respiratory failure, and also severe hypertension[2]. *N. kaouthia* venom contains several pharmacologically active components including cobra venom factor, kaouthiagin, phospholipases, cardiotoxin, neurotoxins and myotoxin. The cobra venom factor triggers the cascade of the alternative pathway of complement activation. Cobra venom factor and kaouthiagin cleave human von Willebrand factor and lead to anti-coagulation. Neurotoxins bind specifically to nicotinic acetylcholine receptors and block postsynaptic neurotransmission, thus causing flaccid paralysis resulting in respiratory failure and possibly death of the envenomed victims[3]. The administration of antisnake venom (ASV), the only specific treatment for snake bite, however, is associated with many drawbacks. Antivenom for ophidian bites is a suspension of antibodies, prepared mainly from horses. Animals are hyper-immunized against the venom of a given species (monovalent) or venoms from several different species at the same time (polyvalent). Infusion of ASV may lead to adverse reactions ranging from early reactions (pruritus, urticaria) to potentially fatal anaphylaxis[4].

Herbal medicine is a major component in all indigenous people's traditional medicine and a common element in Ayurvedic medicine. The use of plants against the effects of snakes bite has been long recognized; more scientific attention has been given since last 20 years. Extracts from plants have been used among traditional healers, especially in tropical areas where there are plentiful sources, as therapy for snakebite for a long time. Tannins comprise a group of polyphenolic compounds widely distributed in the plant kingdom that present the unique ability to form complexes with macromolecules, particularly proteins. Since snake venoms consist mainly of proteins (70-90%) that are responsible for a wide range of clinical signs and symptoms,

tannins show biological properties on anti-snake venom activity[5]. Previous reports suggest that the phytoconstituents like alkaloids, flavanoids, lipids present in the plant are responsible for the antivenom activity[6]. Several studies are carried on antivenom activity of medicinal plants. *Buchanania lanzan* includes in the list of the plants which have anti snake venom activity. Fruit and bark extract of *Buchanania lanzan* is used for the treatment of snake bite in Chhattisgarh region[7]. Some tribals of Rajasthan apply bark paste locally for snake bite poisoning[8]. Keeping the above information in view, in the present study an attempt was made to evaluate the safety and efficacy of ethanolic extract of the plant *Buchanania lanzan* bark for its antivenom activity against *N. kaouthia* venom in validated screening models.

MATERIALS AND METHODS

Collection and authentication of plant material:

The *Buchanania lanzan* for the present study was collected from local region of Tirunelveli, Tamilnadu in the month of July 2013. It was authenticated by Mr. V. Chelladurai, Research officer-Botany (Scientist-C) Central Council for Research in Ayurveda & Sidda, Govt. of India (Retired). The Bark was collected, dried, pulverized into coarse powder and used for extraction. It was packed in Soxhlet extractor and extracted using ethanol as solvent. The temperature was maintained on an electric heating mantle with thermostat control. Appearance of colourless solvent in the siphon tube was taken as the termination of extraction. The extract was concentrated by using rotary flash evaporator. The concentrated extract was then air dried at room temperature, stored in airtight container.

Chemicals:

All the chemicals and solvents were of analytical grade and were procured from Agapee including creatine kinase kit. Agarose was procured from Hi Media. Mispa semi Auto analyzer and UV- Spectrophotometer (UV 1601), Shimadzu corporation, Japan) were used to measure the absorbance.

Experimental animals:

Healthy Wistar albino mice (20-25 g) of either sex were used for the experiment and procured from the animal house of Srinivas College of Pharmacy, Mangalore. They were maintained under standard conditions (temperature 22 ± 2°C, relative humidity 50±5% and 12 hr light/dark cycle). The animals were housed in sanitized polypropylene cages containing paddy husk as bedding. They had free access to standard pellet diet and water *ad libitum*. The Institutional Animal Ethics Committee had approved the experimental protocol before initiation of experiment. The animals were acclimatized for atleast one week before use.

Acute toxicity:

Acute toxicity study of the ethanolic extract of plant *Buchanania lanzan* Bark was performed as per the OECD guidelines 425 at a limit dose of 2000 mg/kg. The doses was administered by oral route in mice as per scheduled in OECD guidelines 425. Animals was observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily thereafter, for total 14 days for sign of toxicity and/or mortality if any. The LD_{50} was calculated by using OECD 425 guidelines.

