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Anti-Venom Activity of *Camellia Sinensis* L. Leaves Extract on *Naja naja* Snake Venom.

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ABSTRACT

In almost many parts of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snakebite. In this study tea leaves which contains several medicinal values were used to detoxify the *Naja naja* venom. The dried and powdered tea leaves were subjected to solvent extraction. From the four different solvents extracts, isopropanolic extracts of tea leaves were screened for antivenom activity against *Naja naja* (cobra) venom via *in vitro* testing. *In vivo* analysis has also shown considerable results against inhibition of coagulant, fibrinolytic and phospholipase activities caused by cobra venom. About 500 mg/kg body weight dosage of plant extract were able to neutralize the lethal activity of 2LD50 of *Naja naja* venom by 83.33%. Edema formation was also neutralized by the extract effectively. The above observations confirmed that tea extracts possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite envenomation.

Keywords: Snakebite, anti-venom, *Naja naja, in vitro, in vivo. Camellia sinensis*

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INTRODUCTION

Snakebite contributes a serious medical and socio-economic problem in many parts of tropical and subtropical countries of the world [1]. The commonest Indian venomous snakes are common krait (*Bungaruscaeruleus*), common cobra (*Naja naja*), saw-scaled viper (*Echis carinatus*) and Russell's viper (*vipera russelli*) [2].Generally, it is very difficult to identify accurately the snake species that is involved in cases of envenomation.It may be identified by the symptoms that is caused by envenomation. This is due to the various types of toxins that are present in the venom of various snake species. Some of which are neurotoxin and cardiotoxin. For example the snake venom of *Naja naja* consists of α-neurotoxin and β-neurotoxin which inhibits neurotransmission. α-neurotoxin or post synaptic neurotoxin binds with high affinity to acetylcholine binding site on skeletal muscle on nicotinic receptor. Thus inhibits the opening of the ion channel and blocking nicotinic transmission causing paralysis. Similarly β-neurotoxin causes early changes in neurotransmitter release. It also causes blockage in K^+ current causes transient facilatory phase produced by β-neurotoxin causing repolarization[3].

More over different families of snake produce venoms with different pharmacological activities; clinical treatment against the snake envenomation often involves use of a polyvalent antibody as antivenin. These polyvalent antivenins have wide range of disadvantages such as the need to be kept at low temperatures and the allergic reactions which occur in some patients (Houghton). Over the years, many attempts have been made for the development of snake venom antagonists especially from plant sources. Since, India has a rich tradition of the usage of medicinal plants; many of them are also used to treat snakebite victims especially in rural areas [4].

Plants are used either single or in combination, as antidotes for snake envenomation by rural populations in India and in many parts of the world. Plants are reputed to neutralize the action of snake venom, with a plethora of plants claimed to be antidotes for snakebites in folk medicine[3]. Several plant species are popularly known as anti-snake venom and have been scientifically investigatedin many plant species [5-10].Generally most of the scientific documentation on antivenomous properties is reported in plant extracts which is specially preferred during medications. But always it is safer to have medicating values on plants which are routinely consumed in daily intake. It has also been reported that the extracts of tea leaves have many medicinal values[11-15].

The infusion of tea leaves was known in ancient China as a detoxifying medicine [16]. Tea polyphenols can also be used to inhibit the local tissue damage induced by snake venoms [17].Yao-Ching *et al.*(2004) [18]reported antivenin activity of melanin extracted from black tea for the first time. Inhibitory effect of *Camellia sinensis*leaves extracts against the neuromuscular blockade of *Crotalus durissus terrificus* venom was also reported [19].The present study is aimed at bringing the anti-venomous potential crude extract of tea leaves on *Naja naja* venom.

MATERIALS AND METHODS

Collection of plant leaves and preparation of extract

Fresh tea leaves of UPASI-9 clone were collected from UPASI Tea Research Institute, Valparai, Tamil Nadu, India. The collected leaves were shade dried, powdered and extraction was done using hexane, ethyl acetate, propan-2-ol and methanol as solvent in Soxhlet's apparatus. After extraction the solvents were evaporated by air dry and the powdered form of crude compounds was used for further assays.

