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<Review>

Snake Venom Phospholipase A_2 and its Natural Inhibitors

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Abstract – Snakebite is a severe medical, economic, and social problem across the world, mostly in the tropical and subtropical area. These regions of the globe have typical of the world's venomous snakes present where access to prompt treatment is limited or not available. Snake venom is a complex mixture of toxin proteins like neurotoxin and cardiotoxin, and other enzymes like phospholipase A_2 (PLA_2), haemorrhaging, transaminase, hyaluronidase, phosphodiesterase, acetylcholinesterase, cytolytic and necrotic toxins. Snake venom shows a wide range of biological effects like anticoagulation or platelet aggregation, hemolysis, hypotension and edema. Phospholipase A_2 is the principal constituent of snake venom; it catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid, which is the precursor of eicosanoids including prostaglandins and leukotrienes. The information regarding the structure and function of the phospholipase A_2 enzyme may help in treating the snakebite victims. This review article constitutes a brief description of the structure, types, mechanism occurrence, and tests of phospholipase A_2 and role of components of medicinal plants used to inhibit phospholipase A_2.
Keywords – Phospholipase A_2 (PLA_2), Glycerophospholipids, Snake venom, Medicinal plants, Natural inhibitors

Introduction

The World Health Organization has tagged venomous snake bite as one of the "Neglected disease conditions in the Tropics" in 2009 (WHO Neglected Tropical Diseases, 2010).[1] The name of snake bites as disease condition was dropped out from the list of Neglected Tropical Diseases in 2013, after sometime World Health Organization (WHO) reconsidered snakebites as Neglected Tropical Diseases on June 9th, 2017.[2] About 90% of snakebites are caused by the 'big four' among the crawlers - common krait, Indian cobra, Russell's viper and saw-scaled viper. India has long believed to have more snakebites than any other country in the world. In India, approximately 35,000 – 50,000 deaths are supposed because of snakebite every year.[3] 0.47% of the total mortality has assigned to snakebites. Snakebite has a higher rate of death in rural areas (97%), which are more common among males (59%) than in females (41%), and peaked at age ranges between 15 – 29 years (25%) during the rainy months of June to September.[4]

Venom is a type of zootoxin that is delivered to prey through sting or bite. Snake venoms contain a mixture of different components with an extended range of biological and pharmacological activities. These components consist of proteins which are higher than 90% of their dry weight, including proteases, phospholipase A_2, hyaluronidase, L-amino acid oxidases, nucleases, esterases, and many others.[5] Certain proteins exhibit enzymatic activity, while many others are non-enzymatic proteins and peptides. Though, the toxicity of snake venom is correlated to the number of toxins present in different venoms. Snake venoms have several toxins and enzyme, which are generally classified as hemotoxin, myotoxins, cardiotoxins, cytotoxins and neurotoxins.[6,7] This review is mainly focusing on the phospholipases A_2 (PLA_2) present in the different snake venom and its potential inhibitors.

Phospholipase A_2 is the principal constituent of venom; it is also found in pancreatic juice, synovial fluid and many other tissues of mammals.[8] Phospholipase A_2 paralyzes the prey by affecting the peripheral nervous system; it also affects the muscular system, as several PLA_2 acts on skeletal muscle which causes massive

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damage to the tissue (myotoxins). However, other effects have also been observed like anticoagulation or platelet aggregation, hemolysis, hypotension and edema.[7,9] Different Phospholipase A_2 show diverse characteristic according to their regulation, localization, structure, mechanism of action and their dependency on divalent metal ions.[10] The Phospholipase A_2 present in venom is a water-soluble enzyme which functions as a hydrolytic agent .[11] It hydrolyzes monomeric, micellar or lipid bilayer phase; but in case of hydrolysis of it acts as phospholipid at higher critical micelle concentration of the lipid and acts poorly on monomeric phospholipid.[12,13]

