Research Article

ISOLATION, PURIFICATION AND CHARACTERIZATION OF ACTIVE COMPOUND FROM *ANDROGRAPHIS PANICULATA* L AND *PHYLLANTHUS AMARUS* L AND TESTING THE ANTI-VENOM ACTIVITY OF THE DI-HERBAL EXTRACT BY *IN-VITRO* AND *IN-VIVO* STUDIES

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ABSTRACT

The mortality associated with snake bites is a serious medical, social and economic problem in many countries and about 35,000 to 50,000 people reportedly die of snakebites in India; hence in this study an attempt has been made to reduce the effect of snake venom of Indian Cobra *Naja naja*. Shade dried leaves of the *Andrographis paniculata* and *Phyllanthus amarus* was extracted with methanol solvents. The compound responsible for the anti venom property was isolated by solvent extractions and silica gel column chromatography. Spectral analysis was performed to determine the photochemical constituents in the plant leaf. The extract was purified to isolate the active compound and was tested for this anti venom activities through *in vivo* experiments with Swiss albino mice and *in vitro* experiment phospholipase activity. *In vivo* experiments carried out with Swiss albino mice was carried out to determine the LD₅₀ potency and Edema forming activity. Methanolic extracts of *Andrographis paniculata* and *Phyllanthus amarus* were tested for their activity on various pharmacological effects like lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity of cobra and krait venoms. The aqueous extract displayed a significant inhibitory effect on the lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity. About 0.16 mg and 0.19 mg of *Andrographis paniculata* and *Phyllanthus amarus* extracts were able to completely neutralize the lethal activity of 2 LD₅₀ of cobra and Cobra venom respectively.

Keywords: *Andrographis paniculata*, *Phyllanthus amarus*, lime flocculation, di-herbal

INTRODUCTION

Andrographis paniculata L. is an erect annual herb extremely bitter in taste in all parts of the plant body. The plant is known in north-eastern India as Maha-tita, literally "king of bitters", and known by various vernacular names. As an Ayurveda herb it is known as Kalmegh or Kalamegha, meaning "dark cloud *Andrographis paniculata* L. grows erect to a height of 30–110 cm in moist, shady places. The slender stem is dark green, squared in cross-section with longitudinal furrows and wings along the angles. The lance-shaped leaves have hairless blades measuring up to 8 cm long by 2.5 wide. The small flowers are borne in spreading racemes. The fruit is a capsule around 2 cm long and a few mm wide. It contains many yellow-brown seeds¹. Since ancient times, *A. paniculata* is used in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for multiple clinical applications. The plant extract exhibits anti typhoid, antifungal, anti hepatotoxic, antibiotic, anti malarial, anti hepatic, anti thrombogenic, anti inflammatory, anti-snake venom, and antipyretic properties to mention a few, besides its general use as an immunostimulant agent². The herb has shown an ability to reduce inflammation (heat) and fight viral infection, and is used as a principal ingredient in traditional Chinese medicinal formulas for lung support from colds.

Phyllanthus amarus

Phyllanthus amarus is an erect annual herb, 10 to 50 cm high, with smooth cylindrical stem 1.5 to 2 mm thick and deciduous horizontal branchlets 4 to 12 cm long and about 0.5 cm thick, with 15 to 30 leaves. The leaves are alternate, on petioles 0.3 to 0.5 mm long, elliptic, oblong or obovate, 5

to 11 mm long and 3 to 6 mm wide, rounded to slightly point at the tip, scarcely oblique on one side at the base. The flowers are alone or usually one male and one (larger) female are in each leaf axel together. The seed capsules on stalks are 1 to 2 mm long, round, smooth, 2 mm wide, With 6 seeds. In many countries around the world plants in the genus *Phyllanthus* are used in folk remedies; therefore this genus is of great importance in traditional medicine³. The genus *Phyllanthus* has a long history of use in the treatment of liver, kidney and bladder problems, diabetes and intestinal parasites. *P. amarus*, *P. nururi* and *P. urinaria* are used in the treatment for kidney/gallstones, other kidney related problems, appendix inflammation, and prostate problems. In a number of countries, the aerial part of *Phyllanthus amarus* is highly valued in traditional medicine for its healing properties⁴. This plant is traditionally used around the world in the treatment of liver ailments and kidney stones. *Phyllanthus amarus* has also shown to work as an antifungal, antibacterial and antiviral agent⁵. In India this plant is used in traditional medicine to treat liver diseases, asthma and bronchial infections⁴. This popular medicinal herb is also a remedy around the world for influenza, dropsy, diabetes and jaundice gives a summary of the ethno botanical use of *Phyllanthus amarus* around the world.