Collection of Snake venom

The venom of *Naja naja* was collected from the Green cross corporation, Erode branch, Tamil Nadu, Indiaand stored in deep freezer. The collected venom was diluted in saline (pH 7.2-7.4) with concentration of 5mg/ml in ratio of 1:100 as stock. Further 1:500 dilution of stock solution was made and it was used as working concentration. The concentration of protein in snake venom was estimated by Lowry *et al.*(1951) [20].

Experimental animals

Healthy adult Swiss albino mice, weighing about 18-22g between 1 and 2 months of age, were used for the study. The study was approved by the Institutional Ethics Committee for animal experimentation and all the studies were carried out at KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India. Mice were housed individually in polypropylene cages, maintained under standard conditions (12 hrs light and 12 hrs dark cycle; 25^{o}C and 45-55% relative humidity).They had been given standard pellet diet supplied by Hindustan Lever Co. All the inhibition studies were carried with five groups where group-I received 20 µl of saline alone, group II received venom alone and group III were injected with venom with standard antivenom. Group IV and V received venom along with different doses of extract where group IV received extract of dose I (250 mg/kg of body weight) and the group V received Dose II (500 mg/kg of body weight) of extract.

Procoagulant activity

Procoagulant activity was carried according to the method of Theakston and Reid [21] as modified by Laing *et al.*(2003) [22]. Fresh human blood was mixed with 0.1M tri sodium citrate (1:9 v/v) and the mixture was centrifuged at 2000 rpm for 5 minutes. The supernatant obtained was used as Platelet Poor Plasma (PPP). Minimum coagulant dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Inhibition studies were carried with MCD value. 30µl of Tris-HCl (pH 7.6) buffer were added to 300 µl of PPP and pre-warmed for 15 minutes at 37°C before use. After 15 minutes venom (MCD value) and extracts in the ratio of 1:10, 1:20, 1:30, 1:40, 1:50 were added and the clot formation was initiated by adding 30 µl of 0.25 M calcium chloride and the clotting time was recorded. Neutralization was expressed as Effective dose (ED), defined as the µl of plant extracts in µg venom which clotting time was increased to three times when compared to clotting time of plasma incubated with MCD of venom alone. Control tubes had either plant extract alone or venom alone along with blood.To study the inhibitory effect of tea leaves on coagulant activity experiments were pre-designed using Design Expert Version 8.0 by two factor interaction method (i.e. extract volume and venom concentration) and the clotting efficiency by tea leaves extracts were analyzed.

Phospholipase (PLA2) activity

PLA2 activity in the *Naja naja* venom was measured on agarose erythrocyte- egg yolk gel plates to define the minimum indirect hemolytic dose (MIHD) according to Gutierrez *et al*.(2009) [23]. 3mm wells were made on 0.8% high EEO agarose where 1.2% of blood and 1.2% of lecithin were added along with different concentrations of venom. The MIHD was the dose of venom that induced a hemolytic halo of 11 mm diameter after incubation for 16 hours at 37ºC. Then the inhibition studies were carried out with MIHD value of venom with different concentrations of plant extracts and 10 mM calcium chloride. The effective concentration or neutralization value of the plant extract is defined as the concentration of extract which reduces the hemolytic halo from 11 mm to 5.5 mm.

Fibrinogenolytic activity

The fibrinogenolytic activity of *Naja naja* venom was determined by modifying the method of Ouyang and Teng (1976) [24]. Inhibition of fibrinogen was carried out in SDS-PAGE (15%) according to the method described by Laemmeli (1970) [25]. The reaction mixture containing bovine fibrinogen (20 µg) and venom (20 µg) in 5 mm Tris-HCl buffer (pH 7.4) and 10 mm NaCl was incubated for 15 minutes at 37°C. For inhibition studies snake venom was pre-incubated with different concentrations of extract and then it was incubated with above mixture for 15 minutes at 37ºC. After pre-incubation reactions were terminated by adding equal volume of sample buffer containing 0.2M Tris-HCl (pH 6.8), 20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue and 10 mM β-mercaptoethanol, followed by boiling at 100ºC for 10 minutes.

