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Insecticidal Activity of a Bioinsecticide from Stem Bark of *Streblus asper* on Red Cotton Bug (*Dysdercus cingulatus*)

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A B S T R A C T

Cotton, the most important fiber crop of India plays a dominant role in its agrarian and industrial economy. Pest management approach of varying degrees of sophistication has been applied for insect control in all cotton-producing areas of our country. During the coevolution of plants and insects, plants have biosynthesized a number of secondary metabolites to serve as defense chemicals against insect attack. Plants appear to produce a wide variety of secondary metabolites as defensive weapons. Such defensive chemicals are far superior to the synthetic pesticides. It concludes that the isolated polyphenolic biopesticide from *Streblus asper* (PBSA) was found to have noteworthy insecticidal activity to *Dysdercus cingulatus* (red cotton bug) with an LD_{50} of 0.894 µg/insect by residual film technique and 0.595 µg/insect by topical application. Significant inhibition in activities of acetylcholine esterase (AChE), antioxidant enzymes and glutathione-S-transferase (GST) and significant increase in the lipid peroxides (MDA) lead to the fact that *Dysdercus cingulatus* become more susceptible to the tested biopesticides. The present study has provided some basic information on the mechanism of insecticidal action of polyphenolic compound that will be useful to the development better management strategies. This may provide a useful beginning for the development of eco- friendly biopesticides.

***Key words*:** Polyphenolic compound, *Streblus asper*, Acetylcholinesterase, *Dysdercus cingulatus*

Environmental pollution by pesticides has been increasing due to their uncontrolled use to manage various pests. Current strategies for the control of insects have relied heavily on insecticides, resulting in the development of resistance in insects [1]. It is not uncommon among farmers to make four or five insecticide applications. Such usage, although aimed at one or two target pests, will usually have an adverse effect on other insects and on the ecosystem, including natural enemies. This may result in pest resurgence and development of insecticide resistance in insects [2]. To avoid these problems, it is necessary to minimize the use of chemical insecticides for insect control. Consequently, an intensive effort has been made to find alternative methods of pest control.

Recent emphasis is on the use of natural insecticides, which are usually of plant origin. Bioinsecticides are highly effective, safe, and ecologically acceptable than synthetic insecticides. Because of their natural origin, biological products are biodegradable and they do not leave toxic residues or by-products to contaminate the environment [3]. Studies on plant-insect chemical interactions in the last few decades have unveiled the potential of utilizing bioinsecticides in the form of secondary plant metabolites as pest control agents. This interest in bioinsecticides is providing an alternative in pest management programmes to the synthetic insecticides [4]. Cotton (*Gossipium hirsutum*), the most important fiber crop of India plays a dominant role in its agrarian and industrial economy. The red cotton bug, *Dysdercus cingulatus* F. is an important pest of cotton. Although synthetic chemical insecticides can control it, the side effects are enormous [5]. Pest management approach of varying degrees of sophistication has been applied for insect control in all cotton-producing areas of our country.

Streblus asper, Lour. (Family- Urticaceae, Sub family- Moraceae) is a traditionally used medicinal Plant in India. Stem bark is reported to be effective against lymphoderma, chyluria and other manifestations of filariasis [6-7]. Insecticidal effects have been shown in extracts of the *S. asper* stem [8] and leaves [9]. *Streblus asper* leaf extract was shown considerable antimicrobial activity [10-11]. Preliminary study has reported that chloroform extract of dried stem bark showed insecticidal activity against red cotton bug, *Dysdercus cingulatus* [12]. In view of this, we have aimed to separate and identify the bioinsecticide from the stem bark of *Streblus asper* and also study the mechanism of insecticidal activity in *Dysdercus cingulatus* (red cotton bug).

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Red cotton bug (*Dysdercus cingulatus*)

Stem part of *Streblus asper*

MATERIALS AND METHODS

Insects: Dysdercus cingulatus

The red cotton bug, *Dysdercus cingulatus* Fabr. (Heteroptera: Pyrrhocoridae) were obtained from our laboratory colony maintained under controlled conditions (temp 28-30°C, r. h 95 ± 2% and a photoperiod of light 12 h; dark 12 h) by feeding soaked cottonseeds. Newly emerged fifth instar insects were used for the experiments. Each treatment contains three replications and fifteen insects were used for each treatment.