Phospholipase A_2 forms a superfamily which has 15 separate, distinguishable groups and several subgroups of PLA_2. Phospholipase A_2 enzymes stay assigned to specific groups based on their molecular weight, sequence, disulfide bonding, and the requirement for Ca^{2+} for action.[14,15] Group numbering was established by utilizing the pre-existing venom designation of I and II and expanding them to include subgroups IA, IB, and IIA designation for the secreted PLA_2 ($sPLA_2$); based on origin Group III for the clearly different PLA_2 is added which is procured from bee venom, and establishing the Group IV (GIV) designation for the cytosolic PLA_2 ($cPLA_2$).[10,15]

Structure of phospholipases A_2 – The molecular weight of PLA_2 is 13,000 Da with 116 – 124 amino acid residues, and six or seven disulphide bonds. It is a small water-soluble protein; it is highly resistant to denaturation because of its strong disulfide bond.[10] The superfamily of phospholipase A_2 includes groups comprising four main types including the secreted $sPLA_2$, cytosolic $cPLA_2$, calcium-independent $iPLA_2$, and platelet-activating factor (PAF) acetylhydrolase/oxidized lipid lipoprotein-associated (Lp) PLA_2.[15,16] Each class of phospholipase A_2 is involved in signal transduction cascade.[17] More than 180 PLA_2 structures are determined while 3D structures of many PLA_2 are studied by NMR and X-ray crystallography.[18] The crystal structure of a group I PLA_2 is monomeric even if the sources are different; the PLA_2 has similar scaffolds in their monomer unit. Some group II PLA_2 are dimeric.[19] The classification (Fig. 1) is given below:

(A) Secreted PLA_2

The first discovered PLA_2 enzyme was a secreted phospholipase A_2 molecule. They have a low molecular weight of 13-15 KDa with a catalytic site consisting of histidine and calcium bound to it and six conserved disulfide bonds with one or two variable disulfide bonds.[15,20]

(B) Cytosolic PLA_2

Cytosolic PLA_2 is composed of calcium-dependent lipid-binding C2 domain and a catalytic a/b hydrolase domain; it has a serine and aspartic acid dyad in the active site which is required for the calcium activity.[21] Group IVA is the first group, IV cytosolic PLA_2 discovered in human platelets.[22]

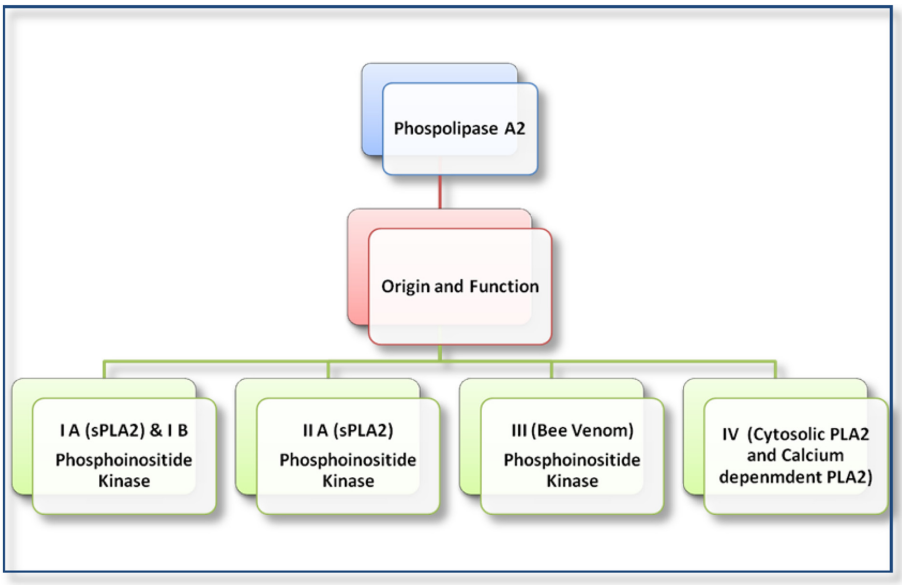

Fig. 1. Classification of major Phopspholipase A_2 groups of snake venom with respect to their distinctive nature in which Group 1A and IB PLA_2 are similar to old world snake venom PLA_2's like cobra. Group II is secretary type of enzyme, whereas Group III are mammalian PLA_2 isolated from Bee venom and group IV are classified differently because of their Ca^{2+} dependent action and cytosolic origin.

