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# *In vitro* anti-venom potential of various *Jatropha* extracts on neutralizing cytotoxic effect induced by phospholipase $A_2$ of crude venom from Indian cobra (*Naja naja*)

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## Abstract

In present study various species of *Jatropha* were evaluated for antidote nature induced by phospholipase $A_2$ ($PLA_2$) cobra venom. Although qualitative phytochemical analysis exhibited less variation across *Jatropha* species studied, substantial variability in terms of $PLA_2$ inhibition by various solvent extracts across the species and between different parts of same plant was observed. Among all samples methanolic extracts of *J. gossypifolia* leaf showed highest inhibition of $PLA_2$ toxicity while some aqueous extracts of *J. foetida* and all aqueous extracts of *J. curcas*, enhanced $PLA_2$ activity. Present results highlight *Jatropha* not only as rich source of secondary compounds with antidote property for snake bite but also potent toxic agents as revealed with increased hemolysis by some aqueous extracts of *J. curcas* and *J. foetida*. Our findings suggest that methanolic leaf extract of *J. gossypifolia* contain potent small molecular antagonist(s) to the snake venom $PLA_2$ which will be very useful to design adjuvant therapies in treatments of snake bites.

## Introduction

Snakebite is one among vital public health issues of tropical countries including India. Most of the mortality cases come from farmers, hunter gathers of rural areas which lack proper sickbays and antivenin supply (Panfoli et al., 2010). *Naja naja* commonly called as Indian Elapid is one among the poisonous snakes with toxin sub-fractions like metalloproteases, phospholipases, hyaluronidases, neurotoxins, and myotoxins which wreak havoc in victim's body. Phospholipase $A_2$ is one of well studied fraction of cobra venom with many pharmacological effects like presynaptic neurotoxicity (Kini and Iwanga, 1986), myotoxicity (Mebs and Samejima, 1980), edema (Vishwanath et al., 1987) cardio-toxicity and hemolysis (Condrea et al., 1980, 1981).

Till date, antibodies produced in horses are the only source of antivenom, a process of time consuming. Moreover, antibodies need to store in low temperature conditions that lack in rural areas of developing countries. Snake bite victims sensitive to horse products produce hyper-sensitive side reactions when treated with antiserum of equine origin (Nazim et al., 2008). So, identification of alternative medicine with no risk and low temperature storage requirements became important task. Plant based antidote sources are known from olden ages for snake bites. *Jatropha* is one among

reported to have antidote property and used in some tribal medicines with no scientific evaluation. Phyto-compounds isolated from *Jatropha* plants are reported to have other therapeutic activities like anti-inflammatory, antitumor, molluscidal, insecticidal and fungicidal activities (Albuquerque, 2006).

The present study is aimed on systematic evaluation of antidote potentials of various solvent extracts of *Jatropha* species.

## Materials and Methods

### *Plant selections, collections and material preparations*

For the present study, three species of *Jatropha* viz., *Jatropha gossypifolia* (J.G), *Jatropha foetida* (J.F) and *Jatropha curcas* (3 lines) are selected. *J. gossypifolia, J. foetida* (collected from Seshachalam Hills, Tirupathi), were identified by Dr. A. Madhusudhana Reddy, Assistant Professor, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh, India. Three distinct varieties of *J. curcas* (these materials were generously provided by Dr. L. Sivarama Prasad, Naturole Bioenergy Ltd., India, which were originally collected from Sai Petro Chemicals, Maharastra (J.C-1); Aleru, Warangal (J.C-2); and Uganda, Africa (J.C-3); the three varieties of *J. curcus* are being presently maintained in Yogi Vemana University, Kadapa) were selected based on genetic diversity shown by molecular genetic studies carried out using RAPD and ISSR profiling (Data not shown). Leaves, stems and roots of each variety were collected separately, thoroughly washed and shade dried.

### *Snake venom*

Lyophilized crude venom of *Naja naja* (Indian cobra) was purchased from "The Irula Snake-Catchers Industrial Cooperative Society (ISCICS), Mamallapuram, Tamil Nadu and the crude venom was stored in airtight containers at 4°C until used.

### *Plant materials sampling and soxhlet extractions*

Equal amount of all dried plant materials (viz., leaf, stem and roots separately) were pulverized to fine powder and subjected to soxhlet extraction with chloroform, methanol and water solvents at elevated temperatures of 40, 45 and 75°C respectively. The extract obtained was concentrated through rotavapour at a constant temperature of 38°C for chloroform, methanolic extracts and 46°C for aqueous extracts with the help of water bath. Solid extracts were weighed and stored in dry environment until further use in opaque containers in refrigerator.

