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Evaluation of venom neutralising capacity of Indian medicinal plants by *in vitro* methods

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ABSTRACT

The aqueous extracts of the fruits of *Emblica officinalis*, leaves of *Ocimum sanctum*, bark of *Azadirachta indica* and bulbs of *Allium sativum* were tested for the snake venom neutralizing capacity using in vitro methods. The venom of *Naja naja* (Cobra) was used to evaluate the antivenom activity of these extracts. The parameters assessed were the neutralization of coagulant activity, fibrinolytic activity and phospholipase activity. All the extracts studied exhibited significant activity in neutralizing the venom effects and among the four extracts tested, *Ocimum sanctum* showed superior activity. The present study suggests the effectiveness of the above extracts in the treatment of snake bite and also supports the use of aqueous extracts by tribals and vaidyas.

INTRODUCTION

Snake bite is a global problem especially in tropical countries like India. It is estimated that deaths due to snake bite in the Indian subcontinent is more than 25000 every year [1]. *Naja naja* and *Viper russelli* are the common snakes in India and a large number of deaths occur due to envenomation by these snakes [2]. The most effective and accepted therapy for snake bite is the immediate administration of anti venom. However this therapy carries an associated risk of anaphylactic shock and serum reactions [3], [4]. In this context, many attempts have been made over the years for the development of snake venom antagonists from plant sources. Many Indian medicinal plants are recommended for the treatment of snake bite [5]. The present study investigates the venom antagonizing activity of four plants used for this purpose by the 'visha vaidyas' (traditional healers of venom) in Kerala.

MATERIALS AND METHODS

Materials

The freeze dried cobra venom was obtained from Irula Snake Catchers Industrial Co-operative Society, Chennai, India and was preserved at 4°C for further use. All the crude drugs were purchased from the local market or collected locally from Kottayam district, Kerala, India.

Preparation of plant extracts [6]

The fresh drugs (20g) were crushed using mortar and pestle separately, placed in a beaker and soaked in 200ml distilled water with continuous stirring for 3hours at room temperature. The extract was filtered through a muslin cloth and filtrate was concentrated at 40°C. The dried residue was suspended in normal saline at a concentration of 0.1% and kept at 4°C.

Inhibition of coagulant activity [7]

The minimum coagulant dose (MCD) of venom was used for the assay. It is defined as the dose of venom which induced clotting of plasma within 60 sec. To determine the MCD, various amounts of venom dissolved in 100µL PBS (pH 7.2) was added to human citrated plasma at 37°C. Plasma incubated with PBS alone served as the control. Then the coagulation time was recorded and the MCD was found out. In the neutralization assay, the MCD of the venom was mixed with various concentrations of plant extracts. The mixtures were incubated at 37°C for 30 min. Then 0.1ml of each mixture was added to 0.3ml of citrated plasma and the clotting times were recorded. In control tubes plasma was incubated either with venom alone or test extract alone. Neutralization was expressed as the effective dose (ED) defined as the ratio of mg of plant extract to the mg of venom in which the clotting time increased three times when compared to the clotting time of plasma incubated with two MCD of venom alone.

Inhibition of fibrinolytic activity [8]

The minimum fibrinolytic concentration (MFC) was defined as the concentration of venom that induced a fibrinolytic halo of 10mm diameter. Neutralization experiments were performed by incubating the MFC of venom with various amounts of plant extracts at 37°C for 1hour. After incubation, 10µl of the mixture was applied to wells in the plaque. After 18 hours of incubation at 37°C, the diameters of fibrinolytic halos were measured. Neutralization was expressed as the effective dose (ED) which is the ratio of mg of plant extract to the mg of venom that caused 50% reduction of the hemolytic halo when compared to the effect of venom alone.

Inhibition of Phospholipase activity [9]

Phospholipase activity was measured using an indirect hemolytic assay on agarose-erythrocyte egg yolk gel plate. Various doses of venom was added to 3mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.25 egg yolk as source of lecithin and 10 mM $CaCl_2$. Plates were incubated overnight at 37°C and the diameters of hemolytic halos were measured. Control wells containing 10µl saline was used to compare the results. The minimum indirect hemolytic dose (MIHD) corresponds to the dose of venom that produced a hemolytic halo of 11mm diameter. The neutralization assay was carried out by mixing the MIHD of venom with various amounts of plant extracts and incubating at 37°C for 30min. Then aliquots of 10µl of these mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Plates were incubated at 37°C for 20 hours. Results were compared with the control samples which contained venom without plant extracts. ED is calculated as the ratio of mg of plant extract to the mg of venom that caused 50% reduction of the fibrinolytic halo when compared to the effect of venom alone.