(C) Calcium Independent PLA_2

The group VI family of PLA_2 enzyme includes the calcium-independent PLA_2. They do not require calcium for their catalytic activity.[23]

(D) The PAF acetylhydrolase/oxidized lipid $LpPLA_2$

The group VII family includes the PAF acetylhydrolase/ oxidized lipid $LpPLA_2$. Unlike other PLA_2, this enzyme can access substrate in the aqueous phase as it contains serine, aspartic acid hydrolase triads and histidine.[24,25]

Mechanism action of phospholipases A_2 – Phospholipases A_2 are hydrolytic enzymes that release fatty acids from the second carbon group of glycerol. Unsaturated fatty acid tails in phospholipids are hydrolyzed at the sn-position by this enzyme which leads to the generation of lysophospholipids and other unsaturated fatty acids[26,27] (Fig. 2). These products of hydrolysis cause a wide range of adverse pharmacological effects by changing the physical properties of cell membranes and by activating downstream signal transduction pathways.

Snakebites and associated symptoms in humans – Snakebite is a serious public health problem in any region of the globe. Lack of effective treatment, precise identification of the biting snake and application of specific antivenoms has lead.[1,28] In many outreached rural areas deal with a dosage of noxious, emetic, infusions to drink and herbs, even mud and stones are also applied to wound of snakebite. Cuts are repeatedly made over and around the bitten site; also on every possible site on the bitten limb in an attempt to release the venom. Anxiety, nausea, vomiting, headache, and fainting are prevalent non-specific symptoms in snakebites.[29] Swelling, redness, and pain are often related to envenoming bites; they arise because of inoculated toxin enzymes of venom move in the lymphatic system earlier it enters the circulation.[30,31] Local bruising, sensation and skin/muscles tissue necrosis may rise as a consequence of cytotoxins in the venom of many snakes, including cobras and most viperids and crotalids.[32] Systemic envenoming characteristically presents with either: haemotoxic reactions resulting from the activity of haemorrhaging, anticoagulants, procoagulants, and haemolytic factors; neuromuscular weakness as an outcome of toxins present in the venoms which blocks AChRs at the neuromuscular junction or neuro-myotoxicity subsequent from toxins that initiate neuro- and/or myo-degeneration.[33] However, coagulopathy is one of the main cause of mortality in snakebite cases across the world, where Phospholipases A_2 acts as one of the strongest anticoagulant.[34] The basic mechanism behind this anticoagulation is driven by the hydrolysis of phospholipids. However, in the absence of phospholipids, the inhibition action of PLA_2 is reported.[35]

Test/Method – Worldwide some specific detection test against PLA_2 enzyme have been reported ranging from simple *in-vitro* biochemical assays, agar plate assays based on biological extracts, ex-vivo assays on animal tissues etc.[36,37] However, tests like 20 min coagulation test are used to determine coagulopathy caused due to snakebite.[38,39] The acidimetric method is used to determine the PLA_2 assay.[40] For this assay, lecithin suspension is prepared with lecithin, calcium chloride, and an equal proportion of sodium deoxycholate. A homogenous mixture is made with a pH 8. PLA_2 from venom or any other source can add to phospholipid containing medium to initiate the hydrolysis. The decrease in pH indicates the formation of fatty acid due to hydrolysis. µmoles of fatty acid released/ minute is measured to express the enzyme activity. The venom or the enzyme PLA_2 is pre-incubated with extracts for half an hour at 37 ºC to study the rate of inhibition.[41,42] Apart from lab testing methods, clinical cohort studies are also performed on patients suffering from envenomation of different Australian and Asian snakes. Here, serum samples of patients were taken, and venom specific enzyme immunoassay (EIA) was performed against PLA_2. This PLA2 assay was found to be positive in both coagulopathic snakes (Russell's viper and Hump-nosed viper) and neurotoxic snakes (kraits and cobras).[42] To treat the people from snakebite, apart from detection of PLA_2, identification of potent is also important. These inhibitors should be examined for their efficiency, efficacy

