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Evaluation of toxic effect of plant extracts having insecticidal effect to Sand fly

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ABSTRACT

The established vector for Kala-azar (VL) *Phlebotomus argentipes* is developing tolerance/resistance against the common insecticide (DDT). However, the insecticidal effect of wild plant i.e. *Clerodendron infortunatum* leaf (Hexane extract) was found effective in killing 63% sand flies that can be enhanced with formulation of lead molecules. Hence, its toxicity evaluation to humans is pre-requisite. Percentage Hemolytic Inhibition activity of the extract was observed with IC_{50} value of 25.80 mg/L, 18.68 mg/L & 15.90 mg/L at 450 nm, 490 nm & 655 nm wavelengths respectively. The extract was found non-toxic and safer to use as a new insecticide against Sand fly.

Introduction

Kala-azar is a major health problem and affecting poorest community in Bihar, India. The insecticide of choice, DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) is now developing tolerance/resistance in some parts of Kala-azar endemic zone of Bihar, India (Dinesh et al., 2010; Singh et al., 2012). Search for insecticidal property of botanicals against this vector may be the alternate, biodegradable eco-friendly tool to control sand flies. Use of botanicals as traditional medicine has been an ancient practice for various diseases. In the last century, plants are reported scientifically to possess various medicinal properties viz., antibacterial (Kumar et al., 2010 a; Kumar et al., 2010 c), antifungal (Kumar et al., 2010 b), anticancer (Rajkumar et al., 2009), anti-inflammatory (SV Kumar et al., 2009), anti-helminthic (Adama et al., 2009), antioxidant (Priya et al., 2010; Oliveira et al., 2011), larvicidal activity (Pitasawat et al., 1998; Oliveira et al., 2011; Sagnou et al., 2012), repellent (Kebede et al., 2010), etc. Roark (1947) described approximately 1,200 plant species having insecticidal value. Several groups of phytochemicals such as alkaloids, steroids, terpenoides, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan et al., 2005).

The selected plant *C.infortunatum* Linn. (Family: Verbenaceae), locally known as 'Bhant' in Hindi is a small shrub occurring throughout the plains of India mostly in mango orchards as wild herb. This plant is an important ethno medicinal plant with several medicinal properties and used widely in Indian folk medicine for the treatment of bronchitis, asthma, fever, burning sensation, diseases of blood, inflammation and epilepsy (Sreevastava, 2007). Traditionally, the plant is used as an antipyretic, antihelmentic, antioxidant, anti-diabetic, anti-inflammatory, anti-venom, anti-fertility activity, antimalaria and antimicrobial. Leaves of the plant are prescribed for tumor, certain skin diseases and scorpion sting (Chowdhury et al., 1994; Rajurkar, 2011; Bhattacharjee et al., 2011). Above cited literature represents *C. infortunatum* as an important source of novel pharmaceutically important compounds and a future candidate as an insecticide. Novel botanical insecticides are looking forward to the alternative sources and in last few decades, botanicals have been studied extensively for their insecticidal activity to develop new lead molecules to add on to Vector Control Programme. Toxicity of the active molecule is of prime consideration in development of insecticide. There are reports of acute and sub-chronic toxicity of the methanol extract of *C. infortunatum* leaf (MECI) in Swiss albino mice (Das et al., 2010). *C. infortunatum* leaves (hexane extract) was found having insecticidal effect against sand fly vector of Kala-azar. Hence, it is essential to find out haemolytic inhibition activity against human erythrocytes.

Materials and methods

Collection of plant and preparation of herbarium & plant material

The plant was collected from Kala-azar endemic areas of Vaishali district of Bihar, Patna, India and identified by a taxonomist from Department of Botany, Science College, Patna, and Bihar, India with Voucher Number- PK90. Herbarium sheet was prepared using standard methods.

Preparation of Plant Extracts:

The plant was dried in shade after washing and surface sterilization with 70% ethyl alcohol under laboratory conditions. Infusion of leaf was prepared by grinding in electric grinder. Crude extract of plant was collected in hexane (w/v) in the ratio of 1:10; (2g/20 ml) by soaking the infusions of parts of plants in Hexane for 48 hrs under laboratory conditions. The filtrate was collected and stored in refrigerator. The residue was treated thrice till clear appearance. The filtrate was dried at 40^0C to obtain crystals. Extract was stored at -20^0C for further bioassay.