Bioinsecticide from Streblus asper

The stem bark of the plant, *Streblus asper* was collected from Nagarcoil Forest (Tamil Nadu, India) and was authentically identified by a qualified botanist. Voucher specimens are deposited in the herbarium of Department of Botany, University of Kerala. Bioinsecticides were extracted from *Streblus asper* according to the procedure reported earlier from this laboratory [12]. Chloroform fraction [fraction C] obtained from column chromatography ((Silica gel, Particle size 0.13-0.25mm,60-120 mesh)) was subjected to silica gel thin layer chromatography using 20 × 20cm plates with thickness 0.5mm, 30% acetic acid as solvent system and dried plates were illuminated under UV light [13]. Two blue spots [spot I with an Rf of 0.482 (compound I) and spot II with an Rf = 0.589 (compound II)] were obtained (Figure 1) and both were eluted in chloroform, solvent was evaporated in vacuum, and the compounds were redissolved in 40% ethanol for testing the insecticidal action on *D. cingulatus*.

Fig 1 TLC spots under UV light

Laboratory toxicity bioassays

Newly emerged fifth instar *Dysdercus cingulatus* were used for studying the insecticidal action. Insects were divided into three groups (15 insects each). A solvent control (ethanol 40% v/v) set was run simultaneously. Insecticidal activity of these compounds was tested by following methods [14-15].

(a) *Topical application*: Varying concentrations of compound I (0.2µg - 1.0 µg/insect) and compound II (1.0 µg - 5.0 µg/insect) were applied topically onto the bug. The experiment was carried out in the petridish using a microapplicator and provided with cotton seeds and cotton. Mortality was recorded after 24 hrs.

(b) *Residue film technique*: For this purpose, compound I (0.2µg - 1.0 µg/insect) and compound II (1.0 µg - 5.0 µg/insect) were uniformly coated inside the upper lid and lower base of the petridish by using an electric fan. Then the bug was confined inside the petridish and provided cotton seeds and cotton. Mortality was recorded after 24 hrs.

Partial chemical identification of the compound with the most insecticidal activity

The compound I with maximum insecticidal activity was subjected to UV spectral analysis in methanol (Perkin- Elmer lamda 15 UV/ Visible spectrometer), IR in KBr (Perkin Elmer) and ^{1}H NMR analysis was done with the help of Regional Sophisticated Instrumentation Centre, Central Drug Research Institute, Lucknow, India.

Mechanism of insecticidal action of the most active compound from Streblus asper

Experimental design

Newly emerged fifth instar *Dysdercus cingulatus* were used for studying the mechanism of insecticidal action. Insects were divided into two groups (10 insects each), a solvent control (ethanol 40% v/v) and a test group with 0.595 µg (LC_{50}) of compound I (Rf = 0.482) in 40% ethanol. After 12 and 24 hours of topical application, a tiny incision in the antennae and the hemolymph was collected directly into a polystyrene tube containing few crystals of phenylthiourea. Hemocytes were removed from the hemolymph by centrifugation at 200g for 15 min. Separated hemolymph was used for various analysis [16]. Whole brain was minced separately and homogenized with normal saline and centrifuged for 10 minutes at 3000 rpm and the supernatant was used for various analyses. Homogenates (as described) were performed in pools of 10 insects and every enzyme activity determination was the average of 6 independent pools of 10 insects [16].

Biochemical assay of acetyl cholinesterase (AChE)

The assay of acetyl cholinesterase (AChE EC 3.1.1.7) activity was done the method of Ellman and Courtney [17]. The activity was expressed as nanomoles of thiocholine liberated per minute per milligram protein.

Estimation of protein

Protein contents of supernatant were determined after TCA (trichloro acetic acid) precipitation by the method of Lowry *et al*. [18] using bovine serum albumin (BSA) as the standard protein. The protein was measured at 670 nm absorbance in a spectrophotometer.