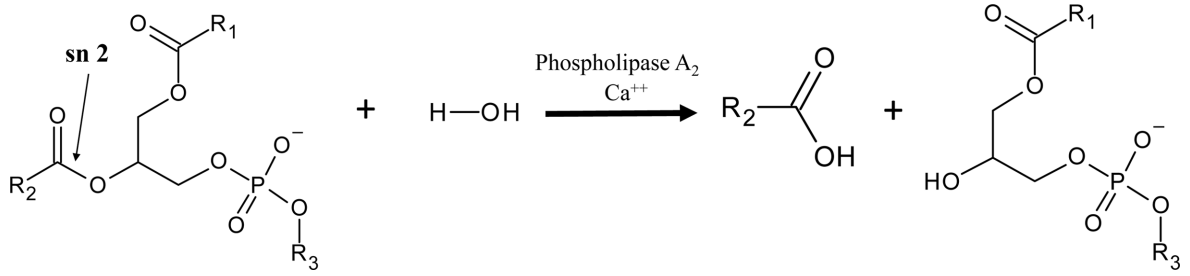

Fig. 2. Mechanism action of phospholipases A_2.

Table 1. Crude extract as inhibitors and their inhibition activity against snake venom phospholipase A_2

S. No.	Crude extract	Medicinal Plants	Inhibition studies	Ref.
1	Aqueous extract of stem bark	*Mangifera indica*	*In vitro* and *in vivo* PLA_2 activity	3
2	Ethanol extract of seeds	*Tamarindus indica*	*In vitro* and *in vivo* PLA_2 activity	61
3	Aqueous extract of leaves	*Morus alba*	*In vitro* and *in vivo* hemorrhagic, myotoxicity and edema inducing activity	60
4	Methanolic extract of seeds	*Vitis vinifera*	*In vitro* and *in vivo* hemorrhagic, myotoxicity and edema inducing activity	63
5	Ethanol and aqueous extract of rhizomes	*Dryopteris chochleata*	*In vitro* PLA2 activity	53
6	Petroleum, chloroform, ethanol (absolute) and methanol extract of stem bark	*Schumanniophyton magnificum*	*In vivo* PLA_2 activity	57
7	Methanol extract of fresh aerial part	*Eclipta prostrata*	*In vitro* and *in vivo* PLA_2 activity	55
8	Crude extract of fresh roots	*Tabernaemontana alternifolia*	*In vitro* and *in vivo* PLA_2 activity	66
9	Ethyl acetate extract of leaf	*Azima tetracantha*	*In vitro* and *in vivo* PLA_2 activity	65
10	Ethanol extract of roots	*Combretum leprosum*	*In vitro* and *in vivo* PLA_2 activity	70
11	Aqueous extract of leaves	*Casearia sylvestris*	*in vivo* activity	79
12	Ethanol extract of whole plant	*Andrographis paniculata*	*In vitro* and *in vivo* PLA_2 activity	80
13	Aqueous extract of leaves, stems, and roots	*Casearia sylvestris*	*In vitro* and *in vivo* PLA_2 activity	81
14	Methanol extract of whole plant	*Ganoderma lucidum*	*In vitro* and *in vivo* PLA_2 activity	82
15	Aqueous extract of leaves	*Schizolobium parahyba*	*In vitro* and *in vivo* PLA_2 activity	83