Hemolytic Inhibition activity assay

Preparation of Red Blood Cells Suspension:

The protocol as described by Kalita et al. (2011) was followed for preparation of Red Blood Cells suspension with slight modifications with the approval of Ethical Committee of the institute, Rajendra Memorial Research Institute of Medical

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Sciences (ICMR), Agamkuan, Patna-800007, Bihar, India. Human blood (2 ml) was collected from a healthy volunteer having blood group 'O' Rh –ve after taking written consent from the volunteer using 5 ml disposable syringe in EDTA vial to avoid coagulation. The blood was centrifuged at 1500 rpm for three minutes. The pellet was washed thrice with sterile Phosphate Buffer Saline solution (1 X, pH 7.2 ± 0.2) by centrifugation at 1500 rpm for 5 minutes until the supernatant was colorless. A final 0.5% RBCs suspension was prepared by suspending 50µl of RBCs suspension in 9950 µl of Phosphate Buffer Saline (PBS). Fresh cell suspension was prepared and used within 6 hours after preparation.

Preparation of stock and test solution of plant extract:

The stock solution (11800 mg/L) of the hexane extract of *C.infortunatum* was prepared in PBS by dissolving 11.8 mg of crude extract in 1 ml PBS. From this stock solution the test solution (1000 mg/L) was prepared by dissolving 847 µl of plant extract in 9153µl of PBS.

Hemolytic Inhibition Activity Assay:

Hemolytic Inhibition assay was conducted in 96-well Microtitre plates with Bio-Rad® ELISA Reader following Hassan et al. (2010) protocol with slight modifications. 100 µl of PBS were added to all wells of columns 3-5 and column 12 except the wells in the first two columns in which 100 µl of H_2O were added. Then, 100 µl of test concentration (1000 mg/L) of the plant extract was pipetted into the first row of columns 3-5. The contents of the first row of columns 3-5 were serially diluted through row eight by aspirating and redispensing three times then transferring 100 µl to the next row. This procedure was repeated until 100 µl were discarded from each column after the last dilution. 100 µl of 0.5% RBCs suspension were added to all wells of columns 1-5 and column 12 of the plate. This process resulted in eight dilutions ranging from 500 mg/L- 4.15625mg/L hexane extract (*C.infortunatum* leaf) fraction of PBS. The first two columns containing H_2O with RBCs were 100% hemolysis positive control wells, while column 12 containing PBS and RBCs alone served as the 0% hemolysis negative control wells. The experiment was conducted in three replicates. Each plate was sealed with aluminum foil and kept for 3 hours at room temperature. Turbidity was measured by reading well optical density at 450 nm, 490 nm and 655 nm using a multi-well plate Bio-Rad® ELISA reader and observing a decrease in optical density associated with RBCs lysis. % Hemolytic Inhibition Activity was calculated using the formulae:

% Hemolytic Inhibition Activity =

$$\frac{[\text{Absorbance (+Ve Control)-Absorbance (Test)}]\ X100}{[\text{Absorbance (+Ve Control)-Absorbance (-Ve Control)}]}$$

(Where, +Ve Control = H_2O + RBCs, -Ve Control = PBS + RBCs, Test = PBS + Plant Extract + RBCs).

The IC50 value was calculated for each wavelength using Graph Pad Prism 6 Software and Transform Log-dose curve was plotted.

Results

The phytochemical analysis of hexane extract of *C.infortunatum* leaves indicated the presence of Alkaloids, Oils & fats, Phytosterol, Terpenoids and Proteins & Amino Acids as major phytochemical groups which need to be evaluated for any toxic effect. However, it has shown 63% insecticidal effect to Sand flies (Unpublished data, Accepted). The Per cent Hemolytic Inhibition Activity of the extract varied with concentration of the extract at all the wavelengths but was almost similar for each concentration at different wavelengths. At 250 mg/L, 125 mg/L and 62.5 mg/L concentrations the Per cent Hemolysis Inhibition is greater than the –Ve control indicating it to be non-toxic with potential of haemoglobin restoration and highly acceptable.

Fig 1

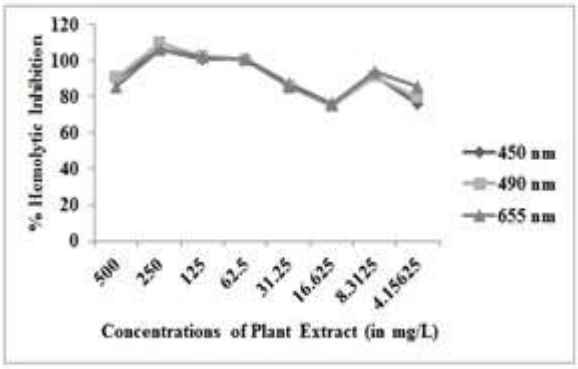

Fig 1: Percentage Hemolytic Inhibition Activity of *Clerodendron infortunatum* leaves (Hexane extract) to 0.5% human erythrocyte suspension at different wavelengths

The IC_{50} value for Per cent Hemolytic Inhibition activity of the extract was found to be 25.80 mg/L (95% CI Top-0.7414 to 0.9862, Bottom-0.7541 to 0.8986), 18.68 mg/L (95% CI Top-0.4349 to 0.7255, Bottom-0.4775 to 0.6032) & 15.90 mg/L (95% CI Top-0.2586 to 0.5383, Bottom-0.3088 to 0.4116) at 450 nm, 490 nm & 655 nm wavelengths respectively.