### *Phytochemical screening and analysis*

Equal amount of each extract (45 extracts in total) obtained from Soxhlet method was used for qualitative phytochemical estimations and identifications of various secondary metabolites like, alkaloids, flavonoids, terpenoids, saponins, tannins and phenolics present in the extracts by using standard methods as described elsewhere (Trease and Evans, 1996), Sofowora (Sofowora, 1993), and Harborne (Harborne, 1998). The positive results were noted as present (+) and negative results as absent (-).

### *Antivenom assay*

### *Preparation of RBC cells from blood sample*

Human blood was collected in glass tubes containing sodium citrate as anticoagulant. The blood samples were centrifuged at 800 rpm for 10 min and plasma was carefully removed by sterile Pasteur pipette. The pellet of red blood cells were washed three times by PBS buffer (0.15 M NaCl, 10 mM phosphate buffer, pH 7.0) and then suspended in a fresh PBS buffer at a density of $1.2 \times 10^9$ cells/mL monitored by a Neubauer chamber. To this 1% of egg albumin was added (Habermann and Neumann, 1954; Gutierrez, 1988) and the resultant mixture was considered as substrate to snake venom $PLA_2$s in the hemolytic assay described below herein.

### *Hemolytic assay*

Toxicity of the venom and its neutralization by the *Jatropha* extracts was determined through indirect hemo-lytic assay (Habermann, 1954; Gutierrez, 1988; Paula et al., 2010) by using human erythrocytes and hen's egg yolk emulsion as substrate. The amount of venom (10 µg/mL) that can produce 70%hemolysis after the incubation was denoted as the Minimum Indirect Hemolytic Dose (MIHD). Experiments were performed by pre-incubating 25 µg of *Jatropha* extract with a MIH dose of *Naja naja* venom for 30 min at 37°C prior to evaluating the hemolytic activity. To this, 5 mL of prepared erythrocyte suspension was added and incubated for further thirty min at 37°C. After 30 min, venom activity on the substrate was stopped by adding 3 mL of ice cold PBS buffer to the reaction mixture. The extent of cell lysis/hemolysis caused by the venom on the RBC cells was measured by calculating the amount of hemoglobin released from the cells. The amount of released hemoglobin from the reaction mixture was estimated from the intensity measured at 540 nm using UV spectroscopy. The hemolysis produced by the venom in the absence of *Jatropha* extracts was taken as 100% and the percentage of inhibition/activation by each *Jatropha* extract used in the present study against snake venom $PLA_2$s was calculated as shown in the following calculations. All measurements were made in triplicates and results are expressed as mean + standard deviation.

% inhibition of $PLA_2$ activity by plant extracts = 100 – 100 x OD of test sample at 540 nm/OD of control sample at 540 nm