Table 2. Purified natural inhibitors and their inhibition activity against snake venom phospholipase A_2

S. No.	Purified natural inhibitors	Source	Inhibition studies	Ref.
1	Schumanniofoside	*Schumanniophyton magnificum*	*In vivo* PLA_2 activity	57
2	Wedelolactone and demethylwedelolactone	*Eclipta alba* (Genetically modified)	*In vitro* PLA2 activity	56
3	AIPLAI (Azadirachta indica PLA_2 inhibitor)	*Azadirachta indica*	*In vitro* PLA2 activity	58
4	Arjunolic acid	*Combretum leprosum*	*In vitro* and *in vivo* PLA_2 activity	70
5	Quercetin-3-O-rhamnoside	*Euphorbia hirta*	*In vitro* and *in vivo* PLA_2 activity	69
6	Sitosterol and stigmasterol	*Eclipta prostrata*	*In vivo* PLA_2 activity	84
7	Rosmarinic acid	*Cordia verbenacea*	*In vitro* and *in vivo* PLA_2 activity	85
8	2-hydroxy-4-methoxy benzoic acid	*Hemidesmus indicus*	*In vivo* PLA_2 activity	86
9	Aristolochic acid	*Aristolochia spp.*	*In vivo* PLA_2 activity	87
10	Biflavonoid morelloflavone	*Garcinia madruno*	*In vitro* and *in vivo* PLA_2 activity	88
11	Turmerin	*Curcuma longa*	*In vitro* and *in vivo* PLA_2 activity	89

and concentration.

Types of phospholipase A_2 inhibitors – Due to the high cost, extensive production period, short life span, and common medical side-effects of currently available anti-venom serotherapy, researchers have attempted to find inhibitors or antidotes from synthetic compounds, modified chemical molecules, herbal extracts and marine compounds.[43,44] Phospholipase A_2 is the suitable target and generally used for inhibitors screening of both natural and synthetic inhibitors.

1. Synthetic inhibitors

By exploiting computational biology tools for drug discovery, different bioinformatic database tools can play a major role in the selectivity of inhibitors which can be validated by *in-vitro* experiments. Inhibitors could be found in the synthetic library of compounds and obtained by chemical/biochemical synthesis, and in combination with *in silico* methodologies. Bioinformatics tools can be used for comparing synthetic compounds and its derivatives which have structural similarity with effective flavonoids like quercetin, that can bind to the active site of a phospholipase A_2. Through Molecular simulation Dynamics, a wide variety of synthetic molecules were examined for inhibition of phospholipase A_2 enzymes,