Lethal toxicity

The median lethal dose (LD50) of *Naja naja* venom was determined according to the method of Theakston and Reid (1983) [21]. Various doses of venom (5 µg to 50 µg) in 0.2 ml of physiological saline were injected into the mice via tail vein, using 3-5 mice for each venom dose. The LD50 was calculated as the venom concentration at which 50% of death occurs within 24 hours of venom injection. For studying the inhibition potential of lethality by tea extracts 2LD50 of *Naja naja* venom were pre incubated with various amount of

plant extracts (250 mg/kg, 500 mg/kg) for 30 minutes at 37°C. After incubation the mixture was injected intravenously into mice. Inhibition studies were carried with 5 groups with 3–5 mice at each antivenom dose as described earlier. The median Effective Dose (ED50) was calculated by probit analysis and defined as extract concentration at which 50% of mortality was inhibited.

Edema- forming Activity

Edema forming activity was carried out according to the method of Lomonte *et al.*(1993)[26] where different concentrations of venom (5 µg to 50 µg) were injected to right footpad of mice along with 20 µl of saline. 3-5 mice were chosen for each venom concentration and the left footpad of mice was injected with 20 µl of saline which served as control. After 1 hour legs were cut at ankle joint and edema formation were determined in both of the legs using formula, weight of edematous leg × 100/ weight of control leg. Minimum edema formation (MED) is defined as the venom concentration which induced the edema ratio of 120% compared to control leg. For inhibition studies venom was pre incubated with plant extracts of different doses (250 mg/kg and 500 mg/kg) for 1 hour and studies were carried out by injecting 3MED of venom. Effective dose of plant extract is defined as the dose of plant extract which reduced the edema ratio to 30% as with MED.

Statistical analysis

All the experiments were carried out for at least three independent observations and the results were expressed as mean ±SE. ANOVA were used for the statistical comparison and the values whose p value < 0.005 were considered as significant value.

RESULTS AND DISSCUSSION

Procoagulant activity

Preliminary screening was carried out with different extracts of tea leaves via all the above mentioned *in vitro* screening assays and the isopropanolic extracts of tea leaves were chosen for the *in vivo* analysis. Venom dose which induced clotting of plasma within 60 seconds was recorded as minimum coagulant dose (MCD). It was observed that 1 µg of *Naja naja* venom is capable of inducing clotting in 58.7±2.08 seconds and thus 1 µg of venom was considered as MCD for coagulant inhibition studies. The model suggested by Design Expert Version 8.0 to study inhibitory effect of isopropanolic extract of tea leaves was quadratic model with R value of 0.8971.

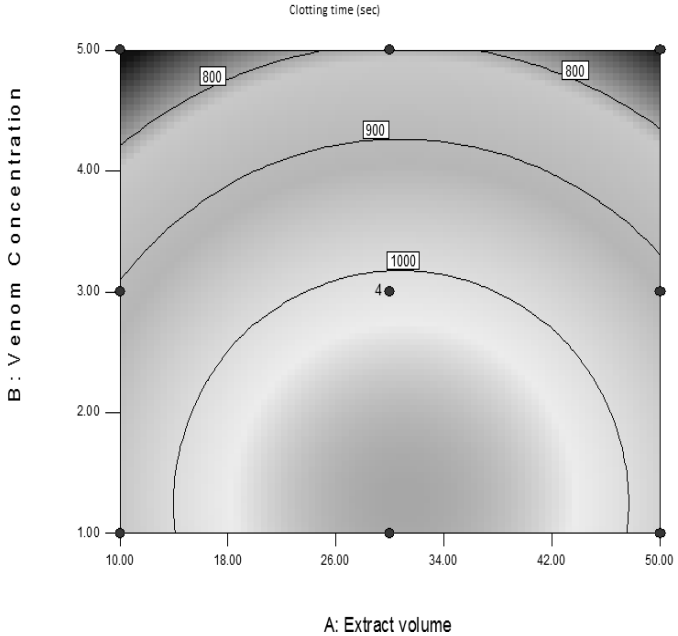

Fig 1a. Contour plot for the effect of tea leaves extract on clotting inhibitory activity