**Table I**

**Phytochemical analysis of *Jatropha* extracts**

| Phytocompound | Alkaloids | Flavanoids | Terpenoids | Saponins | Tannins | Phenolics |
|---|---|---|---|---|---|---|
| Plant[a] | Aqueous extract | | | | | |
| J.G Stem | - | + | + | + | + | - |
| J.G Leaf | - | + | + | + | + | - |
| J.G Root | - | + | + | + | + | - |
| J.F Stem | + | + | + | + | + | - |
| J.F Leaf | + | + | + | - | + | - |
| J.F Root | + | + | + | - | + | - |
| J.C-1 Stem | + | + | + | + | + | + |
| J.C-1 Leaf | + | + | + | + | + | + |
| J.C-1 Root | + | + | + | + | + | + |
| J.C-2 Stem | + | + | + | + | + | + |
| J.C-2 Leaf | + | + | + | + | + | + |
| J.C-2 Root | + | + | + | + | + | + |
| J.C-3 Stem | + | + | + | + | + | + |
| J.C-3 Leaf | + | + | + | + | + | + |
| J.C-3 Root | + | + | + | + | + | + |
| | Methanol extract | | | | | |
| J.G Stem | + | + | - | + | + | + |
| J.G Leaf | + | + | - | + | + | + |
| J.G Root | + | + | - | + | + | + |
| J.F Stem | + | + | + | + | + | + |
| J.F Leaf | + | + | + | + | + | + |
| J.F Root | + | + | + | + | + | + |
| J.C-1 Stem | - | + | + | + | + | + |
| J.C-1 Leaf | - | + | + | + | + | + |
| J.C-1 Root | - | + | + | + | + | + |
| J.C-2 Stem | - | + | + | + | + | + |
| J.C-2 Leaf | - | + | + | + | + | + |
| J.C-2 Root | - | + | + | + | + | + |
| J.C-3 Stem | - | + | + | + | + | + |
| J.C-3 Leaf | - | + | + | + | + | + |
| J.C-3 Root | - | + | + | + | + | + |
| | Chloroform extract | | | | | |
| J.G Stem | + | + | + | + | + | + |
| J.G Leaf | + | + | + | + | + | + |
| J.G Root | + | + | + | + | + | + |
| J.F Stem | + | - | + | - | + | + |
| J.F Leaf | + | - | + | - | + | + |
| J.F Root | + | - | + | - | + | + |
| J.C-1 Stem | + | + | + | - | + | + |
| J.C-1 Leaf | + | + | + | - | + | + |
| J.C-1 Root | + | + | + | - | + | + |
| J.C-2 Stem | + | + | + | - | + | + |
| J.C-2 Leaf | + | + | + | - | + | + |
| J.C-2 Root | + | + | + | - | + | + |
| J.C-3 Stem | + | + | + | - | + | + |
| J.C-3 Leaf | + | + | + | - | + | + |
| J.C-3 Root | + | + | + | - | + | + |

[a]J.G - *Jatropha gossypifolia*; J.F- *Jatropha foetida*; *Jatropha curcas* varieties, J.C-1; J.C-2 and J.C-3 as described in materials

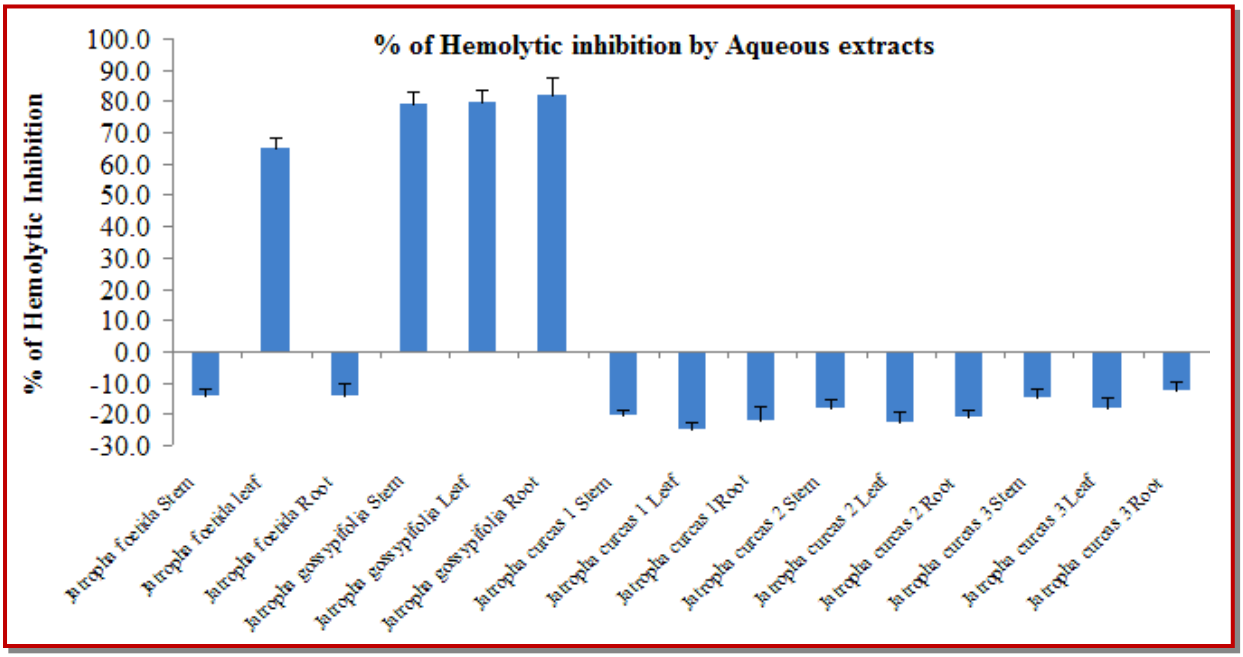


Figure 1: Effect of aqueous extracts of stem, leaf and root obtained from various *Jatropha* herbs used in the present study against hemolysis induced by snake venom $PLA_2$ (Increased hemolysis activity of venom by phytocompound)

## Results

Selection of *Jatropha* lines for present phytochemical and antidote analysis (Table I) was based on the phylogenetic tree constructed from molecular genetic studies of 35 lines using RAPD and ISSR marker profiling (data not shown here) was found to be helpful for initial screening. Our results strengthen the reports of *Jatropha*'s use as an antidote in folk medicine.