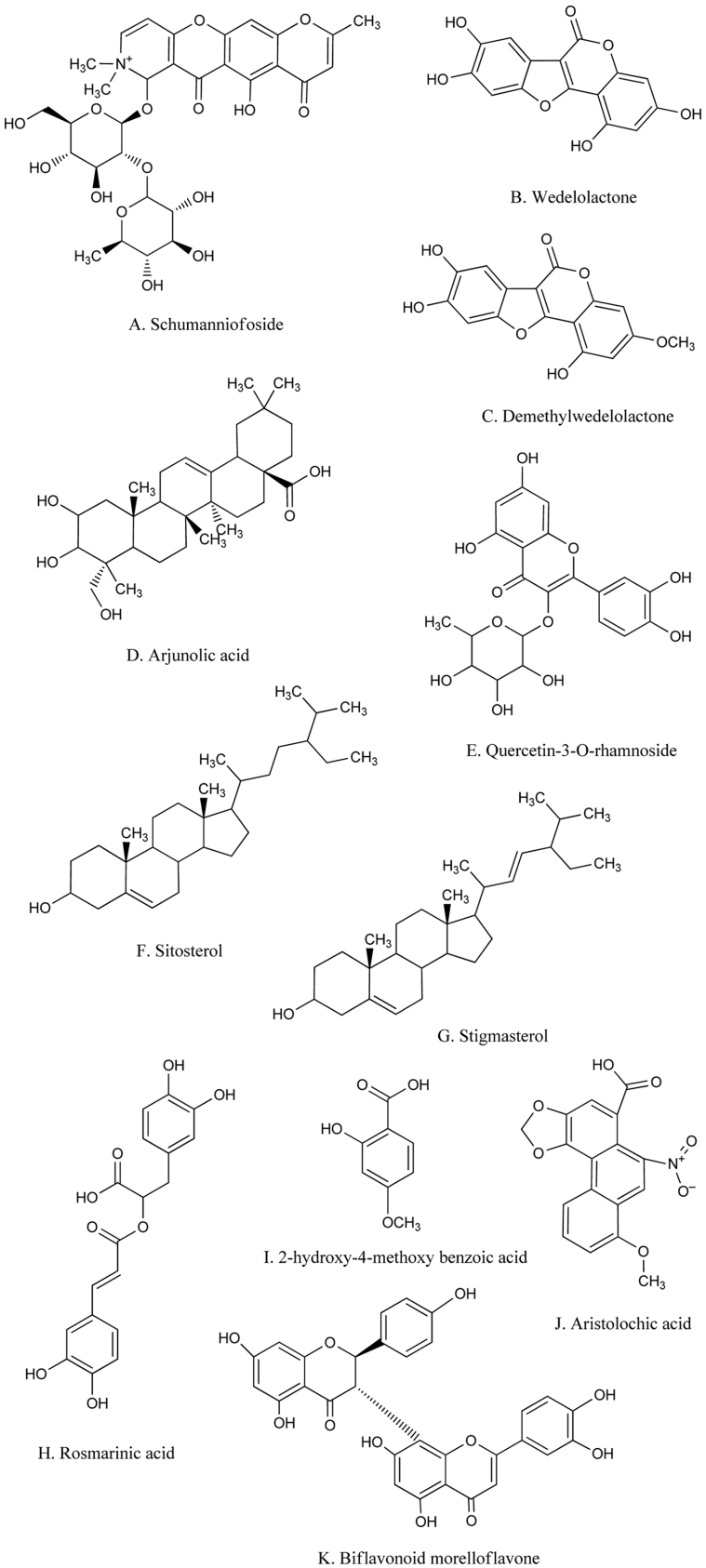

Fig. 3. Structures of plant constituents as natural inhibitors against venom.

with *in vitro* and *in vivo* validation studies, Varespladib was identified as a potent inhibitor.[45] This compound has been proposed as an effective broad-spectrum snake venom inhibitor and used for management of snakebite envenomation. Other inhibitors derived from 2-sulfenyl ethylacetate which inhibited phospholipase A_2 snake venom in micromolar concentrations are some of the inhibitors studied till now.[46]

2. Natural inhibitors

Management of snakebites is still carried out using traditional anti-venom therapy.[47] The usage of medicinal and herbal plants along with their constituents have wide application throughout human life, whose information is inherited by the involvement of countless generations.[48] Generic medications from the plants are ubiquitous by nature because of their use in mild disease treatment, for example, regular fewer, cold and cough etc. These therapies are benefited because of better patient resistance, cost effectiveness, trustworthiness, and a long history of utilization. Several therapeutic plant species or medicinal plants signify a vital source of bioactive constituents which are useful to the treatment of snake bites patient, or indirectly, applicable as boosts to conventional serum therapy.[49,50]

Plants and its components used against phospholipase A_2 – From ancient time the tribal healers utilize several local available plants in ethnomedicinal practices to neutralize the venom, different plant parts like the root, stem, leaves, flower, fruit and even the whole plant are used in this practice.[51,52] The indigenous people make use of the indigenous plant in the rural area to treat venom of scorpion, bee and snake.[44] Plant extracts have become a promising alternative to substitute traditional snake anti-venom, which often is unavailable in emergencies. Each plant has different types of phytochemical constituents which help in neutralizing the venom. The phytochemicals present in plants are flavonoids, terpenoids, saponin, alkaloids, tannins, glycosides, antioxidants, phytosterols etc. and each constituent has its own effect on inhibiting different proteins in venom. Alcoholic and aqueous extracts can be used against snake venom, the alcoholic extracts help in dissolving the polyphenolic components, which makes the extract more effective.

