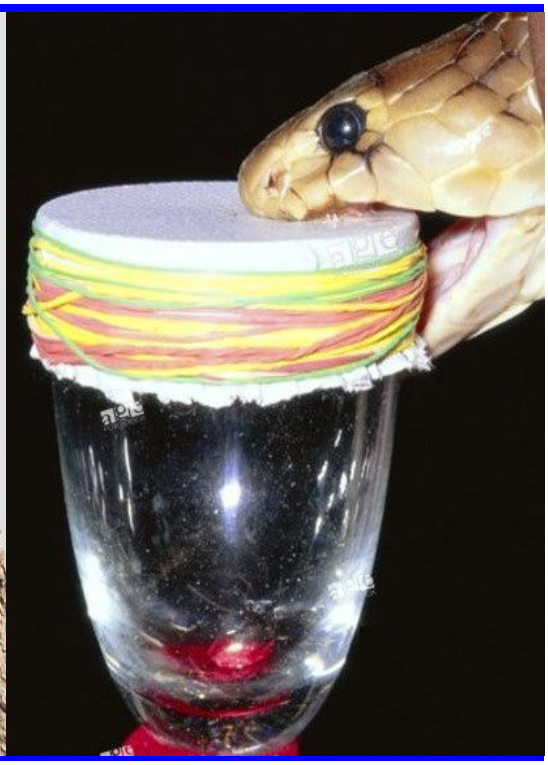

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In vivo studies on detoxifying actions of aqueous bark extract of *Prosopis cineraria* against crude venom from Indian cobra (*Naja naja*)

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Abstract

Detoxification effect of aqueous, methanol and petroleum ether extracts of medicinal plants such as *Aristolochia bracteolata, Mucuna pruriens, Prosopis cineraria* and *Rauvolfia tetraphylla* was systematically screened against lethality of crude venom of *Naja naja* using Swiss albino mice as animal models. We have herein demonstrated that aqueous bark extract of *P. cineraria* has substantial anti-venom potential vis-à-vis other extracts used in the present study. The aqueous extract at the dose of 14 mg/kg b.w. was able to almost completely neutralize the lethal activity of $3LD_{50}$ (1.1 mg/kg b.w.) of the cobra venom and the extract did not cause any types of adverse side-effects to the animal models. The investigation justifies not only the veraciousness of the extract used by traditional healers of Asian subcontinent as antidotes to snake venoms and also suggests that the aqueous extract should contain specific inhibitors to most principle toxic components of the crude venom.

Introduction

A total number of snake species identified is about 2000 to date and nearly 300 species of them are venomous snakes, which prevail in all parts of the world except 'Antarctica' (Karalliedde, 1995; Mohapatra et al., 2011). However, snakebite is being a serious public health problem in equatorial and tropical regions. Particularly in India and neighboring countries, nearly 200,000 snakebite-cases per year have been reported as per a recent survey on medical problems associated with snakebite (Bawaskar., 2004).

Indian cobra (*Naja naja*) is one of the four poisonous snake species found in India and crude venom of the *Naja naja* is rich source of protein toxins exhibiting pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity and tissue lytic activities (Dufton et al., 1988; Harvey, 1991; Sivaraman et al., 1999; Biswajit and Sivaraman, 2013). Though anti-venom to the Indian cobra venom could be developed a century ago, many technical and clinical concerns preclude its availability in all medical centers and utility to a large number of cases, respectively (Williams et al., 2010; Gilon et al., 1989).

Interestingly, numerous plants have been used as antidotes against various snake venoms in the folk medicinal systems of several countries (Mendes et al., 2007; Dal Belo et al., 2008; Ode et al., 2006). The veraciousness of the plants for the treatments of snakebite could be convinced by traditional healers and the folklore is far before the commercial anti-venoms were invented.

In these backgrounds, quite a number of laboratories from several countries have focused their gist of research on identifying plants that are capable of detoxi -fying the snake venoms from various species and also on evaluating their anti-snake venom effect through

modern scientific methods (Fry et al., 2002; Pithayanukul et al., 2009). Moreover, a systematic investigation on understanding the scientific basis of the herbal medicines to snakebite treatments is justified owing to cost-protective and side-effects (such as anaphylaxis, pyrogen) of polyvalent antiserum, only remedy presently available for the treatment of snakebite patients. Thus, the studies on the herbal medicines are believed to pave ways for developing effective first-aid treatments to snakebite and also to identify prototypes of small chemical molecules specifically inhibiting various protein toxins present in the snake venoms.