Phytochemical screening:

Freshly prepared methanol ethanolic extract of plant *Buchanania lanzan* Bark was subjected to preliminary phytochemical screening for detection of major chemical constituents.

Determination of Median Lethal Dose of venom (LD50 or MLD):

Lethal dose 50 (LD50) was determined using mice weighing (20-25g) of either sex. Varying doses of *Naja Kaouthia* venom in phosphate buffer (pH-7.2) were injected intraperitonially into different groups of mice containing 6 in each group. The dose of venom ranging from 0.1 - 0.7 mg/kg body weight of the mice were administered and control group was received only saline. Survival time of each animal in each set was recorded for 24 h. LD50 was calculated by the comparison of doses injected, with the observed survival time within 24 hrs[9].

Neutralization of lethal effect[10]:

The Swiss albino mice (20-25g) of either sex will be divided randomly into 4 groups of six animals each. The different groups will be assigned as below

Group I: Control (Phosphate buffer, *i.p*)

Group II: Venom *(i.p)*+ Ethanolic Extract of *Buchanania lanzan* (200 mg/kg) *(p.o)*

Group III: Venom (*i.p)* + Ethanolic Extract of *Buchanania lanzan* (400 mg/kg) *(p.o)*

The mice(n=6) were administered with ethanolic extract (200 & 400mg/kg) of *Buchanania lanzan* through oral one hour prior to administration of 1 to 3.0 folds of MLD of venom by intraperitonial route; all the animals were observed for mortality for 24 hrs.

Inhibition of Myotoxicity[11]:

The Swiss albino mice (20-25g) of either sex will be divided randomly into 4 groups of six animals each. The different groups will be assigned as below

Group I: Normal control (phosphate buffer pH 7.2)

Group II: Positive control (1.2µg/g) of *N. kaouthia venom* (2 MLD) by i.m. route

Group III: Ethanolic Extract of *Buchanania lanzan* (200mg/kg) + 2MLD of *N. kaouthia* venom(Supernatant of mixture by i.m. route)

Group IV: Ethanolic Extract of *Buchanania lanzan* (400mg/kg) + 2MLD of *N. kaouthia* venon (Supernatant of mixture by i.m. route)

Quantitation of plasma creatine phosphokinase *(CPK)* activity was used as the indicator for the measure of Inhibition of myotoxic effect of venom. 2 MLD (1.2µg/g) doses of *N. kaouthia* venom were pre-incubated with different concentrations of EEBL (200 & 400mg/kg) for 1 h at 37°C. The mixture was centrifuged and supernatant was taken and administered to mice by i.m. route (0.2 ml). Control animals received a similar injection of venom only. Mice from each group were anaesthetized after 4 h and blood samples were obtained from the retro-orbital puncture of mice. Collected blood sample was centrifuged at 3000 rpm for 10 min. and Plasma was collected for measurement of plasma CPK activity. Absorbance was read at 340 nm wavelength.

Phospholipase A2 Activity[12]:

Determination of Minimum hemolytic dose (MHD) of *N.Kaouthia* venom:

300 µl of packed sheep erythrocytes washed four times with saline solution, 300 µl of 1.2% egg yolk solution in saline solution and 250 µl of 0.01 M CaCl2 solution were added to 25 ml of 1% (w/v) of agar at 50ºC dissolved in PBS pH 7.2. The contents were mixed well and was added to Petri dish and allowed to gel. Later, wells of 3 mm diameter were made and filled with 15 µl venom samples and incubated at 37ºC for 20 hr. The diameters of hemolytic halos were measured. To determine the minimum hemolytic dose (MHD) of *Naja kaouthia* venom, 15 µl of solutions containing different amounts of venom concentration (from 1 to 50 µg), were added into the wells. 15 µl of Phosphate buffer of pH 7.2 was added to control well. After 20 h of incubation at 37ºC, the diameters of hemolytic halos were measured.

The minimum hemolytic dose (MHD) was defined as the amount of venom that induced a hemolytic halo of 11-mm diameter.