For the forty five extracts obtained (3 solvents x 5 plants x 3 parts of each plant–stem, leaf and root) qualitative phytochemical analysis exhibited less variation among the species of *Jatropha* viz., *gossypifolia* (J.G- stem, J.G- leaf and J.G- root), *foetida* (J.F- stem, J.F- leaf and J.F- root), and *curcas* (J.C- stem, J.C- leaf and J.C- root), and almost no varietal variation was found within the species (varieties – J.C-1) of *curcas* and also no significant variation of phytocompounds between different parts of same *Jatropha* plants is observed (Table I).

The selection of plants for the present study, based on phylogenetic analysis was found to be worth full as the same kind of variation has been reflected in both phytochemical composition and antiophidian property of various *Jatropha* extracts. Owing to the records of using *Jatropha* as antidote for snake bite in folk's medicine, investigation on different species of *Jatropha* phytocompounds inhibiting $PLA_2$ was carried out. All the aqueous extracts of *J. curcas*, roots and stem extracts of *J. foetida* were found to be lethal as the extracts increased hemolysis when compared with positive control which has only venom (MIHD) (Figure 1). Interestingly, it was also found that these extracts alone did not cause any membrane lytic effect on red blood cells (results not shown). Of all the aqueous extracts, *J. gossypifolia* leaf, root and stem showed high hemolytic inhibition of 85.9, 84.5 and 81% respectively (Figure 1). Variation was found in terms of antivenom activity across aqueous extracts of *J. foetida* plant parts viz., stem, leaf and root. Only leaf extract of *J. foetida* showed anti-venom property in terms of $PLA_2$ inhibition whereas stem and root aqueous extracts of the plant showed enhanced-hemolytic activity resembling *J. curcas* extracts when incubated with venom.

Methanolic extracts of all three varieties of *J. curcas* showed good inhibition within a range from 81 to 88.3% and the variation among different parts of a same plant was also negligible (Figure 2). However, extracts of various parts from *J. gossypifolia* and *J. foetida* showed high variations in the degree of their inhibition potentials against hemolytic activity of $PLA_2$. Roots of *J. foetida* (74.5%) and leaf of *J. gossypifolia* (89.8%) showed high inhibitions. Of all methanolic extracts examined in the present study, leaves extracts of *J. gossypifolia* showed strongest inhibition of $PLA_2$ (Figure 2).

All chloroform extracts of *J. curcas* showed very less inhibition of venom hemolysis which were in the range of 2.6 to 22.4%. In *J. foetida*, leaf showed very less inhibition of 9.1% and root extract showed high inhibition of 81.7%. In *J. gossypifolia* the leaf showed the highest of 87.5% $PLA_2$ inhibition followed by stem and root (Figure 3).

Of all three species of *Jatropha* (*J. foetida, J. gossypifolia* and *J. curcas),* and within three selected lines of *curcas*

