Neutralization Potentials of Portulaca oleracea Leaf Extract against Naja nigricollis Venom Phospholipase A2

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Abstract— Snake bites are considered a neglected tropical trauma that affects thousands of people worldwide. Although anti-venom immunotherapy is the only treatment available against snake envenomation, it is associated with many side effects. As a result of this, plants are been studied in folk medicine to be used as an alternative. Portulaca oleracea is a well-known medicinal plant in Nigerian ethnomedicine for the management of many diseases. Investigations concerning its phytochemicals and pharmacological characteristics have been carried out and its potency against snake envenomation has been evaluated. In this study, we futher evaluate its venom neutralizing (antidote) properties against Naja nigricollis venom in an in vitro and in vivo assay using mice. LD100 of N. nigrocollis venom was calculated from its LD50 result (1414.21 µg/kg body weight of mice). The egg yolk coagulation method of Ahmed et al was used to study the Phospholipase A2 neutralizing potentials of the aqueous leaf extract of Portulaca oleracea on N. nigrocollis venom. To study the antivenom potentials of the ethanolic leaf extract of Portulaca oleracea, eighteen (18) male and female albino mice weighing between 15-35 g were randomly divided into six (6) groups of three (3) mice each. Group 1 received normal saline + venom, groups 2-5 received venom + P. oleracea ethanolic leaf extract at 0, 5, 10 and 15 mins delay respectively, while group 6 received venom + a combined ethanolic leaf extract of Portulaca oleracea and Euphorbia hirta. N. nigricollis venom was administered intraperitoneally at a dose of 2828.42 µg/kg body weight of mice and Portulaca oleracea extract was orally administered at a dose of 250 mg/kg body weight at different time interval in the in vivo assay. The result for the LD100 of N. nigricollis venom showed a 100% mortality. Results for the Phospholipase A2 neutralizing potentials of the aqueous leaf extract of Portulaca oleracea on N. nigrocollis venom showed that 1ml of 500 µg/ml of the aqueous leaf extract of Portulaca oleracea neutralized the toxic effects of 1ml of 200 µg/ml of Naja nigricollis venom. The result of the effects of time-lag after the administration of N. nigricollis venom and Portulaca oleracea extract showed a 0% survival in the group 1 (control), 100% survival in group 2, 33.3% survival in group 3, 66.7% survival in group 4, 0% survival in group 5 and a 66.7% survival in group 6. This study showed that the ethanolic leaf extract of Portulaca oleracea showed anti venom neutralizing potentials in animal models. The above results indicate that the plant extract possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purpose in case of snake bite envenomation.

Index Terms— Antivenom, Ethnomedicine, Naja nigricollis, Neutralize, Phospholipase A2, Portulaca oleracea, Snake bite.

1. INTRODUCTION

SNAKEBITE remains a major health hazard that leads to high mortality rate worldwide. The number of snakebites that occur each year may be as high as five million. They result in about 2.5 million poisonings and 20,000 to 125,000 deaths [1]. The frequency of bites varies greatly in different parts of the world. They occur most commonly in Africa, Asia and Latin America with rural areas more greatly affected.

Although anti-venom immunotherapy is the only treatment available against snake envenomation, it is associated with many side effects which include; anaphylactic shock, pyrogen reaction and serum sickness. These are possible outcomes of the action of antigenic proteins present in higher concentrations in anti-venom [2]. In addition, they do not neutralize the local tissue damage [3]. Also, anti-venoms (antisera) are not available in remote areas and they are quite expensive.