Neutralization of Phospholipase activity by EEBL[13]:

Samples of constant amount of [15µg (1MHD)] venom were incubated with various amount of EEBL for 30 min at 37^0C. Then aliquots of 15µl of the mixtures (venom + EEBL) were added to wells in agarose-egg yolk-sheep-erythrocytes gels plates and incubated 37^0C for 20 h. Control samples [15µg (1 MHD)] contained venom without extract. Plates were incubated at 37^0C for 20 hr. Neutralization was expressed as the % Inhibition that reduced 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Inhibition of *in vitro* Human Red Blood Corpuscles (HRBCs) lysis[14]:

Blood was collected from healthy human volunteers by vein puncture using EDTA as anticoagulant. The collected blood was washed three times with saline and 1% HRBC will be prepared. Lyophilized venom of *Naja kaouthia* was dissolved in physiological saline solution to make a stock solution of 100µg/ml.

Then combination of 1ml of venom (100µg), 1ml of phosphate buffer (pH 7.4) and1ml of 1% HRBCs was taken in various tubes. To these tubes different concentrations of the extracts of *Buchanania lanzan* (20, 40, 60, 80, 100, 200, 400µg/ml) was added. The drugs were prepared using physiological saline while the control sample was free of extract. The mixtures was incubated at 37ºC for 30 minutes and centrifuged at 1000 rpm for 3 minutes. The supernatant was collected from the tube and absorbance was measured at 540 nm using spectrophotometer. The control was taken as 100% lysis and the percent inhibition of haemolysis was calculated according to the equation.

RESULTS AND DISCUSSION

The successive extraction of powdered *Buchanania lanzan* bark was carried out using ethanol by soxhlet extraction method. The alcoholic extract obtained was dark brown in colour.

Table 1: Extraction Details

Solvent	Color and Consistency	Method	Percentage Yield
Ethanol	Dark brown	Hot percolation	13.5%

Preliminary phytochemical screening

The alcoholic extract was subjected to preliminary phytochemical testing to determine the presence of phytoconstituents. The results were shown in Table No. 2.

Table 2: Preliminary phytochemical screening

Sl. No.	Test	Results
1.	Alkaloids	+ve
2.	Carbohydrates	+ve
3.	Flavonoids	+ve
4.	Glycosides	+ve
5.	Saponins	+ve
6.	Steroids	+ve
7.	Tannins	+ve
8.	Protein	-ve
9.	Volatile Oil	-ve

Acute oral toxicity study

Ethanolic extract of *Buchanania lanzan* Bark was subjected to toxicity studies. Extract was administered orally into mice and observed up to 24 and 72 hours. Animals did not show any mortality or toxic symptoms at a dose up to 2 g/kg body weight. 200 and 400mg/kg doses were selected for further study.

Median lethal dose (LD50) of *Naja kaouthia* venom:

Median lethal dose (LD50) of *Naja kaouthia* venom was assayed by injecting different doses of venom in 0.1 ml physiological saline i.p. to Swiss albino mice of both sex weighed between 20-25 g. It was found to be 0.6mg/kg for i.p. route.

Neutralization of lethality induced by venom in mice:

100% neutralization of lethality of one fold venom was seen in 400mg/kg treated mice. 200mg/kg treated animals showed 66.66% and 33.33% neutralization of lethality for 2.0 and 3.0 fold of snake venom respectively. There were 83.33 and 66.66% neutralization in 2.0 and 3.0 folds respectively seen in high dose of plant extract. Results are depicted in Table No:3.

Inhibition of myotoxic effect of snake venom

Injection of *N. kaouthia* venom induced myonecrosis as measured by serum creatine phosphokinase (CPK) activity which increased from 161.5 ± 1.555 units/liter in normal mice (untreated control) to 2412 ± 122.0 units/liter (venom treated without extract). The ethanolic extract of *Buchanania lanzan* at dose of 200mg/kg decreased the CPK activity about 16.2%. However, the ethanolic extract of *Buchanania lanzan* at dose of 400mg/kg significantly decreased the CPK activity about 36.3%. Data are shown in Figure 1.