Data analysis

LC_{50} values were analyzed using probit analysis performed with the statistical software SPSS/Windows (SPSS 10.0. LNK). Activities of AChE, SOD, catalase and GST and concentrations of GSH and TBARS were analyzed using analysis of variance (ANOVA). The data were analyzed by using SPSS (version 10.0) for Windows and expressed as means ± SEM.

RESULTS AND DISCUSSION

Insecticide toxicity

(Table 1-2) give the mean percentage mortality rate of insects treated with compound I (Rf = 0.482) & compound II (Rf = 0.589) according to the average number of insects for each case both in residual film technique and topical application respectively. The mean percentage mortality of *D. cingulatus* was observed to be increased in a concentration dependent manner in both cases. Topical application showed a better mortality rate than residue film technique (Table 1-2).

Table 1 Mean percentage mortality of *D. cingulatus* treated with graded doses of Compound I (Rf = 0.482) by topical application and residue film technique

Dose (µg)	Mean percentage mortality	
	Topical application	Residue film technique
0.2	21.92 ± 1.12	8.67 ± 0.85
0.4	35.10 ± 1.02	16.63 ± 0.56
0.6	50.39 ± 0.953	28.21 ± 0.78
0.8	65.62 ± 1.34	42.69 ± 0.54
1.0	78.66 ± 1.45*	58.25 ± 0.80*
40% ethanol	0	0

*Represents the group which shows maximum % mortality
Values expressed as mean ± SEM, for n = 6 experiments

Table 2 Mean percentage mortality of *D. cingulatus* treated with graded doses of compound II (Rf = 0.589) by topical application and residue film technique

Dose (µg)	Mean percentage mortality	
	Topical application	Residue film technique
1	2.38 ± 1.23	0.92 ± 0.055
2	4.15 ± 1.58	1.74 ± 0.12
3	6.87 ± 1.74	3.13 ± 0.57
4	10.80 ± 1.98	5.32 ± 0.98
5	16.12 ± 2.10	8.59 ± 1.45
40% ethanol	0	0

Values expressed as mean ± SEM,
For n = 6 experiments

The LD_{50} values of compound I (Rf = 0.482) and compound II (Rf = 0.589) are given in (Table 3). The maximum insecticidal activity was shown by the compound I (spot I: Rf - 0.482) with an LD_{50} of 0.894 µg/insect by residual film technique and 0.595 µg/insect by topical application. But the compound II showed lesser insecticidal activity with an LD_{50} of 10.51 µg/insect by residual film technique and 8.99 µg/insect by topical application (Table 3).

Table 3 LC_{50} values of compound I (Rf = 0.482) and compound II (Rf = 0.589) by topical application and residue film technique

Polyphenolic rich fraction	LC_{50} (µg/insect)	
	Topical application	Residue film technique
Compound I (Rf = 0.482) from TLC	0.595 ± 0.04	0.894 ± 0.08
Compound II (Rf = 0.589) from TLC	8.99 ± 0.98	10.51 ± 1.58

Partial chemical identification of the most active compound

The UV spectrum (Fig 2) showed a λ max at 282 nm. Markham [13] has tabulated the characteristic absorption maxima of flavanoids which shows that flavanones and dihydro flavanones absorb in this region. The IR spectrum (potassium bromide pellet method) of the spot I obtained from TLC showed a broad band centered at 3444 cm^{-1}, which indicates the presence of OH groups in the molecule. A strong peak is observed at 1735 cm^{-1}, which is assignable to the stretching vibration of a C==O group, may be of an ester carbonyl. The spectrum also seems to suggest the absence of any benzoid derivatives as the 900-600 cm^{-1} region, where the δ C—H vibrations of aromatic C—H hydrogens would appear, is mostly featureless (Fig 3). The ^1H NMR spectrum in chloroform ($CDCl_3$), 300 MHz (Fig 4) indicates that isolated fraction contains compound(s) which have a hydroxyl group, seem to be non-aromatic, carry MeO- substituent(s) and several alkane hydrogens, including one or more methyl groups. There appears to be no CH_3CO groups in the compound. So, the compound/s belongs to flavanone class. The polyphenolic content was found to be 526 mg/kg in compound I of dried stem bark of *Streblus asper* (Fig 5).