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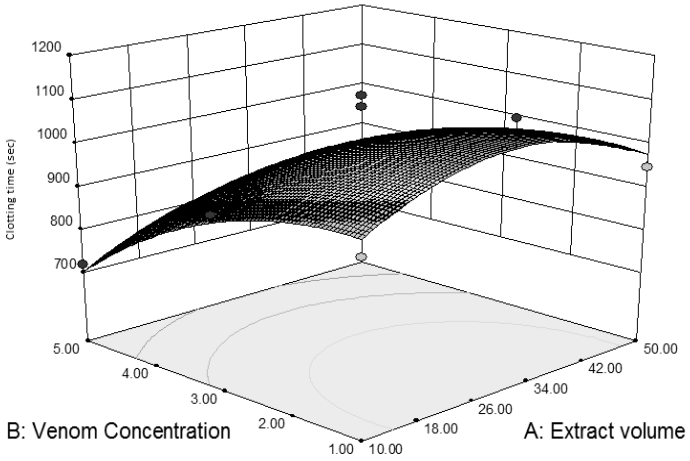

Fig 1b. 3D plot for the effect of tea leaves extract on clotting inhibitory activity

From Fig 1a and 1b it was clear that 10 µg of isopropanolic extract concentration were effective against 1 µg of venom concentration (MCD value) which delayed the clotting time up to200 sec. It was also observed that upon increasing the extract concentration clotting time also increases and by increasing venom concentration, clotting time decreases. The extract shown better result than the effect of *Mimosa pudica*, where 1.4 mg root extract inhibited the clot formation completely against 60 µg of cobra and krait venom [2]. Although neutralization dose of tea extracts were not identified effective concentration of extract was found to be 10 µg of the extract.

Phospholipase (PLA2) activity

MIHD of *Naja naja* is the venom concentration that induced a hemolytic halo (HH) of 11 mm diameter after incubation for 16 hours at 37°C. *Naja naja* venom (5µg) forms HH of 11.23±0.31 mm in 0.8% on agarose erythrocyte- egg yolk gel plates. Thus 5 µg of cobra venom is considered as MIHD. 50 µg of extract concentration reduced hemolytic halo up to 5.1±0.26 mm was found to be effective concentration of extract. All the results were in accordance with the results of Pithyunkal *et al.*(2010) [27] who reported that methanolic extract of tea leaves with concentrations of 101 µg and 303 µg inhibited PLA2 enzyme activity completely. Phospholipaseis mainly involved in destruction of lecithin on erythrocyte membrane. Although the studies were carried out indirectly, reduction of hemolytic halo on erythrocyte- egg yolk gel plate shown the inhibition of PLA2 enzyme by tea leaves extract effectively.

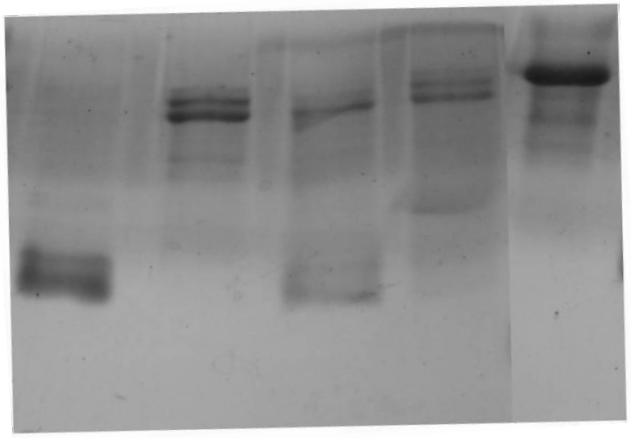

Fig 2.SDS-PAGE (15%) - Fibrinolytic inhibition of *Naja naja* venom by tea extract.

Lane 1:*Naja naja* venom, Lane 2: Fibrinogen, Lane 3: *Naja naja* venom and fibrinogen (10 µg each), Lane 4:*Naja naja* venom, fibrinogen and extract (10 µg, 10 µg and 20 µg respectively), Lane 5: BSA as a marker (66 KDa),

Fig 2.demonstrates that propanolic extract of tea leaves can also inhibit the destruction caused by *Naja naja* venom towards fibrinogenic α and β segments. 20 µg of extract was effective against fibrinolytic cleavage caused by venom. Similar result was reported by Pithyunkal *et al.* (2010) [27] where 22.5 µg of tea extract inhibited fibrinolytic activity of *Naja naja* venom (16.5 µg). Inhibition of fibrinogenolytic in cobra venom by tea extract could prevent from extra cellular damage (ECM) thereby preventing diffusion to toxins to inner cellular components. Isopropanolic extract of tea leaves were effective in all *in vitro* test at minimum concentration, it was chosen for *in vivo* analysis.