MATERIALS AND METHOD**Preparation of Venom**

Lyophilized snake venom of *Naja naja* (Indian cobra) was collected from King Institute of Preventive Medicine and Research. One gram of lyophilized venom was dissolved in 100 ml of 0.90 % saline and centrifuged at 2500 rpm for 10

minutes. The supernatant was used as venom and stored at 4°C for further use.

Medicinal Plants and Preparation of Extracts

Collection of the Plant Materials

The plant material was collected around the local garden in King Institute Of Preventive Medicine and Research by verifying the flower and leaf size and colour as described in previous literature. The plants at the flowering stage were cut at the base leaving behind about 10–15 cm of stem for plant regeneration. The samples were submitted in the sample section of DAT at King Institute of Preventive Medicine and Research and they were assigned the voucher number: DAT020312 and DAT030312 for *Andrographis paniculata* and *Phyllanthus amarus* respectively.

Crude Extract Preparation

Andrographis paniculata and *Phyllanthus amarus* leaves were dried for 10 days under shade powdered using a homogenizer and separated using a percolator. The plant powder was mixed with methanol in the ratio of 1:10 and left in the shaker for 72 h of 115 rpm in the shaker. Then by using soxhlet apparatus methanol and the crude extract were separated. There after the extract was filtered using Watman no A-1 filter paper. The dried extract was stored in dessicator at room temperature for further use. The plant extracts were expressed in terms of dry weight.

Isolation and Purification of the Active Compound

Column Chromatography

Column was packed with slurry of silica gel (mesh size, 60–120) with chloroform. Then dried methanol extract (4 g) of *A. paniculata* and *P. amarus* was first dissolved in methanol and carefully applied at the top of prepared column. Immediately after application of sample, a gradient of chloroform and methanol (mobile phase) was used as eluent to collect fractions of methanol extract of *A. paniculata* and *P. amarus*. The column was run with a gradient of chloroform : methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90) finally 100 % methanol and 10 fraction (F1 – F10) were collected. The wavelength and the absorbance values of the collected samples were determined using spectrophotometer (Hitachi 1900) and the values were tabulated. The fraction that corresponds to higher absorbance value is taken for further experiments. Therefore, from all the collected fractions, solvent was removed by evaporation at room temperature. Equal volume of both the extract was mixed together to be used as the di-herbal extract.

Phytochemical Analysis

Kjeldahl test

0.7 g of the sample i.e. the plant powder is weighed and mixed with 10 g of anhydrous ammonium sulphate. A pinch of copper sulphate is added that acts as a catalyst. To this mixture, 30 ml of concentrated sulphuric acid is added which will help the nitrogen to convert into ammonium sulphate. This gets charred due to the organic compounds present in the sample and is heated in a burner. After sufficient heating, the mixture is being cooled by adding distilled water to it and the heat liberated is reduced by exposing the flask to running tap water. The whole contents are transferred to a 2 litre round bottomed flask for further distillation process. To the mixture, 1:1 alkali (NaOH) is added and 30 ml of 0.1 N HCL is added in the condenser with methyl red as an indicator. During the process of distillation, all the nitrogen compounds

get converted to ammonium chloride and a titration against 0.1 NaOH confirms the quantity of protein present in the sample using the formula. Percentage of protein content in sample is the Titer value of nitrogen multiplied by the protein conversion factor and then dividing by the amount of the sample taken.