Table: IC50 Value for the % Hemolytic Inhibition Activity of *Clerodendron infortunatum* leaves (Hexane extract) to human erythrocyte at different wavelengths.

Wavelengths	IC_{50} Value (in mg/L)	R^2 Value	Std. Error		95% Confidence Interval	
			Top	Bottom	Top	Bottom
450 nm	25.80	0.0294	0.06	0.03	0.74 to 0.99	0.75 to 0.90
490 nm	18.68	0.0328	0.07	0.03	0.44 to 0.73	0.48 to 0.60
655 nm	15.90	0.0388	0.07	0.03	0.26 to 0.54	0.31 to 0.41

The Transform Log-Dose curve showed that the extract was non-toxic causing either very low hemolysis or no hemolysis being very close to –Ve control.

Fig 2

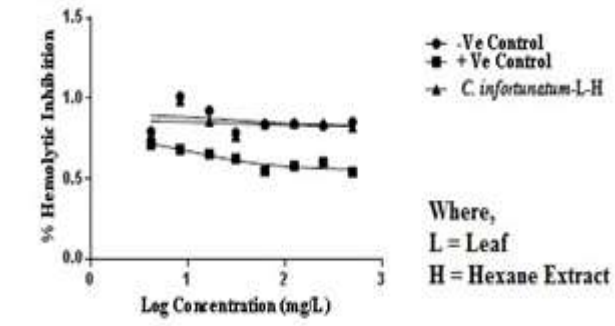

Fig 2: Transform of Log-Dose Vs response Curve for the % Hemolytic Inhibition Activity of *Clerodendron infortunatum* leaves (Hexane extract) to human erythrocyte at 450 nm wavelength.

Fig 3

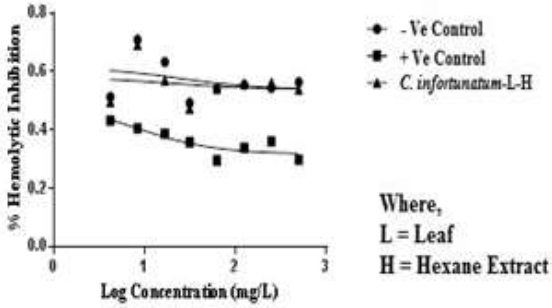

Fig 3: Transform of Log-Dose Vs response Curve for the % Hemolytic Inhibition Activity of *Clerodendron infortunatum* leaves (Hexane extract) to human erythrocyte at 490 nm wavelength.

Fig 4

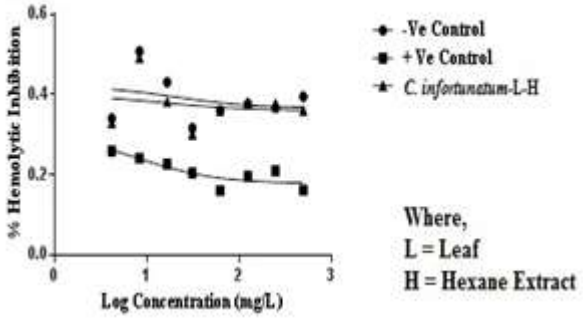

Fig 4: Transform of Log-Dose Vs response Curve for the % Hemolytic Inhibition Activity of *Clerodendron infortunatum* leaves (Hexane extract) to human erythrocyte at 655 nm wavelength.

Discussion

Earlier studies on phytochemical analysis of plant have shown the presence of saponin, clerodin (a bitter diterpene), fixed oil which consists of Glycerides of Lenoleic, oleic, stearic and lignoceric acid (Bhattacharjee et al., 2011). The methanol extract of the plant was found to contain triterpenes, steroids and flavonoids (Sannigrahi et al., 2009). These phytochemical compounds are the key compounds in the medicinal value of the plant.

Studies conducted on the methanol extract of *C. infortunatum* reported that the plant extract restored the hematological parameters by increasing the number of RBC count and hemoglobin content as compared to that of Ehrlich Ascites Carcinoma control and was found to be nontoxic to Swiss Albino mice (Das et al., 2010) which were in concordance to our study.

The low IC_{50} value for % Hemolytic Inhibition Activity revealed that the plant extract was highly acceptable and non-toxic to human beings at all the concentrations. Hence, the plant extract can be further analyzed and harnessed for the active molecule to develop alternative insecticide. Therefore, the plant extract can be used as a suitable safer candidate for future alternate to chemical insecticides, adding a new weapon in the arsenal of Vector Control Programme.

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