Lethal toxicity

In *in vivo* analysis LD50 of *Naja naja* venom was found to be 14 µgas it induced the 50% mortality inmice after 24 hours. For lethality inhibition studies 2LD50 of venom were chosen to determine effective dose of extract in inhibiting lethality.

Table 1. Determination of Effective dose (ED50) of isopropanolic tea leaves extract

Group (n=6)	Dose of the drug	Mortality after 24 hrs	% of survival after 24 hrs
I	Control	0/6	100
II	Venom alone	6/6	_
III	Venom + Std	0/6	100
IV	Venom + 250 mg/kg body weight of extract	4/6	33.33
V	Venom + 500 mg/kg body weight of extract	1/6	83.33

From Table 1 it is observed that, Group I (control), who does not received any drug had survival rate of 100% after 24 hours where Group II which received venom of 28 µg (2LD50) suffered 100% mortality. Group III which received venom with standard anti-venom had survival rate of 100%. Group IV which received venom with 250 mg/kg body weight of extract had survival rate of 33.33% but Group V (Dose II) which received venom with 500 mg/kg body weight of extract had survival rate of 83.33% after 24 hours and Dose II acts as effective dose (ED50) in inhibition of lethality caused by *Naja naja* venom. Extract were also seen to inhibit several clinical symptoms like blistering, inflammation, local tissue destruction completely caused by the cobra venom. Hung *et al.*(2004) [28] showed an antagonistic effect of 3 mg per mouse of melanin extracted from black tea (MEBT), when administered intra peritoneal (i.p) immediately after venom administration in the same place of venom injection. They showed that mixture of compounds present in tea extracts can be very effective in reducing local effects caused by the snakebite. But no reports were available for the inhibition of lethality due to snakebite by tea leaf extracts. Significantly, Dose II of the extract were capable of inhibiting lethality up to 83.33% in a group of six mice injected with 28 µg of cobra venom via tail vein.

Edema- forming Activity

MED is defined as the venom concentration which induced the edema ratio of 120% compared to control leg. 5 µg of cobra venom is capable of inducing edema ratio to 121.47% and is considered as MED. For inhibition studies 3MED value of venom is used to determine the efficacy of tea extract at maximum venom dose in inhibiting edema. The edema formation ratio was measured to be 161.01% in group of animals which received 3MED of venom of alone (15 µg). Animals received venom along with standard antivenom had a reduction in edema formation to116.6%.Animals which received venom along with Dose I and Dose II of plant extract, the edema was reduced to 116.31% and 101.77% accordingly. Effective dose of plant extract is defined as the dose of plant extract which reduced the edema ratio to 30% as with MED. Almost all the drug treated animals had edema reduction up to 30% with MED. Dose II inhibited edema formation greater than standard antivenom, because commonly antivenom were derived by immunizing horse, this acts as foreign protein to animals [24]. This antivenom may sometimes results in increased edema formation and can be overcome by treating snakebite with plant extracts like tea leaf extract.

CONCLUSION

Although various forms of antivenin are commercially available for the treatment of snake bite but these antivenin tend to cause various side effects as discussed earlier. So, always plant derivatives occupy prior position in treatment of snake bite. Moreover very rare and endangered plants have been reported to contain antivenom property. Through this study tea which is very common in every house has been taken for studying antivenom property and concordant positive results have been obtained for antivenom activity against cobra venom. Initially, three *in vitro* screening tests were conducted with four different extracts of tea leaves and isopropanolic extracts were screened and suggested for *in vivo* analysis. *In vivo* tests were carried out in Swiss albino mice and reported that tea extracts of dose 500mg/kg of body weight were found to inhibit lethality by 83.33% where the standard antivenom inhibits lethality by 100%. It was also found that isopropanolic extract of tea leaves can also reduce edema formation considerably compared with commercial antivenom. Through this study we can conclude that tea extracts can be used as naturally derived drug for the treatment of snake bite. But exact compound which is responsible for antivenom properties of tea leaves extract and mechanism of compounds inhibiting the venom proteins are yet to be studied.

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