ISOLATION, PURIFICATION & CHARACTERIZATION OF ACTIVE COMPOUND FROM ANDROGRAPHIS PANICULATA.L AND TESTING ITS ANTI-VENOM AND CYTOTOXIC ACTIVITY BY *IN-VITRO* & IN-*VIVO* STUDIES

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Abstract

The active compound present in *Andrographispaniculata L.* – Andrographolide which was isolated and purified using column chromatography, TLC and the characterization of andrographolide was done using FT IR and NMR studies. Furthermore, in vivo and in vitro studies confirm the anti venom property of the plant and the cytotoxic levels determined by the use of cell lines has its major start towards the cellular level study.

Keywords: *Andrographispaniculata.L,* Andrographolide, medicinal plant.

I. INTRODUCTION

Snake bite is a serious problem in tropical and subtropical countries like India. According to World Health Organization (WHO), poisonous snakes are responsible for at least 5 million human fatalities annually (Chippaux, 1998). The largest number of fatal snake bites occurs in South East Asia (Aubert, 1996). Based on the hospital records David (2005) estimated that annually ~ 50000 people die due to snake envenomation in India.

II. METHODS AND MATERIALS

A. Preparation of Venom

Lyophilized snake venom of Naja naja (Cobra) was collected from King Institute of preventive medicine and research (KIPM&R). One gram of lyophilised venom was dissolved in 100 ml of 0.90% saline and centrifuged at 2500 rpm for 10 min. The supernatant was used as venom and stored at 4°C for further use.

B. Isolation of Plant Extract

Andrographis paniculata leaves were collected from the KIPM&R campus. The leaves were dried for 7 days under shade, powdered using a homogeniser and separated using a percolator. The plant powder was packed and placed in the thimble of soxhlet apparatus and was extracted with methanol (60-80°C for 72 hrs) and the extract was then concentrated In rotary evaporator for further drying purpose. The dried extract was stored in dessicator at room temperature for further use.

1. Column Chromatography

Column was packed with slurry of silica gel (mesh size, 60-120) with chloroform. Then the dried Methanol extract (4 gm) of *A. paniculata* was first dissolved in Methanol and carefully applied by pipette at the top of prepared column. Immediately after application of sample, a gradient of Chloroform and Methanol (mobile phase) was used as eluant to collect fractions of Methanol extract of *A. paniculata*. The column was run with a gradient of Chloroform : Methanol (98:2, 95:5, 90:10, 80:20, 70:30, 50:50, 30:70, 20:80, 10:90, 5:95, 2:98) finally 100% Methanol and 12 fractions (F1-F12) were collected. The Optical Density of the collected samples was determined using nano drop spectrophotometer and the values were tabulated.

The fraction that corresponds to higher O.D value is taken for further experiments. Thereafter, from all the collected fractions, solvent was removed by evaporation at room temperature and the crystals are set to characterization studies.

2. Kjeldahl Method of Protein estimation

0.7 g of the sample i.e. the plant powder is weighed and mixed with 10 gms 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 0.1N NaOH confirms the quantity of protein present in the sample using the formula, Percentage of Protein Content in sample = (Titre value of Nitrogen * protein conversion factor) / Amount of the sample taken.

3. Shinoda Test for the confirmation of flavanoids:

To 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 mins and is observed for a pink colouration if the sample contains flavanoids.

4. Salkowski Test

To 0.5 gm 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 colouration at the interface indicates the presence of terpenoids in the active drug.

III. IN VITRO STUDIES

A. Limes Flocculation Test

5mg of the 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 1ml and the tubes were labeled from T_1 to T_4 with one tube as the control. To the tubes labeled from T_1 to T_4, venom was mixed at different concentrations i.e. 0.1%, 0.01%, 0.001% and 0.0001%. The water bath was set at 37°C and the tubes were incubated in the water bath for 30 mins to observe any flocculation present in the tubes due to the neutralizing effect of Andrographolide against snake venom.