Snake Venom is a complex mixture of many substances such as; toxins, enzymes (hydrolytic growth factors, activators and inhibitors) with a wide spectrum of biological activities [4]. Snake venoms are also known to cause various physiological changes in organs of different animals. The composition of snake venom determines the physiological effects on their target and it varies with snake type, age and environment where there are found. The components of snake venom are mostly enzymes which include; L-amino acid oxidase, Alanine amino transferase, Phospholipase A2, 51 Nucleotidase, Phosphodiesterase, Deoxyribonuclease, Ribonuclease I, Adenosine triphosphatase, Amylase, Hyaluronidase, NAD-Nucleotidase, Glusamine ammonium lyase and Kininogenase which are found in all snake types and Lactate dehydrogenase, Lysophospholipase, Acetylcholinesterase, Alkaline Phosphatase, Acid phosphatase, Factor-X activator, Heparinase, α-fibrinogenase, β-fibrinogenase, α-β-fibrinogenase, Fibrinolytic enzyme, Prothrombin activator, Collagenase and Elastase which are found in some species [5].

PLA2 which has been found to be present in all snake venoms causes hemolysis by lysing the phospholipidcell membranes of red blood cells. PLA2 has shown to be responsible for many pathophysiological disorders like cardio toxicity, neurotoxicity, edema, necrosis, hemolysis, and anti-coagulation [6]. Due to the fact that PLA2 is widely present in all snake venoms, it is a good molecular target for the development of drugs against snake bite. To neutralize deleterious effects of PLA2 activities numerous enzyme inhibitors have been tested previously and natural antidotes (medicinal plants) have been considered the most reliable source to neutralize snake venom Phospholipase A2 (PLA2) [7].

The World Health Organization (WHO) has estimated that up to 80 percent of people in the developing world are dependent on traditional system of medicines primarily because of their easy accessibility, wide affordability and cultural familiarity.

Over the years, many attempts have been made for the development of snake venom antagonists from plant sources. Some works have been done in other to find out the phytochemicals in some plants that have ethno botanical usage for snake envenomation [8]. Some plants have shown to possess anti-snake venom potentials. Plants like Annona senegalensis, Moringa oleifera, Alluim cepa, Allium sativum have been used by the Fulani Herdsmen in Taraba State for the management of snake bite [9]. Extracts of Uvaria chamae has also shown to neutralize some biological effects of Naja nigricollis snake venom in rats [10].

Recently, work has been carried out in the department of Biochemistry, ANSU, Uli on phytochemical composition of Portulaca oleracea. In silico work has been carried out which revealed that some of the phytochemicals obtained from this plant have strong affinity for snake venom PLA2 compared with the control ligand used [8]. The phytochemical constituents of Portulaca oleracea that have been isolated include; flavonoids [11], alkaloids [12], fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals. Research has shown that this plant is a good source of omega-3 fatty acid. Its LD50 result showed no mortality and is > 5000 mg/kg in mice [13]. Its pharmacological effects include; antibacterial [14], anti-ulcerogenic [15], anti-inflammatory [16], antioxidant and wound-healing [17] properties. Aside the insilico work done on this plant, few work have been done to ascertain its snake venom neutralizing potentials. An in vivo work has been carried out using the ethanolic leaf extracts of Portulaca oleracea which showed a neutralizing effect on different time-lag of the LD50 value of Naja nigricollis snake venom in mice [13]. The aim of this study therefore is to carry out an in vivo and in vitro assessment of the anti-snake venom neutralizing potentials of aqueous and ethanolic leaf extracts of Portulaca oleracea in mice.

2. MATERIALS AND METHODS

Chemicals, solutions and equipment: All chemicals used in the present study were of analytical grade and purchased from QULIKEM, India. Centrifuge (Heraeus Christ GMBH Estrode), Analytical balance, measuring cylinder, micropipette, mortar, pestle, beakers, retord stand, burette, syringes and deep freezer.

Collection, Identification and Extraction Procedure of the Plant Sample

The leaves of Portulaca oleracea were harvested from Girls High School, Agulu, Anaocha Local Government Area in Anambra State and was identified by Mrs. Emezie, A. U., a pharmacologist in the department of pharmacology, school of pharmacy, Nnamdi Azikiwe University, Agulu campus. Its voucher number is PCG 474/A/028.