RESULTS

Inhibition of coagulant activity

The minimum coagulant dose of cobra venom was determined by testing increasing concentrations of venom (10-100µg) to find the concentration that effected the clotting of human citrated plasma in 60sec. The activity of plant extracts in neutralizing this effect was assessed by observing the absence of clotting in 60 sec when various doses were incubated with a fixed dose of venom. All the plant extracts used in the study showed good inhibition of coagulant activity. The ED values were found to be 30µg, 18µg, 26µg and 22µg for aqueous extracts of Amla, Tulsi, Neem and Garlic respectively (Table 1).

Inhibition of fibrinolytic activity

Venom induced fibrinolytic activity was determined by modified plaque assay. The minimum fibrinolytic activity was defined as the amount of venom that produced fibrinolytic halos of 10mm diameter. The fibrinolytic activity of venom was effectively antagonized by all the plant extracts tested. The ED values for Amla, Tulsi, Neem and Garlic were found to be 0.7mg, 0.5mg, 0.9mg and 0.6mg respectively (Table 2).

Inhibition of phospholipase activity

Phospholipase activity was measured using indirect hemolytic assay on agarose-egg yolk gel plate. Minimum indirect hemolytic dose was determined by applying increasing doses (1-50µg) venom in the wells made in the agarose gels. 15µg of cobra venom produced 11mm hemolytic halo. This shows that cobra venom has phospholipase A_2 enzyme which has the ability to cause lysis of sheep RBC's. The various plant extracts tested have shown good inhibition of this enzyme activity in a dose dependant manner. The ED values were found to be 12µg, 9µg, 12µg and 10µg for Amla, Tulsi, Neem and Garlic respectively (Table 3).

Table 1. Inhibition of venom induced coagulant activity by plant extracts

Plant extract used	MCD(µg)	ED (µg)
Amla	60	30 ± 2.0
Tulsi		18 ± 1.6
Neem		26 ± 1.8
Garlic		22 ± 1.8

Results are expressed as the mean of 4 observations

Table 2. Inhibition of venom induced fibrinolytic activity by plant extracts

Plant extract used	MFD (µg)	ED (mg)
Amla	15	0.7 ± 0.05
Tulsi		0.5 ± 0.05
Neem		0.9 ± 0.10
Garlic		0.6 ± 0.05

Results are expressed as the mean of 4 observations

Table 3. Inhibition of venom induced phospholipase activity by plant extracts

Plant extract used	MIHD (µg)	ED (µg)
Amla	15	12 ± 1.2
Tulsi		9 ± 1.6
Neem		12 ± 1.6
Garlic		10 ± 1.4

Results are expressed as the mean of 4 observations

DISCUSSION

Deaths following snakebite are common problem throughout the world especially in India. The only available treatment for this is the use of snake venom antiserum, despite several side effects. Therefore, more scientific attention has been given since last 20

years [10] to find a safer alternative to antiserum therapy. The use of plants for the treatment of snakebite has long been recognized and many Indian plants have been recommended for this purpose [11] out of which few have been examined for the neutralization of venom. In the present study, we evaluated the potential of aqueous extracts of Amla fruit, Tulsi leaves, Neem bark and Garlic bulbs in neutralizing the coagulant, fibrinolytic and hemolytic activities of cobra venom in vitro. All these plant extracts showed significant potential in inhibiting the venom induced activities in plasma and blood. There were reports that triterpenoids present in plants may involve in the venom activation process [12] and the activity exhibited by these drugs may be due to their triterpenoid content. The aqueous extracts were selected for the study because the 'vishavaidyas' used only aqueous extracts to treat the ill-effects of snake bite. Earlier research on plants as antivenom also supports the use of aqueous extracts [13]. In all the assays, the smallest ED values were obtained for Tulsi leaf extract. It is noteworthy that these studies were performed using the crude extracts and thus an increase in the extent of activity can be expected with the use of highly purified extracts or isolated constituents.

CONCLUSION

Results of the present study conducted on the few important in vitro activities of venom reveal the presence of antivenom principles in the aqueous extracts of Amla, Tulsi, Neem and Garlic. The efficacy of these extracts also justifies the traditional use of aqueous extracts in the treatment of snake bite. However, further research including the isolation of antivenom compounds and in vivo assays is needed to establish these plants as a remedy for snakebite.

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