B. Procoagulant Activity

Human blood was collected and sodium citrate was added to the blood (anti coagulant), mixed gently and centrifuged for 15 mins at 5000 rpm. The resulting supernatant would contain the citrated plasma and the rest of the pellet was discarded.

0.5 ml of the citrated plasma was distributed in different tubes and different concentrations of venom i.e. 1%, 0.1%, 0.01% and 0.001% was added and the coagulating time was noted by starting the timer. The minimum coagulating dose (MCD) was determined as the concentration of the venom to coagulate the plasma within 60 seconds. Plasma incubated with PBS alone served as a control. In case of the neutralization assays, constant amount of venom was mixed with various dilutions of plant extract. The mixture was incubated for 30 mins at 37°C. 0.1 ml of the mixture was added to 0.3 ml of citrated plasma and the clotting times were recorded. In control tubes, plasma was incubated with venom alone and plant extracts alone. In neutralization assays, constant amounts of venom was mixed with various dilutions of the active drug contained in the plant extract which was pre incubated at 37°C for 30 mins. Neutralization was expressed as effective dose (ED), defined as the ratio μl antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

C. Hemolytic Assay

Blood was collected from a healthy volunteer. Sodium citrate was added to the collected human blood, mixed gently and centrifuged for 15 mins at 5000 rpm. The supernatant was discarded and the settled RBCs were taken for the study. 1% of agarose was added to 25 ml of PBS, heated and allowed to dissolve. 0.25 ml of RBCs were mixed with 0.25 ml of the egg yolk and poured in the dissolved agarose solution at 55°C. The mixture was poured onto a petri plate and allowed to solidify. After solidification, 2 wells were punched where in the 1st well was loaded with 0.1% venom, 2nd well loaded with the preincubated mixture of plant extract (1 mg/ml) and venom. The plate was incubated at 37°C for 24 hrs and was observed for hemolytic hallows corresponding to the wells.

D. Cell Toxicity Studies

The sub cultured L6 cell lines were washed with PBS to remove any dead or old cells. 1% TPVG was added to the monolayer and incubated in a CO_2 incubator for 5 mins 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 microtitre plate. The plate was incubated for 48 hrs until monolayers 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 mins. 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% CO_2 incubator. The cell morphology was assessed having an interval of 24 hrs, 48 hrs and 36 hrs after which MTT assay was performed. The same procedure was followed for both the cell lines.

E. MTT Assay

The media present in the 96 well mictrotitre plate was dumped off gently. The cells were washed with PBS to wash off any remaining media. 5 mg of MTT was dissolved in 1 ml of distilled water; from which 20µl of was added to all the wells.

The plate was incubated at 37°C for 5 hrs at 5% CO_2 supply. After an incubation of 5 hrs, the MTT was converted into formazin crystals by the viable cells and the dead cells remain unchanged. 100µl of DMSO was added to all the wells. The formazin crystals formed in the viable cells turn pink in colour after the addition of DMSO whereas the non viable cells retain the colour of formazin.

The absorbance values of the samples loaded in the wells were determined using an ELISA plate reader at a wavelength of 620 nm. The readings were tabulated and a graph was plotted to assess the level of cytotoxicty rendered by *Andrographis paniculata L.* towards L6 and Vero cell lines.

IV. IN VIVO STUDIES

A. LD 50 Potency

Lethal potency test is calculated for the venom of *naja naja* by the method of Spearmer and Carper. LD 50 is calculated as the 50% probability by the analysis of deaths occurring within 24 hrs of venom injection. 6 numbers of mice weighing 18 – 20 g were 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 different concentrations were injected via the intra peritoneal (tail vein) site to the mice. The mice were observed after 24 hrs to assess the death ratio.