In the present study, we have examined anti-snake venom activities of plants such as *Aristolochia bracteolata*, *Mucuna pruriens*, *Prosopis cineraria* and *Rauvolfia tetraphylla* against crude venom of *Naja naja*. Aqueous, methanol and petroleum ether extracts of roots of *A. bracteolata*, seeds of *M. pruriens*, barks of *P. cineraria* and roots of *R. tetraphylla* have been prepared and solubility of the extracts in saline and as well saline containing crude venom (1.1 mg/mL) were tested using spectrophotometric methods (Sivaraman et al., 1998). The *in vivo* effect of the plant extracts (showing solubility in saline) on cobra venom have been studied using Swiss albino mice (with equal ratio of both genders) as animal models. The systematic experiments revealed that aqueous bark extract of *P. cineraria* has remarkable anti-venom potential vis-à-vis other extracts used in the study. The anti-venom potential of *P. cineraria* is unprecedented in the literature and we have also brought into fore a comparative analysis on the anti-venom activities of *P. cineraria* with that of other plants tested against the venom, as reported in the literature to date. In addition, implications of the study on identifying/designing lead antagonists to various protein toxins present in the venom of Indian cobra have been concisely discussed.

Materials and Methods

Selections, collections and extractions of medicinal plants

Based on surveys conducted with quite a number of traditional health healers and tribal people from various parts of south east India, roots of *A. bracteolata*, seeds of *M. pruriens*, barks of *P. cineraria* and roots of *R. tetraphylla* were selected for the present investigation. Barks of *P. cineraria* and roots of *A. bracteolata* were collected from Nalla malla forests, Andhra Pradesh and seeds of *M. pruriens* and roots of *R. tetraphylla* were collected from Mamallapuram, Tamil Nadu, India during autumn seasons of 2011 and 2012. All the four plants were authenticated by botanists at SASTRA University, Thanjavur, Tamil Nadu and voucher specimen for each of the plants have been deposited in the herbarium of the university.

The freshly collected materials of the 4 plants were washed, shade-dried at room temperature and pulverized to coarse powder. Aqueous and methanol extracts of the plants were prepared using maceration and soxhelt extraction methods, respectively. In maceration method, about 300 g coarse powder of the plant material was soaked in 1,000 mL double deionized water containing 0.05% (v/v) chloroform for 72 hours with intermittent mixing at every 2 hours in room temperature (25 ± 1°C). The suspension was filtered using Grade 1 Whatman filter paper (purchased from Sigma-Aldrich) and presence of phytochemical mixture was obtained in solid form upon evaporating the solvent at a defined temperature for appropriate time periods (Ibrahim et al., 1996). In Soxhlet method, about 300 g of coarse powder of plant material was systematically suspended in 1,500 mL methanol and refluxed at 65°C at a stretch of 60 - 70 hours (depending on the plant materials). Methanol extracts of the plants were concentrated in solid form upon removing the solvent from the solution by using distillation methods (Abubakar et al., 2000).

The raw materials of the plants used for preparing methanol extracts were further subjected to similar extraction procedure described above in order to prepare petroleum ether extract of the plants. All the plant extracts were stored in airtight containers at 4°C until used and amounts of the extracts are expressed in terms of their dry weights. All chemicals used in the study were of analytical grade.

In vivo animal studies

Lyophilized crude venom of *Naja naja naja* (Indian cobra) was purchased from "The Irula Snake-Catchers Industrial Cooperative Society (ISCICS), Mamallapuram, Tamil Nadu. Swiss albino mice (24 animals of each gender) with average weight of 31 g were used as test animals and the animals were housed in standard cages at room temperature of 25°C under 12/12 hours light/dark cycles throughout the experiments. The total numbers of 48 animals were divided into eight groups (Group I–VIII) such that each group had six (3 male and 3 female mice) animals. The Group I, II and III–VIII were used as healthy control group, envenomed control group and test groups, respectively; the Group I animals were treated with saline only; the Group II animals were treated with saline containing $3LD_{50}$ (1.1 mg/kg b.w.) of crude venom of Indian cobra; Group III to VIII were treated with saline containing $3LD_{50}$ of the crude venom pre-incubated with 14 mg/kg b.w. of plant extracts (Group III, IV, V, VI, VII and VIII were treated with aqueous root extract of *A. bracteolata*, methanol root extract of *A. bracteolata*, aqueous bark extract of *P. cineraria*, methanol bark extract of *P. cineraria*, aqueous root extract of *R. tetraphylla* and methanol root extract of *R. tetraphylla*, respectively) for 30 min at temperature of 310 K. In all the cases, the

mode of administrations for test samples was intra-peritoneal (i.p.) injection. The number of mice survived in each group was monitored for the time period of 14 days and survival time periods of all experimental animals were also precisely calculated.