For the ethanolic extraction: The leaves were dried at room temperature (25 0C) for a couple of days and later blended into fine powder using dry blender. 250 g of the powder was macerated in 1litre of ethanol in an air tight plastic container and allowed to stand for 48hrs. The extract was filtered successively using muslin cloth and Wattman filter paper No. 42. The extract was then concentrated to dryness over a water bath at 50 oc.

For the aqueous extract: The fresh leaves were pounded in a mortar using a pestle. 0.02 g of the pounded substance was dissolved in 40 ml of clean water to get a concentration of 500 µg/ml. The solution was then filtered into a beaker through a funnel stuffed with cotton wool.

2.1 Laboratory Animal

Albino mice were purchased from the animal house of the Department of Pharmacology, School of Pharmacy, Nnamdi Azikiwe University, Agulu Campus, Anambra State, Nigeria. This study was approved by the Department of Biochemistry, Anambra State University, Uli, according to the institutional ethics. These animals were used as approved in the study of snake venom toxicity. The mice were maintained under normal laboratory condition of humidity, temperature (25+1 0C) and light (12 hours night / day cycle) with access to clean water. An ethical clearance for animal use was obtained for this research work.

2.2 Snake Venom

Lyophilized Naja nigricollis venom was purchased from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria Nigeria.

3. EXPERIMENTAL DESIGN

3.1 Determination of Naja nigricollis Venom Phospholipase A2 Activity:

Twenty five (25) ml of ethanol (99.7%), 1 ml of venom (200 μg/ml) and 0.1 ml of 5% CaCl2 solution were added in same order to two vessels, one containing 1ml of diethyl ether and the other containing 1ml of 1.2% egg yolk (dissolved in diethyl ether) as a source of lecithin. The mixture was titrated immediately with ice cold 0.02 N methanolic NaOH. The coagulation of the sample shows the activity of PLA2 [18].

3.2 Assessment of Snake Venom Phospholipase A2 Neutralizing Potential of Plant Extract:

A mixture containing 1.2% egg yolk (dissolved in diethyl ether) as a source of lecithin, different concentration of aqueous extract of Portulaca oleracae [7.8 to 500 μg (0.02 g of plant extract dissolved in 40ml of distilled water to get a concentration of 500 μg/ml and 2-fold serial dilution to get subsequent concentrations)] in 1ml, 1ml venom [200 μg/ml (0.025 g of lyophilized venom dissolved in 12.5 ml of normal saline to get a concentration of 20 0μg/ml)] and 0.1 ml of 5% CaCl2 solution were added and the reaction vessel was swirled or shaken until the reaction mixture becomes homogenous. The reaction mixture was allowed to stand under room temperature for 4 hrs. At the end 25 ml ethanol and 0.3 mL of cresol red were added and titrated with ice cold 0.02 N methanolic NaOH. A blank was prepared by addition of the same volumes of ethanol, venom and CaCl2 to the ether in same order and was titrated immediately. The hydrolysis capacity of PLA2 exhibited by reacting 200 μg/ml of venom with lecithin, was considered as100% phospholipase activity, and served as control [18].

3.3 In vivo snake venom toxicity neutralizing potential of Portulaca oleracea plant extract on envenomed mice:

Eighteen albino mice were randomly divided into six groups of three mice each.

Group 1: Control group that received snake venom and normal saline.

Group 2: Envenomed mice that received plant extract at zero minute delay.

Group 3: Envenomed mice that received plant extract at five minutes delay.

Group 4: Envenomed mice that received plant extract at ten minutes delay.

Group 5: Envenomed mice that received plant extract at fifteen minutes delay.

Group 6: Envenomed mice that received combined extracts of Euphorbia hirta and Portulaca oleracea at fifteen minute delay.

The venom was administered intraperitoneally at a dose of 2828 µg/kg body weight of mice and the extract was administered orally at a dose of 250 mg/kg body weight of mice at different time

interval.