B. Edema Forming Activity

The edema forming activity of *Naja naja* venom was determined by Lemonte *et al* and Camey *et al.* Group of 2 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. Edema 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. The thickness of each foot pad was measured for every 30 mins after the venom injection using a screw gauge. The ability of the *Andrographis paniculata L.* extract to reduce the edema thus formed is also determined. The plant extract is pre incubated with venom for 30 mins at 37°C. Then a group of 2 mice were injected subcutaneously in the right foot pad with 0.1 ml of the mixtures whereas the left foot pad was injected with 0.1 ml of PBS alone. The control mice was injected with 0.1ml of venom in the right foot pad and 0.1 ml of PBS in the right foot pad. After 1 hr, the edema was calculated as described by Yamakawa *et al.*

V. RESULTS & DISCUSSION

After running the methanolic extract in column chromatography using methanol and chloroform, the studies showed that the 6^{th} component that had a much lighter colour showed a greater absorbance which concluded that it had active component and we crystallized it.

The confirmation test such as Shinoda test, Salkowski test confirmed the presence of flavonoid group in our purified product. The protein content of the dry leaf was 6.6% of the total weight which was found out by substituting the protein factor value to the nitrogen value found out by Kjeldhal method. Based on the TLC performed, the R_F value of brown coloured andrographolide compound was found to be 0.88.

In case of *in vitro* studies of the neutralizing activity of the active compound against snake venom, was done similar to the test done in Lime Flocculation

test of tetanus. Dark green microscopic flocculation was observed. The most suitable concentration of extract to neutralize 0.1% venom was found to be 1 mg/ml. In case of pro coagulant activity, the ED_{50} value of *Andrographis paniculata.L* was found to be 1 mg.

Hemolytic assay was done to determine the effect of venom and plant extract on RBCs. The zone thickness of venom was found to be 0.9 cm, wherein the plant had no zone formation. In half an hour pre incubated venom extract mixture, the zone was 0.15 cm and in case of fresh mixture, it was found to be 0.4 cm.

In *in vivo* studies carried out, the LD 50 potency rate of cobra venom was found to be 9.4% g/mouse. In case of edema formation, the purified sample had a much better neutralizing activity with increase in time. Screw gauge was used to measure the edema formation. The pre incubated extract and venom mixture showed a reduction of 0.006 mm in the edema formation when compared to pure venom that was injected. In case of PBS that was used as a control, there was a minimal swelling in the footpad of mice irrespective of time interval. In studies to determine the cytotoxic effect of plant extract on cell line, L6 and Vero cell lines were used. The cytotoxic/inhibitory concentration for L6 was found to be 60% g/ml. Wherein the plant extract was toxic towards Vero cell lines from a conentration 10% g/ml.

VI. CONCLUSION

All these studies gave a positive result that *Andrographis paniculata.L* possess a potent antivenom property due to its major component Andrographolide and they have no hemolytic and cytotoxic activity up to 60% g/ml concentration.

REFERENCES

[1] Alam MI and Gomes A (2003) Snake venom neutralization by Indian medicinal ants (Vitex negundo and Emblica officinalis) root extracts. J. Ethnopharmacol. 86, 75–80.

[2] S. Meenatchisundaram et al, (Mar 2009), 'Studies on antivenom activity of Andrographis paniculata and Aristolochia indica plant extracts against cobra venom by in vivo and in vitro methods', Vol.2, No.4.

[3] N.V.L Sirisha Mulukuri(2011),'Isolation of Diterpenoid Lactones from the Leaves of Andrographis Paniculata and Its Anticancer Activity', vol.3, no 3, pp. 39-42.

[4] Dimpy Das Borooah (July 2011), 'column chromatography for isolation of andrographolide from in vitro grown medicinal plant-andrographis paniculata', vol: 2: issue-3.

[5] Sudhanshu Saxena (2000), 'High-Performance Thin-Layer Chromatographic Analysis of Hepatoprotective Diterpenoids from andrographis paniculata', vol. 11, pp. 34–36.

[6] Ranjana I, brahmane (2010), 'anti red scorpion venom activity of andrographis paniculata', vol. 3, issue 4.

[7] Sharma, A., Krishna, L. and Handa, SS. (1992), 'Standardization of the Indian crude drug Kalmegh by high pressure liquid chromatographic determination of andrographolide', PhytochemAna: Vol. 3: 129-131.