Significance of the survival timings were determined statistically by subjecting the data to ANNOVA and data depicting $p<0.05$ were considered significant in the analysis (Steel et al., 1960). Studies performed using animal models of the present work were carried-out at Animal House, SASTRA University and Department of Siddha Medicine, Tamil University, TN, using standard and approved experimental protocols (ethical clearance (226/SASTRA/IAEC/RPP) is taken for the research work from Institutional Animal Ethical Committee of SASTRA University).

Results and Discussion

Solvents such as water, methanol and petroleum ether, which are polar, semi-polar and non-polar respectively, were used to extract all types of phytochemicals from various selected parts of the 4 medicinal plants, *A. bracteolata*, *M. pruriens*, *P. cineraria* and *R. tetraphylla*. As mentioned in the 'Methods and Materials' section, coarse powder prepared from the roots of *A. bracteolata* and *R. tetraphylla*, from barks of *P. cineraria* and from seeds of *M. pruriens* were used for preparations of various solvents extracts of the plants. Table I shows dry weights of various extracts of the 4 plant materials and some of their physical characteristics. The data suggest that the amounts of aqueous extracts of the plant materials from *A. bracteolata*, *M. pruriens*, *P. cineraria* and *R. tetraphylla* were about 10.3, 14.8, 3.4 and 5.9% (w/w), respectively. Similarly, the amounts of methanol extracts of the *A. bracteolata*, *M. pruriens*, *P. cineraria* and *R. tetraphylla* were about 10.7, 3.4, 9.9 and 12.3% (w/w), respectively. However, the amounts of petroleum ether extracts of all the four plants were found to be around 0.2 mg per gram crude powder (i.e. about 0.02% w/w) of the plant materials. Moreover, solubility and turbidity of the 12 solvent extracts in saline solution (used as a medium for dissolving crude venom and plant materials for the animal experiments) and saline containing $3LD_{50}$ of crude venom (toxic control) was examined using spectrophotometry at 600 and 280 nm, respectively. From the optical measurements, it was found that petroleum ether extracts of all the plants used in the study and both of the aqueous and methanol extracts of *M. pruriens* seeds were only sparingly soluble in the medium. Owing to the poor solubility of these extracts, only 6 (aqueous and methanol extracts of *A. bracteolata*, *P. cineraria* and *R. tetraphylla*) of the 12 solvent extracts prepared from 4 medicinal plants were chosen for further systematic examinations on understanding biological significances of the extracts against lethality induced by *Naja naja* venom by using animal models. It is important to mention that the selected 6 extracts are fully soluble in saline at the concentration of 2 mg/mL and also do not precipitate protein toxins of crude venom dissolved at concentration of 100 µg per mL saline. These stringent criteria facilitated, as demonstrated below, for authenticating presence of drug-likeness small molecular compounds antagonizing principle toxic components of *Naja naja* crude venom.

Table I

Experimental details for various solvent extracts prepared from 4 medicinal plants and physical characteristics of the solvent extracts

Plant name (Part used)	Extraction method	Solvent used for extraction[$]	Coarse powder used[a] (g)	Amount of extract obtained (g)	Color of extract/Solubility of extract in saline
A. bracteolata (Root)	Maceration	Water	300	30.8	Dark brown/Soluble
A. bracteolata (Root)	Soxhlet	Methanol	300	32.0	Olive green/Soluble
A. bracteolata (Root)	Soxhlet	Petroleum ether	X	≈ 0.05	Gray/Insoluble
M. pruriens (Seed)	Maceration	Water	300	44.4	Black/Insoluble
M. pruriens (Seed)	Soxhlet	Methanol	300	32.2	Brown/Insoluble
M. pruriens (Seed)	Soxhlet	Petroleum ether	X	≈ 0.02	Lime/Insoluble
P. Cineraria (Bark)	Maceration	Water	300	10.1	Maroon/Soluble
P. Cineraria (Bark)	Soxhlet	Methanol	300	29.6	Metallic brown/Soluble
P. Cineraria (Bark)	Soxhlet	Petroleum ether	X	≈ 0.03	Light green/Insoluble
R. Tetraphylla (Root)	Maceration	Water	300	17.7	Dark Brown/Soluble
R. Tetraphylla (Root)	Soxhlet	Methanol	300	37.0	Saffron red/Soluble
R. Tetraphylla (Root)	Soxhlet	Petroleum ether	X	≈ 0.04	Brown/Insoluble