4. RESULTS

4.1 Naja nigricollis PLA2 Activity

The result of the PLA2 activity of Naja nigricollis showed no coagulation in the vessel that contains no egg yolk and coagulation with a 9.8 titer value in the vessel containing egg yolk.

TABLE 1

IN VITRO ASSESSMENT OF PLA2 ASSAY

Concentration of Plant Extract (µg/ml)	Ave Titer Value (ml)	Ave PLA2 neutralizing capacity (%)
500	3.4	57.5
250	4.2	47.5
125	4.9	38.8
62.5	5.5	31.3
31.25	5.9	26.3
15.63	6.8	15
7.8	7.2	10

Blank (without egg yolk) = 5.5

Control (with egg yolk) = 8.0

4.2 In Vitro Assessment of PLA2 Neutralization Assay:

The result of the effects of the aqueous leaf extract of Portulaca Oleracea on PLA2 of Naja nigricollis venom is as presented in table 1.

4.3 Effects of Time-lag After the Administration of 2828 µg/ml of Snake Venom and 250 mg/ml of Plant Extract on the Survival Rate of Mice:

TABLE 2

ADMINISTRATION OF SNAKE VENOM AND NORMAL SALINE (CONTROL)

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of normal saline administered (ml)	Result after 24hrs.
1 (f)	30.5	0.031	0.4	0.8	Died
2 (m)	26.0	0.026	0.4	0.7	Died
3 (m)	18.1	0.018	0.3	0.5	Died

LD100 is meant to kill 100% 0f the experimental animals in this group. So, this group of mice had a 0% survival and a 100% death after the administration of N. nigrocollis venom and normal saline.

TABLE 3

ADMINISTRATION OF SNAKE VENOM AND PLANT EXTRACT AT ZERO MINUTE DELAY

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of extract administered (ml)	Result after 24hrs.
1 (f)	25.7	0.026	0.4	0.7	Survived
2 (m)	26.3	0.026	0.4	0.7	Survived
3 (m)	23.7	0.024	0.3	0.6	Survived

The plant extract was effective after the zero minute delay. There was 0% death and 100% survival.

TABLE 4

ADMINISTRATION OF SNAKE VENOM AND PLANT EXTRACT AT FIVE MINUTES DELAY.

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of extract administered (ml)	Result after 24hrs.
1 (m)	24.8	0.025	0.4	0.6	Died
2 (f)	24.5	0.025	0.4	0.6	Survived
3 (f)	22.1	0.022	0.3	0.6	Died

33.3% of the experimental animals in this group survived while 66.7% died maybe due to some health factors. Though the male mouse looked weak and sick even before the plant extract was given to him.

TABLE 5

ADMINISTRATION OF SNAKE VENOM AND PLANT EXTRACT AT TEN MINUTES DELAY

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of extract administered (ml)	Result after 24hrs.
1 (m)	20.5	0.021	0.3	0.5	Survived
2 (f)	22.6	0.023	0.3	0.6	Survived
3 (f)	21.3	0.021	0.3	0.5	Died

The plant extract was effective after the ten minutes delay. There was a 66.7% survival and a 33.3% death rate and the mouse died after 12hours of envenomation and treatment.

TABLE 6

ADMINISTRATION OF SNAKE VENOM AND PLANT EXTRACT AT FIFTEEN MINUTES DELAY

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of extract administered (ml)	Result after 24hrs.
1 (m)	24.3	0.024	0.3	0.6	Died
2 (f)	20.6	0.021	0.3	0.5	Died
3 (f)	25.9	0.026	0.4	0.7	Died

From this group, the design shows that time of treatment/ plant administration is of essence because there was a 0% survival and a 100% death after fifteen minutes delay.

TABLE 7

ADMINISTRATION OF SNAKE VENOM AND COMBINED EXTRACT OF PORTULACA OLERACEA AND EUPHORBIA HIRTA AT 15 MINUTES INTERVAL.