The ethanol and aqueous extract of *Dryopteris chochleata* show phospholipase A_2 inhibition against *Naja naja* venom.[53,54] The purified butanol extracts of *Eclipta prostrata* were evaluated against *Calloselasma rhodostoma* (Malayan pit viper) venom phospholipase A_2.[55] Producing secondary metabolites from genetically modified *Eclipta alba* using *Agrobacterium rhizogenes*, and this genetically modified plant has pharmacological properties and myotoxic activities against phospholipase A_2 of snake venom.[56] The methanol extract of *Schumanniophyton magnificum* from stem bark inactivates venom of *Naja melanoleuca*.[57] AIPLAI (*Azadirachta indica* PLA_2 inhibitor) plant compound purified from the leaf extract of *A. indica* shows inhibition against cobra and Russell's viper venoms phospholipase A_2 enzymes.[58] Therapeutically important plants *Nicotiana tabacum, Solanum incanum, Carissa spinanrum, Calpurnia aurea, Croton macrostachyus* and *Cynodon dactylon* revealed phospholipase A_2 inhibition activity.[59] Flavonoids are widely present polyphenolic compounds in plants; they inhibit PLA_2 activity, arachidonic acid release leading to the formation of arachidonic acid metabolites, particularly rutin. *Strychnosnux vomica* Linn seed extracts neutralize the lethal, hemorrhagic, defibrinating and phospholipase A_2 activity of *Daboia russeli* venom, as well as the deadly, cardiotoxic, neurotoxic and PLA_2 activities of *Naja kaouthia* venom.[51] *Morus alba* plant leaf extract has been completely eliminated the *in vitro* proteolytic, hyaluronolytic activities along with edema, hemorrhage and myonecrotic activities were also neutralized efficiently against the Indian *Vipera/ Daboia russelii* venom.[60] The *Tamarindus indica* seed extract revealed the enzyme inhibition activities against phospholipase A_2, protease, hyaluronidase, l-amino acid oxidase and 5'-nucleotidase of venom in a dose-dependent manner.[61] The pharmacological effects and enzymatic neutralization ability of bark extract of *Anacardium occidentale* against *Vipera russelii* venom hydrolytic enzymes such as phospholipase, protease, and hyaluronidase in a dose-dependent manner.[62] The *Vitis vinifera* (grapes seed) methanolic extract have the proteolytic activities and also efficiently neutralized the haemorrhage, and myonecrotic properties against the Indian Daboia/ Vipera russelli venom.[63] The anti-venom potential of stem bark aqueous extract of *Mangifera indica* has shown the enzyme inhibition activity against *Daboia russellii* (Russell's viper) venom enzymes such as phospholipase, protease, hyaluronidase, 5' nucleotidase, ATPase and alkaline phosphomonoesterase.[3] The folk medicine from Brazilian plant *Pentaclethra macroloba* against various effects induced by phospholipase A_2 of snake venoms.[64] The ethyl acetate leaf extract of *Azima tetracantha* has shown inhibition against *Bungarus caeruleus* and *Vipera russelli* venom enzymes such as 5' nucleotidase, phospholipase A_2, phosphodiesterase, acetylcholinesterase, phosphomonoesterase, and hyaluronidase in a dose-dependent manner.[41,65] The methanolic extract of *Tabernaemontana alternifolia* from root was found to