Phospholipase A_2 (PLA_2) activity:

N. kaouthia venom produced 11 mm diameter haemolytic halo at a dose of about 15µg. 15µg *N. kaouthia* venom was taken as minimum hemolytic dose (MHD). This shows that *N. Kaouthia* venom have the enzymes (phospholipase A2) that

has the ability to lyses sheep RBC's. *Buchanania lanzan* extract was capable of inhibiting phospholipase A2 dependant hemolysis of sheep RBC's induced by *N. Kaouthia* venom in a dose dependant manner. It was found that 0.225 mg of ethanolic extract of *Buchanania lanzan* (EEBL) significantly inhibited half of the halos 5.300 ± 0.09129 (51.4%). The 0.300 mg of EEBL was able to produce significant inhibition 3.075 ± 0.1250 (71.8%) of halos produced by the phospholipase A2 dependent hemolysis of sheep RBC's induced by *N. Kaouthia* venom, experiment was carried out in triplicate. In contrast, saline solution did not induce haemolysis. When egg yolk was not added to the gels there was no hemolysis, indicating that hemolysis was only of the indirect type, i.e. due to PLA_2 activity in the venom.

Table 3: Neutralization of lethality by EEBL

Group	Treatment	Venom (µg/g)	Folds of Neutralization in terms of LD_{50}	No. Of Deaths /No. Of mice used	% Neutralized
1	Ethanolic Extract 200 mg/kg	0.6	1.0	01/06	83.33
1	Aqueous extract 750mg/kg	1.2	2.0	02/06	66.66
1	Aqueous extract 750mg/kg	1.8	3.0	04/06	33.33
2	Ethanolic Extract 400 mg/kg	0.6	1.0	00/06	100
2	Aqueous extract 1000mg/kg	1.2	2.0	01/06	83.33
2	Aqueous extract 1000mg/kg	1.8	3.0	02/06	66.66