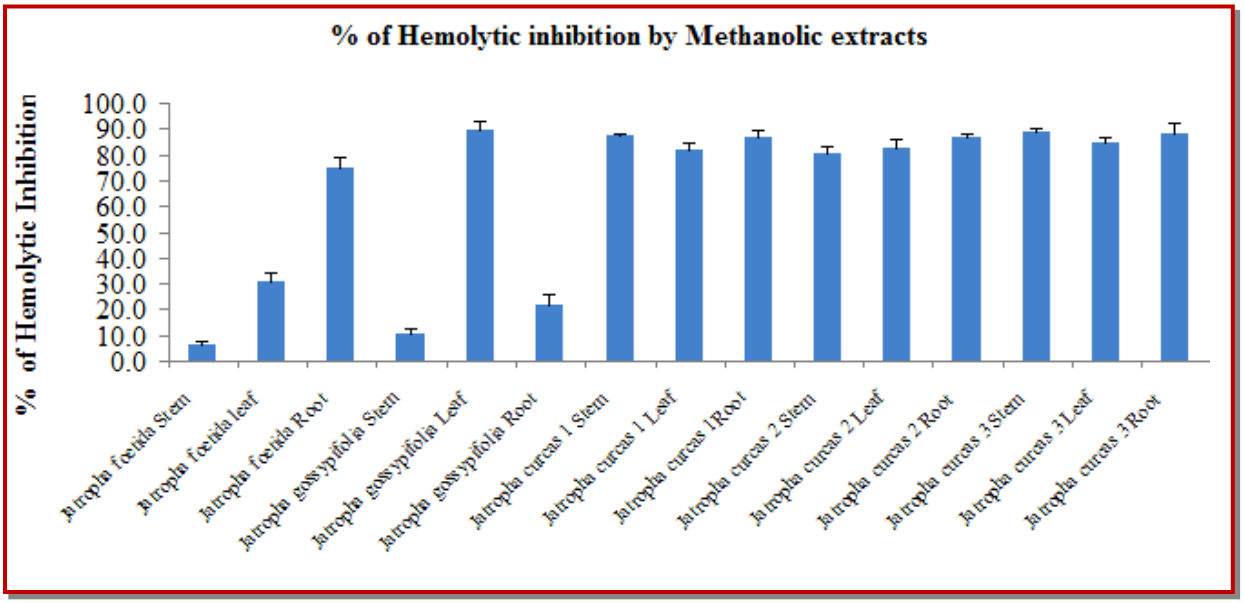


Figure 2: Effect of methanolic extracts of stem, leaf and root obtained from various *Jatropha* herbs used in the present study against hemolysis induced by snake venom $PLA_2$

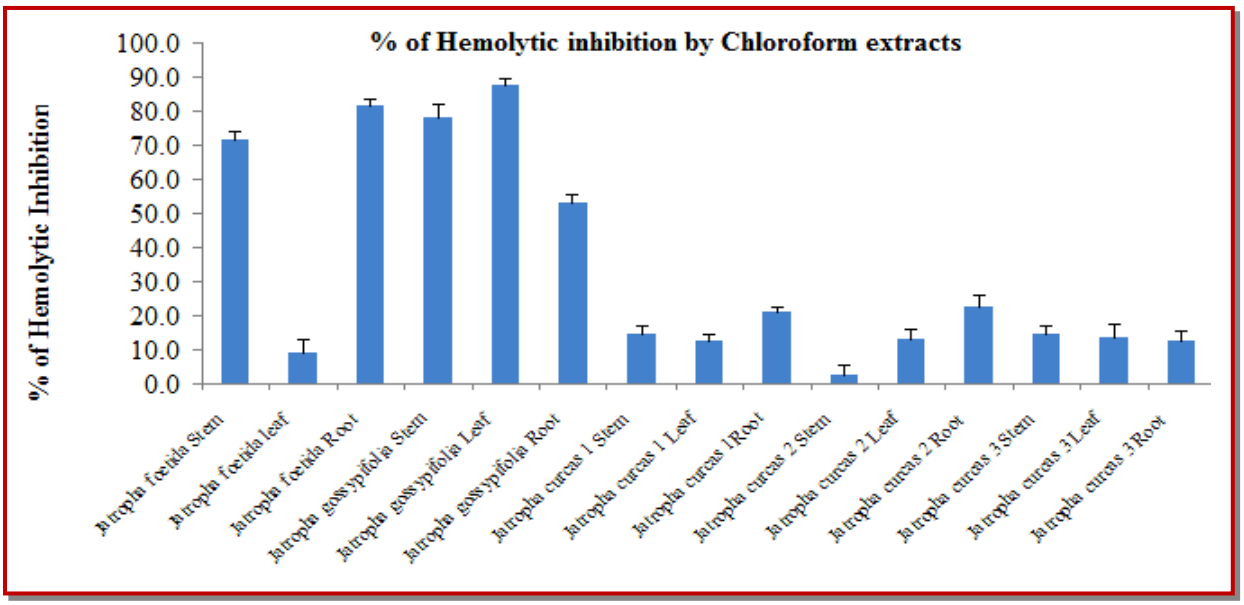


Figure 3: Effect of chloroform extracts of stem, leaf and root obtained from various *Jatropha* herbs used in the present study against hemolysis induced by snake venom $PLA_2$