Shinoda test

To the 5 mg of the plant sample, few magnesium turnings were added. To this, few drops of conc. HCL is added drop wise and left for incubation at room temperature for 2 minutes and is observed for a pink coloration if the sample contains flavanoids.

Salkowski test

To 0.5 g each of the extract, was added 2 ml of chloroform. 3 ml of conc. Sulphuric acid is carefully added over to form a layer. A reddish brown coloration at the interface indicates the presence of terpenoids in the active drug.

In vitro studies

In vitro studies were carried out to assess the neutralization effects of the active drug against the venom of Naja naja.

Limes Flocculation Test

5 mg of the di-herbal plant extract was weighed and dissolved in 5 ml of DMSO; making the concentration 1 mg/ml. The mixture was distributed in 5 tubes each containing 1 ml and the tubes were labelled from T1 to T4 with one tube as the control. To the tubes labelled from T1 to T4, venom were incubated in the water bath 30 minutes to observe any flocculation present in the tubes due to the neutralization effect of *Andrographis paniculata* and *Phyllanthus amarus* against snake venom.

Phospholipase Activity Test

The Phospholipase Activity Method was determined by Gutierrez *et al*⁶. 1 % of agrose was added to 25 ml of PBS (pH 8.1), heated and allowed to dissolve. 0.25 ml of RBCs were mixed with 0.25 ml of egg yolk and poured in the dissolved agarose solution at 55°C. The mixture was poured on to a 6 petri plate and allowed to solidify. After solidification, 2 wells were punched in 2 petri plates where 1st well was loaded with 0.1 % of venom (positive control) 2nd well was loaded with plant extract alone (negative control). In the remaining plates one well was punched in each plate. This was loaded with the pre-incubated mixture of di-herbal plant extract (1 mg/ml) and venom in varying concentration. The plates were incubated at 37 °C for 24 h and was observed hallos corresponding to the wells. Neutralization expressed as ratio mg antibodies /mg venom able to reduce by 50 % the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Cell Viability Assay

The cell viability was assessed for the drug in non cancerous cell lines like L6. The sub cultured cell lines were washed with PBS to remove any dead or old cells. 1 % TPVG was added to the monolayer and incubated in a CO₂ incubator for 5 minutes and then washed off. Fresh MEM media was added to the culture flask and flushed well so as to completely suspend all the cells and plate them on a micro titre plate. The plate was incubated for 48 h until mono layers of cells develop. After the formation of monolayer, drug dilution was performed to assess the toxicity of the active compound. 2

mg of the drug was weighed and dissolved in 0.25 ml of DMSO and vortexed well for 5 minutes. 9 ml of MEM was added to the drug solution and filtered using a syringe filter and the dilutions were prepared. The diluted plant sample was added to all the wells having a drug control and cell control as negative and positive controls. The cells contained in the wells were incubated at 37°C in a 5 % CO₂ incubator. The cell morphology was assessed having an interval of 24 h, 48 h and 36 h after which MTT assay was performed.

In vivo studies

The neutralization of the plant extract containing the active compound was assessed by various in vivo tests in Male Swiss albino mice. Swiss albino mice of 18-26 g will be used as the experimental model. The animal's will be kept in ventilated cages and will be fed with commercial rat chow swatter. Four groups of mice were taken each group consisting of six mice.

Group 1: This group is served as PBS as Control.

Group 2: This group is served as Venom as Control.

Group 3: This group is injected with pre-incubated venom and di-herbal extract mixture at 37°C for 30 minutes.

LD₅₀ Potency

Lethal potency was determined by Theakston and Reid 1983⁷. Group of Mice weighing 18-20 g taken in each batch: having 5 batches on the whole to assess the lethal potency test. Various concentrations of venom were made up to 5 ml with normal saline. The venom under the different concentrations were injected via the intra peritoneal (tail vein) site to the mice. The mice were observed after 24 h to assess the death ratio. The median Effective Dose (ED₅₀) calculated from the number of deaths within 24 h of injection of the venom /anti venom mixture. The ED₅₀ was expressed as anti venom/mouse and calculated by probity analysis