THE EXTRACTS OF BOTH PLANTS WERE GIVEN AT A RATIO OF 1:1.

(sex)	Weight of animal (in g)	Weight of animal (in kg)	Volume of venom administered (ml)	Volume of extract administered (ml)	Result after 24hrs.
1 (f)	28.3	0.028	0.4	0.7	Survived
2 (f)	25.7	0.026	0.4	0.7	Died
3 (m)	15.7	0.016	0.2	0.4	Survived

The mice were envenomated and were treated with the combined extract of Portulaca oleracea and Euphorbia hirta after fifteen minutes and there was a 33.3% death and a 66.7% survival. This result shows that the combination of both plants is more effective than the single plants at fifteen minutes delay.

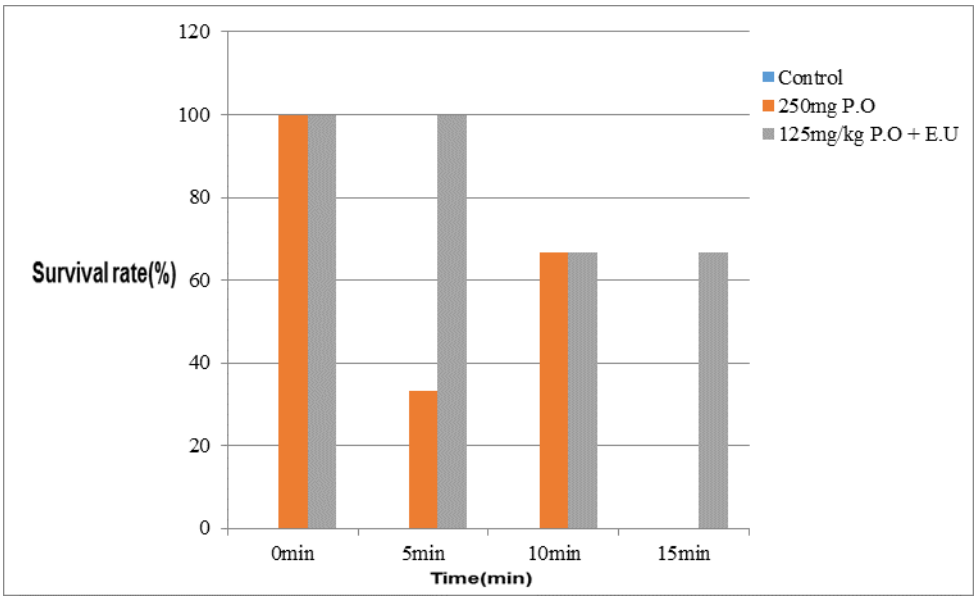

Fig 2: A Bar Chart of % survival rate against the dose or treatment in time.

A Bar Chart of % survival rate against the dose or treatment in time.

The bar chat shows a 0% survival rate in the control of all the groups, a 100% survival in the zero minute delay group, a 0% survival in the fifteen minutes delay group of the single plant dose and a 66.7% survival rate in the fifteen minutes delay group of the combined plant extract.

5. End Sections

5.1 Discussion

Snake bite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria. Although anti-venom (which is prepared from animal sera) immunotherapy is the only treatment available against snake envenomation, it is associated with many side effects which include; anaphylactic shock, pyrogen reaction and serum sickness. These are possible outcomes of the action of antigenic proteins present in higher concentrations in anti-venom [2]. Also, antivenom (antisera) do not neutralize the local tissue damage [3], are not available in remote areas and are quite expensive. Although, the use of plants against the effects of snake bites has been recognized, more scientific attention has been given to it since last 2 decades [19].

Several plants have been used in folk medicine throughout the world as treatment against snakebites [20], [21], [22], [23], [24], [25], [26], [10], [13]. Till date few plants materials have been evaluated in well controlled assays and only a few of them have been found to be effective against Snake envenomation.