combat and neutralize phospholipase A_2 of *Naja naja* venom.[66] The ethanolic root extract of *Coix lacryma-jobi* exhibited anti-venom activity and neutralization of phospholipase A_2 against Indian cobra *Naja naja* venom.[67] The root extracts from *Cyclea peltata* were reported to have significant compounds such as heptadecanoic acid, tetradecanoic acid, hexadecanoic acid, and octadecadienoic acid which can counteract the toxins (acetylcholinesterase, protease and phospholipase A_2) present in *Naja naja* venom.[68] Quercetin-3-*O*-rhamnoside identified from *Euphorbia hirta* extract has revealed the snake venom inhibition activities such as protease, phospholipase A_2, hemolytic activity and hemorrhage inducing activity against *Naja naja* venom.[69] The *Combretum leprosum* extract and its arjunolic acid component shown *in vivo* and *in vitro* inhibition effects against *Bothrops jararacussu* and *Bothrops jararaca* venoms enzymes such as phospholipase A_2, protease, collagenase, hyaluronidase.[70]

Many scientific reports explain the mechanisms of snake venom neutralization including inactivation or precipitation of the toxic venom proteins especially phospholipase A_2, enzyme inhibition, chelation, adjuvant action, antioxidant activity or a synergistic interaction.[71] Most significant mechanism of snake venom neutralization involves inhibition of the active enzymes such as phospholipase A_2, metalloproteases, and hyaluronidases by plant secondary metabolites such as flavonoids, polyphenols, tannins, saponins, xanthenes, terpenoids, steroids, quinonoids, and alkaloids.[72] In this situation, the secondary plant metabolites interact with the snake venom proteins or enzymes by non-specific binding proteins through hydrogen bonding with hydroxyl groups in the protein molecules producing chemically stable complexes.[73]

Marine components against phospholipase A_2 – Phospholipase A_2 inhibitor from marine organisms works as anti-inflammatory agents which neutralize different snake venom.[74] Marine natural compounds as monoalide and its derivatives like scalaradial and related compounds such as Variabilin, a marine furano-terpene, pseudopterosins, vidalols, and 1, 4- dicarbonyl moieties are masking a group of terpenoids these compounds are said to inhibit the PLA_2 enzyme.[75,76] Many natural components isolated from marine algae such as *Chlorophyta*, *Phaeophyta* and *Rhodophyta* have been found to be effective inhibitors of bee venom derived PLA_2.[77] Marine components with anti-inflammatory activity and explicitly calcium-dependent phospholipase A_2 inhibitors are probable to provide meaningful and comprehensive information associated with marine-derived anti-inflammatory agents.[78] Calcium-dependent phospholipase A_2 inhibitor components were found in marine sponges and others were isolated from hard and soft corals, starfish-like crown-of-thorns, jellyfish, sea anemones, sea cucumbers and marine snails.[78]

Conclusion

In summary, the classification, structure and functions of phospholipase A_2 have been studied over the centuries. The identification of PLA_2 enzyme as the main constituent of snake venom has emphasized its importance for the treatment of snakebites. The significant element in any snake venom is phospholipase A_2 enzyme which causes significant pharmacological effects by interfering with the physiological processes of the victim. For that purpose, different types of PLA_2 have been studied to discern their detailed structure, action mechanism and biological functions. Study of PLA_2, its structure, types, and characteristic will help the researchers to find components that will work against PLA_2; the pharmacological issues can maximize, and better-targeted delivery system can develop. The synthetic and natural inhibitors are studied for the neutralization or inhibition of PLA_2 by using different assays mentioned in the review. Inhibitor of PLA_2 that has entered the body of victim helps in reducing the threat to lives. Medicinal plants remain the alternative therapeutic medicine for world populations, particularly in rural or tribal areas where snakebites are frequent.

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Conflict of Interest

The author declares that there is no conflict of interests regarding the publication of this manuscript.

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