Edema Forming Activity

The edema forming activity of *Naja naja* venom was determined by Camey *et al*⁸. Group of mice were injected subcutaneously in the right foot pad with 0.1 ml of venom dissolved in PBS. The right foot pad of the mice was injected with 0.1 ml of PBS alone to serve as a control. The thickness of each foot pad was measured for every 30 minutes after the venom injection using a screw gauge. The ability of the *Andrographis paniculata* and *Phyllanthus amarus* extract to reduce the edema thus formed was determined. The di-herbal plant extract was pre-incubated with venom for 30 minutes at 37°C. Then a group of 2 mice were injected subcutaneously in the right foot pad with 0.1 ml of PBS alone. The control mice were injected with 0.1 ml of venom in the right foot pad and 0.1 ml of PBS alone. After 1 h, the edema was calculated as described by yamakawa *et al*⁸. Edema forming activity was calculated as a percentage of increase in the thickness of the right foot pad injected with venom compared to the control in the left foot pad.

RESULT

Preparation of the crude extract

The dried powder of *Andrographis paniculata* of *Phyllanthus amarus* was weighed 120 g each and was cold macerated separately in methanol for 48 hours in a shaker (Figure 1). The crude extracts were obtained after removing the methanol using soxhlet apparatus, which weighed about 20 g. The crude extract was stored in the dessicator for further use.

Isolation of active compound

Column Chromatography

The active compound of both the plant is a flavanoid and is isolated by means of Column chromatography with silica gel (mesh 60-120) as the solid phase and methanol: chloroform being the mobile phase. 10 fractions were eluted which were set to colorimetric readings to identify the active compound present in the fractions showing the high absorbance value. The maximum absorbance was found at fraction 6:04 (60:40), hence that fraction is considered as the active compound and equal volume of both the extract were mixed to prepare 1 mg/ml di-herbal stock extract for further studies.

Photochemical Analysis

The protein content present in the sample was analysed by kjeldahl's method of protein estimation and was found out to be 6.6 % and 5.3 % for *Andrographis paniculata* and *Phyllanthus amarus* respectively. Shinoda test was performed to confirm the presence of flavonoids. After the addition of magnesium turnings and concentrate Hcl to the sample, a pink coloration was observed indicating the presence of flavnoids. Salkowski test was carried out to confirm the presence of terpenoids in the sample. A reddish brown coloration at the interface was observed after the addition of chloroform and concentrate Sulphuric acid to 0.5 mg of the drug.

In vitro studies

Limes Flocculation Test

10 tubes that were distributed with the drug dissolved in DMSO in a concentration of 1 mg/ml along with various concentrations of venom were incubated at 37°C in water bath and were observed for any floccules as an interface for neutralization. 1st 5 tubes consisting of di-herbal plant extract was labelled is Set 'A'. The first tube in each Set was served as the blank which contained only the plant extract. After incubation the samples for 30 minutes at 37°C, flocculation was observed in all the tubes except for blank. This proved the neutralization effect of the compound present in the plant against the venom. The concentration of flocculation varied with different tubes. The increase in concentration was directly proportional to the increase in the venom concentration, i.e. more amount flocculation was observed in the second tube having a venom concentration of 0.1 % than the other tubes, 0.01 %, 0.001 %, 0.0001 % which had relatively low flocculation. This dilution; where neutralization is maximum; the tube that contains the drug concentration as 1 mg/ml and the venom concentration as 0.1 %.

Phospholipase activity

The plates after incubation were observed for the presence of hallows indicating the lysis of RBCs by the snake venom. After an incubation of 24 h, hallows were formed and the diameter of the zones were measured. The hemolytic zone formed around the snake venom was measured to be 1.9 cm. The zone formed around the pre incubated sample of the plant extract and venom was reduced considerably showing the neutralization effect which when measured was found to be 0.7 cm. This reduction in the hemolytic zone formation was about 60 % of neutralization by the plant extract (Figure 2).