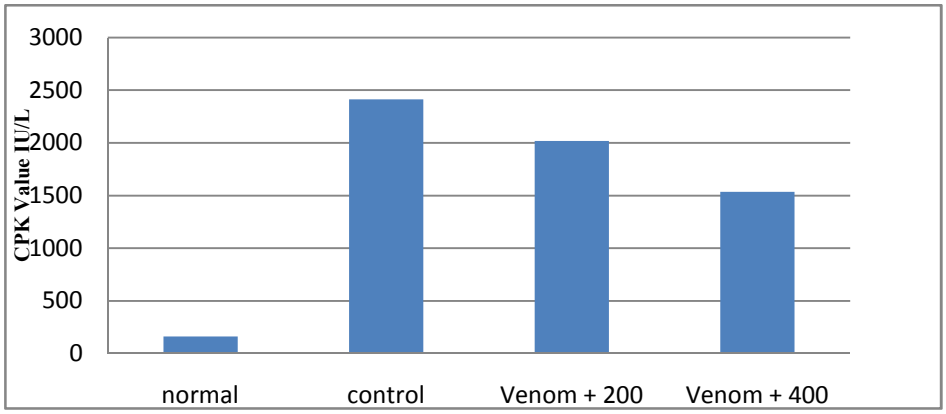

Figure 1: Inhibition of myotoxicity induced by *N. kaouthia* venom

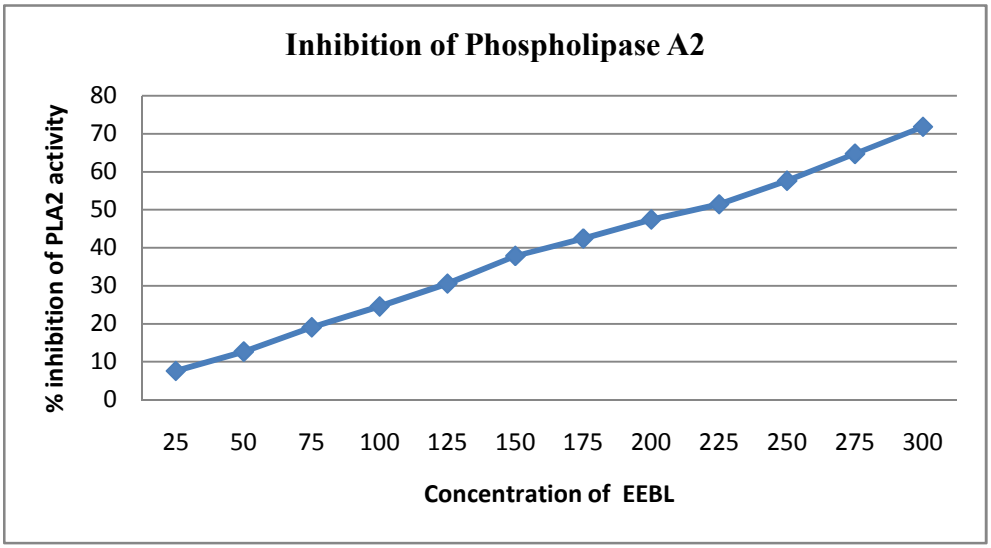

Figure 2: Inhibition of Phospholipase A_2 (PLA_2) activity

Effect of EEBL on Human RBC (HRBCs) lysis induced by *N. kaouthia* venom

When snake bites, the lysophospholipids were produced and disrupt cellular membrane, in turn causes lysis of red blood cells. Direct addition of snake venom into Human RBCs produced hemolysis results in release of hemoglobin. This free hemoglobin measured colorimetrically at 540nm. Effect of extract of *Buchanania lanzan* in neutralizing direct hemolytic activity was successfully carried out. At a concentration of 200 µg/ml of EEBL, hemolysis produced by the venom was significantly neutralized to almost 50% of produced by venom alone.

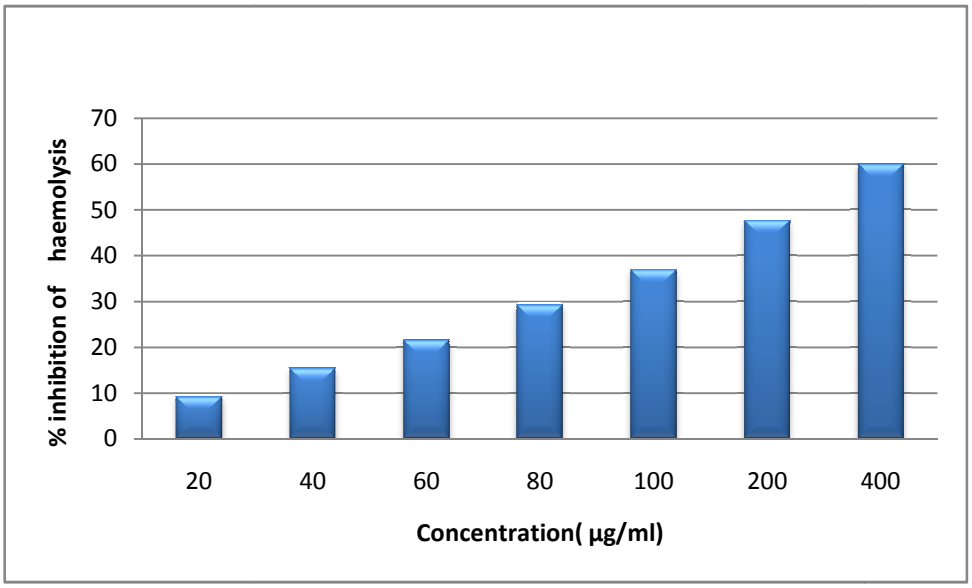

Figure 3: Effect of EEBL on HRBCs lysis induced by *N. kaouthia* venom

Venoms contain proteins, lipids, steroids, aminopolysaccharides, amines, quinines, neurotransmitters and other compounds, and are capable of causing many effects. Elapid venoms have higher concentrations of esterase, such as acetylcholinesterase, that exerts effects on the nervous system[1]. Proteolytic enzymes like trypsin are account for much of the digestive reactions of snake venoms. Phospholipases A and B degrade lipids to free fatty acids and can cause damage to the cell membrane causing lysis and apoptosis[15]. PLA_2 also displays various pharmacological activities such as neurotoxicity, hemolytic activity, myotoxicity, anticoagulant and antiplatelet activities. Several cytotoxins have been isolated from the venom of *N. kaouthia*. Snake venom metalloproteinases (SVMPs) are the only venom components that possess hemorrhagic activity[16].

Antivenom for ophidian bites is a suspension of antibodies, prepared mainly from horses. Animals are hyper-immunized against the venom of a given species (monovalent) or venoms from several different species at the same time (polyvalent). Infusion of antisnake venom (ASV) may lead to adverse reactions ranging from early reactions (pruritus, urticaria) to potentially fatal anaphylaxis[17].