[$]Water containing 0.05% (v/v) chloroform was used for preparing aqueous extracts; [a]The letter 'X' denotes the plant material obtained after subjecting 300 g fresh coarse powder to methanol extractions

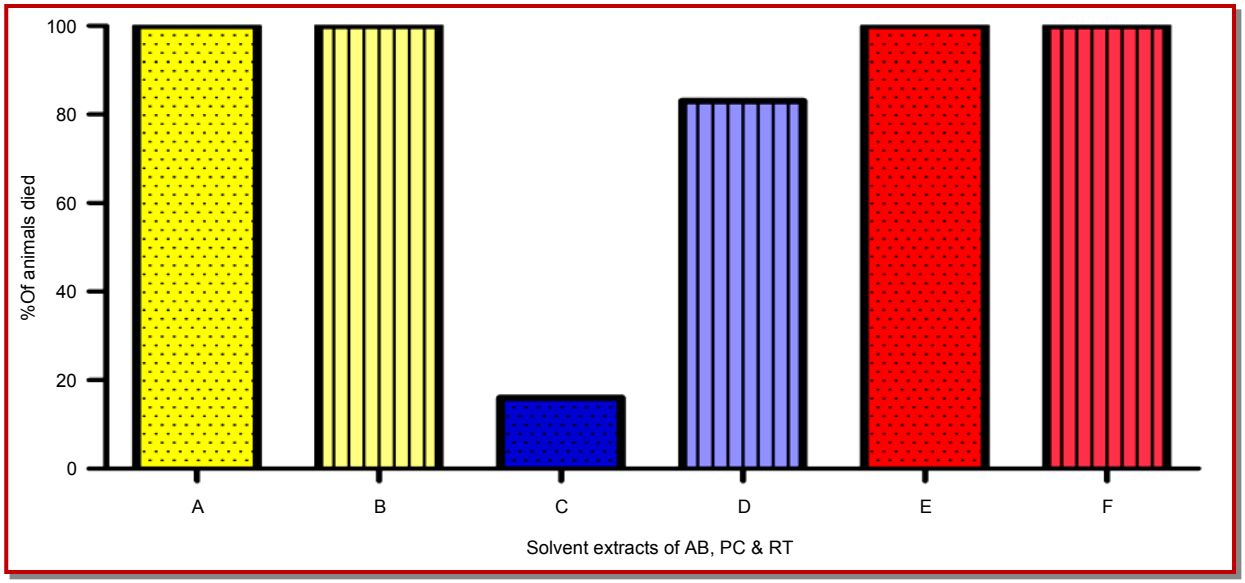

Figure 1: Overall *in vivo* anti-venom effect of plant extracts (A – aqueous extract of *A. bracteolata*; B - Methanol extract of *A. bracteolata*; C – aqueous extract of *P. cineraria*; D - Methanol extract of *P. cineraria*; E – aqueous extract of *R. tetraphylla*; F - Methanol extract of *R. tetraphylla*) against lethality induced by *Naja naja* crude venom is schematically represented as observed from the studies of animal models using Swiss albino mice.

Lethal dose (LD_{50}) of Indian cobra venom used in the present study was found to be 0.37 ± 0.06 mg/kg b.w. (data not shown), which is in good agreement with LD_{50} (0.28–0.80 mg/kg) values reported for the cobra venoms (obtained from different regions of the country) by various research groups in India (Meenatchisundaram et al., 2010; Premendran et al., 2011). Animals in the 'envenomed control' group were subjected to administration of $3LD_{50}$ and survival time of the animals was found to be 52 ± 8 min. Animals in the test groups were administrated to $3LD_{50}$ of the cobra venom pre-incubated with a plant extract at the dose of 14 mg/kg b.w. through i.p. mode of injections (refer to method section).