Cell viability Assay

The L6 cells after 36 h were assayed with MTT, to determine the neutralizing effect of the di-herbal plant extract. There was a considerable viability observed at the dilutions starting from 0.001 % of the extract and continue till 0.1 % dilution in L6 cell lines. Therefore there were formazin crystals observed in the wells containing cell control, and dilutions starting from 10 to 60. After the addition of DMSO to the wells, the viable cells were able to take up the dye and give the out a pink colouration; wherein the neat and other wells having dilutions above 70 retained the yellow colour of MTT dye. Based on the absorbance obtained, the maximum cell viability was found to be 69 % for 1 % dilution when compared to the control.

In vivo test

LD₅₀ potency test

The lethal toxicity (LD₅₀) of Cobra venom was assessed using 18 g, Balb/c strain mice. About 10 µg of Cobra and 3 µg of cobra venom were found to be LD₅₀ for 18 g of mice. The neutralization of lethality was done by mixing constant amount of venom (2LD₅₀) with various dilutions of Plant extracts and incubated at 37°C for 30 minutes prior to injection. It was found that 0.24 mg of di-herbal plant extract was able to completely neutralize the lethal activity of 2LD₅₀ of Cobra venom.

Figure 1: Preparation of the crude extract

Figure 3: Cell viability assay (Left: 0.001 % venom, Right: venom incubated with 0.1 % di-herbal extract)

DISCUSSION

Column chromatography fraction showed good result then the crude extract. Further purification by HPLC of the fraction showed the presence of active compound. Confirmatory test for flavonoids and terpenoids showed the presence of those components in the fractions. Protein estimation also showed the presence of protein the plant

Edema forming activity

After the injection of venom, the pre-incubated mixture contains the drug in a concentration of 1 mg/ml and venom in a concentration of 0.1 % at 37 degree Celsius for 30 minutes and the control sample having PBS into the foot pad of mice. Edema formation was observed in the di-herbal plant extract after 1 hour after injection. Control mice edema and plant extract with venom edema was compared. There is reduction in the edema size when plant extract was injected (Figure 4). In edema forming activity, the mice immunized with Cobra venom showed increase in footpad thickness. About 7 µg of cobra and 2 µg of Cobra venom induced edema formation within 3 h which is considered as 100 % activity. The edema was reduced up to 30 % when 2.8 mg of plant extract per mg venom was given. There was no further reduction in the percentage of edema even when there was an increase in anti venom dose. Neutralization studies can be performed by incubating of venom and di-herbal plant extract prior to testing (pre incubation method). The results showed that the di-herbal plant extracts was capable of neutralizing the lethality induced by the venom. Plant extracts was capable of inhibiting PLA2 dependent hemolysis of sheep RBCs in a dose dependent manner. Edema-forming activity was assessed for Cobra venom. Plant extracts was found to be effective in neutralization of edema induced by venoms. There was a significant decrease in the edema (footpad thickness) when there was an increase in the anti venom (plant extract) dose.

Figure 2: Phospholipase Activity

Figure 4: Edema forming activity (left: venom, right: incubated venom and plant extract)

compound. The studied and investigation clearly shows the presence of a factor that inhibits the venom action. Commercializing this drug in rural areas would be a great remedy for the people who fall a victim for snake bites, prevalent in the rural areas. Anti-venom currently produced is highly costly when compared to plant anti-venom. Alternative use of plant as source of drug would surely help

the people to recover from the venomation by snake bite. Plant anti-venom is reliable as it is a natural source, and it is cost effective. Moreover, side effects in ASVS are more when compared with that of plant, which has major or no side effects. The combined methanolic extracts of *Phyllanthus amarus* and *Andrographis paniculata* plants were tested for anti venom activity against Cobra venom. The di-herbal plant extracts effectively neutralized the Cobra venom induced lethal activity. About 0.24 mg of di-herbal plant extract was able to completely neutralize the lethal activity of 2LD₅₀ of *N. naja* venom. Various pharmacological activities including edema, haemorrhagic, coagulant, fibrinolytic and phospholipase activities were studied and these pharmacological activities were significantly neutralized by both the plant extracts. The above observations confirmed that *P. amarus* and *A. paniculata* plant extracts when combined possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite envenomation.

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