In many countries, plant extracts have been traditionally used in the treatment of snakebite envenomation. The use of plants against the effects of snakes bite has been long recognized; more scientific attention has been given since last 20 years. However, in most cases, scientific confirmation of their antiophidian activity is still needed. It is believed that around 80% of the world population use plants as first therapeutic help in cases of envenomation.

The bark of *Buchanania lanzan* known **contains 13.4% tannins.** Tannins comprise a group of polyphenolic compounds widely distributed in the plant kingdom that present the unique ability to form complexes with macromolecules, particularly proteins. Since snake venoms consist mainly of proteins (70-90%) that are responsible for a wide range of clinical signs and symptoms, tannins show biological properties on anti-snake venom activity. Fruit and bark extract of *Buchanania lanzan* is used for the treatment of snake bite in Chhattisgarh region[7].Therefore, in the present investigation, an effort has been given to evaluate the neutralization capacity of ethanolic extract of *Buchanania lanzan* bark against lethality, myotoxicity, phospholipase A_2 and HRBC lysis of *Naja kaouthia* venom.

Acute oral toxicity study of *Buchanania lanzan* bark extract was performed. It was found to be safe upto 2g/kg. Dose of *N. kaouthia* venom required to produce 50% lethality (LD50) was found to be 6 µg/mouse. Lethality produced by *N. kaouthia* venom was significantly neutralized by various doses of plant extract. On increasing the folds of LD50 of venom, neutralization was found to be decreasing. Lethality by *N. kaouthia* snake venom was mainly produced due to presence of neurotoxins. Snake venom contains myotoxic components results in the degeneration of skeletal muscle by breaking down of muscle fibers. Myotoxicity is characterized by elevation in the serum levels of muscle-derived enzymes, such as creatine kinase (CK). Measurement of plasma creatine phosphokinase (CPK) activity was used as the indicator for the measure of inhibition of myotoxic effect of venom. Treatment of snake venom increased the creatine level from 161.5±1.555

upto 2412±122. However, co-administration of 200mg/kg and 400mg/kg of EEBL reduced the creatine level up to 16.2 and 36.3% respectively.

The PLA_2 enzyme in snake venom digested the lecithin in egg yolk and changed to lysolecithin form in 60 minutes at 37°C. The red blood cells was lysed by lysolecithin and formed as hemolytic halo. 15µg venom mixed with various concentration of plant extract significantly reduced the diameter of hemolytic halo. It was found to be 0.225 mg of EEBL significantly inhibited almost 50 % (51.4%) of the hemolytic halos. Direct addition of snake venom into Human RBC produced hemolysis result in release of hemoglobin. Absorbance of supernatant was measured after centrifugation. Toxic control (venom only) group showed high level of absorbance since it produce maximum hemolysis. Co-administration with EEBL significantly reduced the absorbance which denotes the inhibition of hemolysis.

Previous reports suggest that the phytoconstituents like tannins, alkaloids, flavanoids, lipids present in the plant are responsible for the antivenom activity[5,6]. The active phytoconstituents like tannins and flavonoids present in the extract might responsible for the antivenom activities of the barks of *Buchanania lanzan*.For the development of potent antagonist against snakebite from the plant extract, further investigation is needed to develop active constituents. The mechanism behind antisnake venom activity of EEBL has to be performed to produce potent herbal drug against *N. kaouthia* snake venom.

CONCLUSION

Present investigation revealed that the *Naja kaouthia* envenomation activity was neutralized by alcoholic extract of *Buchanania lanzan* bark. Extract at a dose of 200 and 400mg/kg significantly neutralized the toxic effects of *N. kaouthia* snake venom. Various *in vivo* and *in vitro* models were used to determine the antivenom property of the plant extract by using two dose levels and it showed a significant neutralization of lethality, myotoxicity, PLA2 activity, and hemolytic activity. Results denote that the alcoholic extract of the bark of *Buchanania lanzan* at 400mg/kg dose ranges showed a significant antivenom activity. The active phytoconstituents like tannins, alkaloids, flavanoids and lipids present in the extract might responsible for the antivenom properties of the barks of *Buchanania lanzan*.

From these observations, it is concluded that EEBL may possess antisnake venom activity against toxicity induced by *N. kaouthia* venom. However, further study is needed to determine the mechanism of action and to find out the active components responsible for its antivenom activity.

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