collected from diverse geographical conditions, *J. gossypifolia* leaf extracts was found to be good choice as antidote for *Naja naja* venom due to its high inhibition potential against $PLA_2$ activity vis-à-vis that of other extracts as demonstrated above. Among aqueous, methanolic and chloroform extracts of *J. curcas*, only methanolic extracts were showing high anti-venom properties as inhibition was less in case of extracts from chloroform and negative inhibition could be seen with aqueous extracts from all three varieties of *curcas*. The high inhibition exerted by the methanol extract of *J. gossypifolia* leaf against the *Naja naja* venom strongly suggest for presence of phytocompounds possessing therapeutic potentiality with respect to $PLA_2$ inhibitions in the extracts.

All chloroform extracts of *J. curcas* showed very less inhibition of venom hemolysis which were in the range of 2.6 to 22.4%. In *J. foetida*, leaf showed very less inhibition of 9.1% and root extract showed high inhibition of 81.7%. In *J. gossypifolia* the leaf showed the highest of 87.5% $PLA_2$ inhibition followed by stem and root (Figure 3).

## Discussion

The present study is aimed to identify *Jatropha* species with potent antidote property. Of the three species selected for present study, our results clearly indicate all the species of *Jatropha* can't be used as source of antidote for snake venom as indicated from our results based on increased hemolysis activity of snake venom when incubated with aqueous extracts of *J. curcas and J. foetida*. Though *Jatropha* is used in local / folk medicine as antidote for snake bite, our observations indicate that all species of *Jatropha* and all parts of the plant cannot be taken granted as antidote as a few of them are capable of enhancing venom activity in terms of hemolysis and consequently may lead to lethal. Our results highlight that overlooking or adulteration of plant materials with other plant materials at species level or with different parts of the same plant may not only reduce therapeutic efficiency of extracts but may also increase toxic effects as seen in the case of aqueous extracts of *J. curcas* and *J. foetida.* Moreover, the above anti-hemolytic studies and phytochemical analysis clari -fied that even the phytocompounds are qualitatively found similar in different parts (stem, leaf and root) of the same plant, their quantities may vary from part to part as extracts of different parts of a plant showed a great degree of variations in their venom inhibition potentials. Hence, choosing of a plant species, part of the plant and extract of the part are of great importance because of great variation in phytochemical distribution of which some are found to have therapeutic value as in present case of *J. gossypifolia* extracts and some exhibit lethal properties as seen in case of aqueous extracts from all *J. curcas* varieties and also stem and root extracts of *J. foetida.*

The observations clearly indicate that the aqueous extracts of *J. curcas* and *J. foetida* must have micro/ macro compounds or ions that may aid to enhance phospholipase $A_2$ activity of snake venom. There are reports on increased activity of phospholipase $A_2$ when the enzymes combine with some of the divalent ions like $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$ etc., These ions are reported to enhance the activity of phospholipase $A_2$ by inducing conformational changes in the active site and substrate-binding site of the $PLA_2$ and the resultant conformation of the enzyme may either promote or suppress its biological activity (Jiang et al., 1989). The presence of quercetin-like compounds may also be a reason for increased hemolytic activity and they can form a lipid-raft like domains (Chiou et al., 2012).

From a scientific approach carried out in the present study to identifying potent plant-based antidote(s) to crude venom of *Naja naja*, *J. gossypifolia* leaf was found to be more effective source for compounds with anti-venom properties to inhibit $PLA_2$ that causes destabilizing the plasma membranes of cells and there by affecting cardiac, nervous and circulatory systems.

The extracts that were found to be a promising natural antidote, may presumably act by forming complexes with metal ions and proteins which in turn may change the conformation of active site and there by inhibiting the action of phospholipases $A_2$.The use of this kind of natural antidotes may reduce the risk of anaphylactic shocks in patients sensitive to equine products. Also, the natural antidotes have appreciable technical advantages when compared to commercial anti-venoms that need to be stored at very low temperatures (below 273 K) in a highly sophisticated refrigerator which are technically sound and they are presumably cost protective to have at all parts of rural areas of developing, undeveloped and even for developed countries. Work is in progress on identifying potent compounds responsible for anti-venom activities from methonalic leaf extract of *J. gossypifolia* at molecular/ atomic level resolutions. In these backgrounds, we strongly believe that the outcomes will be very useful to design adjuvant therapies for treatments of snake bites.

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