The search for bioactive molecules in plants used in folk medicine has been growing in the past few years. This study shows that Naja nigricollis venom can inhibit or induce metabolism in mice and has also shown that Portulaca oleracea, a medicinal plant, neutralized some biological effects induced by Naja nigricollis venom [13].

The phytochemical constituents of P. oleracea has shown the presence of saponin, alkaloid, tannin, flavonoid, cardiac glycoside, terpenoids, protein and starch as its active phytoconstituents with saponin as the major constituent. The presence of these constituents in most plants has been reported to have proven its medicinal usage in health issues [27].The phytochemical analysis of Portulaca oleracea has also shown that it is a good source of omega-3 fatty acid and vitamins which means that it is good for consumption.

Pharmacologically, it is a good antibiotic, antioxidant, anticancer, antimicrobial, anti-inflammatory, antiulcerogenic, and hepatoprotective agent.

The LD50 result for the ethanolic leaf extract of Portulaca oleracea plant showed no mortality or abnormal behavior in mice after 24hrs [13].

The LD50 result of Naja nigricollis venom showed no mortality on the groups of mice that were envenomed with a lower dose of 10-1000 mg/ml but the experimental animals in the groups that received a higher dose range of 2000-5000 mg/ml of Naja nigricollis venom all died within 24 hrs [13] which tells us how toxic this venom can be when it gets in contact with one's biological system.

Ethanolic leaf extract of Portulaca oleracea neutralized the toxic effects at different time intervals in mice envenomed with the LD50 result of Naja nigricollis venom [13]. This plant therefore appears to be a promising chemical agent for use as first aid treatment, or in combination with antiserum.

The result for LD100 of Naja nigricollis venom (2828 µg/ml) used on mice had a 100% mortality on the mice in the

control group within 24 hrs (table 2). This shows how poisonous the Naja nigricollis venom used is.

The positive effects of the plant extract was seen in the zero minute delay with a 100% survival of the experimental animals after 24 hrs.

Time is of essence when administering this plant extract because all of the experimental animals died (0% survival) at 15minutes delays (table 6), while the five and ten minutes delay recorded a 33.3% and 66.7% survival respectively (tables 4 and 5).

Portulaca oleracea + Euphorbia hirta combined plant extracts neutralized the toxic effects of the venom at the fifteen minutes delay as shown in table 7.

PLA2 which has shown to be present in all snake venom [5] is a good target when considering treatment against snake envenomation and it was neutralized by the aqueous leaf extract of Portulaca Oleracea in an in vitro assay. The result of the PLA2 activity of Naja nigricollis venom showed no coagulation in the vessel that contains no egg yolk and coagulation with a 9.8 titer value for the vessel containing egg yolk. This is due to the toxic clotting factors present in snake venoms which acted on the lipids in the egg yolk.

The result of the effects of the aqueous leaf extract of Portulaca Oleracea on PLA2 of Naja nigricollis venom showed that as the concentration of the plant extract decreased, the PLA2 neutralizing capacity also decreased.

The different experimental designs carried out showed that the aqueous and ethanolic leaf extract of Portulaca Oleracea is effective on envenomed mice and is more effective when combined with Euphorbia hirta, another medicinal plant. Phytochemical analysis of Euphorbia hirta revealed the presence of alkaloids, flavonoids, saponins, tannins, phytosterols, polyphenols and cardiac glycoside in the leaves of the plant [28].

5.2 Conclusion

From this study, it can be concluded that the extract of Portulaca oleracea is effective in neutralizing the toxic effects of Naja nigricollis venom. Time of treatment is of essence because the longer the time taken after the envenomation, the more disastrous the toxins in the venom damages the body cells.

Further experiment could address the fractioning of the Portulaca oleracea extract in order to identify the bioactive compounds responsible for these observations, their efficacy, safety and the mechanism of action which could possibly lead to the development of pharmaceutical formulations for treating snake bite accidents-victims.

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