Figure 1 shows overall percentage of animals died in each of the test groups within the time span of 24 hours. The aqueous and methanol extracts of both of the *A. bracteolata* and *R. tetraphylla* did not protect the animals from the lethal dose of the venom and all the animals belonging to the groups died within 24 hours of the treatments. However, survival time of the envenomed animals treated with the aqueous extract of *A. bracteolata*, aqueous extract of *R. tetraphylla*, methanol extract of *A. bracteolata* and methanol extract of *R. tetraphylla* were estimated to be 156 ± 6, 201 ± 22, 80 ± 10 and 53 ± 11 min, respectively. Of the four extracts, except methanol extract of *R. tetraphylla*, other three extracts aid to increase the survival time of the envenomed animals comparing to that of animals treated only with lethal dose and significance of the increased survival times were also confirmed at confidence level of $p<0.05$ by using method of one-way ANNOVA. Interestingly, envenomed animals belonging to the group treated with methanol extract of *P. cineraria* and as well the group treated with aqueous extract of *P. cineraria* showed complete life protections for 1 and 5 (out of 6) animals, respectively. However, a mouse that managed to survive more than 24 hours from the group treated with methanol extract of *P. cineraria* died in the time span of around 48 hours (as there was only one survival animal from the group, statistics of the survival time is not given). Strikingly, life styles of the envenomed animals treated with aqueous extract of *P. cineraria* were monitored for at least 14 days from the treatments and we found that the animals that could survive did not develop any toxicity signs such as muscle contractions, akinesia, dyspnea and sedation suggesting that the aqueous extract of *P. cineraria* must contain highly efficient anti-venom compound(s) interacting with principal protein toxins and neutralizing them. These data obviously reveal that aqueous extract of *P. cineraria* has remarkable anti-venom potential vis-à-vis other solvent extracts obtained from the plants of *A. bracteolata*, *R. tetraphylla* and *P. cineraria* used in the present study.

Antivenin activities of a few medicinal plants such as *Andrographis paniculata, Andrographis lineata, A. bracteolata, Clerodendrum viscosum* and *Mimosa pudica* against the crude venom of Indian cobra have been reported using animal models in the literature (Kumar et al., 2010; Sakthivel et al., 2013; Richard et al., 2006; Meenatchisundaram et al., 2009, 2010). However, it is not a straightforward task to carryout a comparative analysis on the outcomes of the works as the studies are differing from each other in their treatments (pre-treatment/post-treatment/pre-incubation/etc.), modes of adminis-

trations (p.o./i.v./i.p./etc.), lethal doses (LD_{50}/$2LD_{50}$/$3LD_{50}$) and animal models (mice/rats/etc.). It has been demonstrated that though the alcoholic extract of *Andrographis paniculata*, ethyl acetate leaf extract of *A. lineata* and alcoholic root extract of *Clerodendrum viscosum* were unable to completely protect test animals envenomed with Indian cobra venom, the extracts facilitated to remarkably increase the survival time of the animals. Mixed aqueous leaf and root extracts of *A. bracteolata* protected 80% of test animals envenomed with LD_{50} of *Naja naja* at a dose level of 200 mg/kg (p.o.) and aqueous root extract of *Mimosa pudica* has been shown to have remarkable protection against the cobra venom at a dose level of 8 mg/kg (i.v). In these contexts, complete protection, without development of any adverse symptoms, of more than 80% of the animals envenomed with $3LD_{50}$ (a high lethal dose of the crude venom used in the animal models reported to date) observed upon treatment with aqueous bark extract of *P. cineraria* at a dose level of 14 mg/kg (i.p) is very impressive results and uncovers substantial effect of the extract on neutralizing toxic components of the snake venom. Thus, to date, leaves of *A. bracteolata*, roots of *M. pudica* (reported in the literature) and barks of *P. cineraria* (present study, the extract also does not cause any side-effect) are only promising sources for identifying lead compounds that are capable of inhibiting principle toxic components of the cobra venom. In these connections, understanding phyto-chemicals compositions of the plant extracts having efficient anti-lethal activities and pharmacokinetics of each of the metabolites of the extracts may pave a way for developing polyherbal formulations in a highly efficient and cost-effective manner for the first-aid treatments of snakebite cases in the subcontinent of Asia. Identification and evaluating the anti-lethal potential of phytochemicals present in the aqueous bark extract of *P. cineraria* are right now under progress in our laboratory. Moreover, the present study and several other reports in the literature on the anti-snake venom potential of various medicinal plants used by folk healers of the subcontinent justify the folklore on the snakebite treatments and bring scientific basis for the anti-lethal actions of the medicinal plants.

Conclusion

This study shows that aqueous bark extract of *P. cineraria* at the dose level of 14 mg/kg could almost completely protect (>80%) the test animals from $3LD_{50}$ lethal dose of the cobra venom.

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