Mechanism of insecticidal action of the most active compound from Streblus asper: Acetyl cholinesterase (AChE) activity

The activity of acetyl cholinesterase (AChE) was significantly inhibited in both hemolymph and brain of red cotton bug treated with active compound from *Streblus asper* on topical application after 12 and 24 hours when compared to control insects. The activity was significantly inhibited after 24 hours topical application when compared to 12 hour exposure (Table 4).

Table 4 Activity of acetyl cholinesterase in haemolymph and brain of red cotton bug (*Dysdercus cingulatus*) exposed to the most active compound from *Streblus asper*

	After 12 hours				After 24 hours			
	Haemolymph		Brain		Haemolymph		Brain	
	Control	Test	Control	Test	Control	Test	Control	Test
AchE (U^1)	28.16±0.803	12.95[a]±0.584	42.36±1.75	27.96[a]±1.02	27.53±0.679	7.89[ab]±0.5511	42.84±1.27	20.33[ab]±0.694

[1]Unit = nanomoles of thiocholine liberated per minute per milligram protein
Values expressed as mean ± SEM, for n = 6 experiments.
[a] test group is compared to control group at $p \leq 0.05$.
[b] test group after 12 hours is compared to test group after 24 hours at $p \leq 0.05$

The use of plant products as insecticides is gaining importance in recent years in view of the environmental and health hazards posed by synthetic organic insecticides. It is reported that the methanolic extract of *Alpinia oxyphylla* possess insecticidal action against larvae of *D. melanogaster* [19]. Results obtained by Saidana *et al*. [20] indicated that the methanolic extract of tunisian halophyte, *Tamarix boveana* caused significant and early mortalities and growth inhibition of the insect *Trogoderma granarium* at the concentration of 50 µg/disc. The previous study in laboratory reported that various extracts of the stem bark of *S. asper* possess insecticidal activity against *Dysdercus cingulatus* [12]. The LC_{50} value of the crude

methanolic extract is 5.56 µg. Four fractions obtained from methanolic extract of *Streblus asper* showed insecticidal action with increasing order of water (LC_{50} 10.15 µg) < n- butanol (LC_{50} 8.97 µg) < n- hexane (LC_{50} 5.89 µg) < chloroform (LC_{50} 2.01 µg). The most active chloroform fraction of *Streblus asper* on further partial purification by silica gel column chromatography and testing the insecticidal action clearly showed that the fractions rich in polyphenolic content (fraction C, D & E) exhibited the highest insecticidal action [12]. The present study showed that on further purification of fraction C by preparative TLC gave two spots (compound I and compound II). The maximum insecticidal activity was shown by the compound I (spot I: Rf - 0.482) with an LD_{50} of 0.894 µg/insect by residual film technique and 0.595 µg/insect by topical application. The increase in insecticidal action upon further purification of chloroform fraction may be due to the accumulation of the polyphenolic compounds. ^{1}H NMR, UV and IR spectral analysis of compound I with maximum insecticidal activity showed that the compound may be in the class of flavanone family [21]. This is in support with the finding that polyphenols isolated from aqueous extract of *Annona squamosa* (Custard apple) showed 80% insecticidal activity against the stored grain pest pulse beetle, *Callosobruchus chinensis* [22]. There are so many reports are available which show that polyphenolic compounds are having insecticidal activity [23-25].

Mode of action of the major insecticides involve mainly in the nervous system, especially inhibition of acetylcholinesterase, an enzyme of critical importance in the transmission of nerve impulse [26]. The measurement of the activity of acetylcholinesterase in red cotton bugs exposed with bioinsecticide was carried out in this perspective. In the present study the polyphenolic bioinsecticide exposure have been demonstrated to reduce the activity of acetyl cholinesterase significantly in red cotton bugs. The results are in agreement with Maazoun *et al*. [27] who reported that *Urginea maritima* bulbs extract exhibited inhibitory acetylcholine esterase (AChE) activity in in the rice weevil, *Sitophilus oryzae* (L.). Our results are also in support with the findings of Rajashekar *et al*. [28], Elbert *et al*. [29], Acheuk *et al*. [30] which showed insect repellent activity, contact toxicity and acetyl cholinesterase inhibition upon exposure of crude plant extracts.

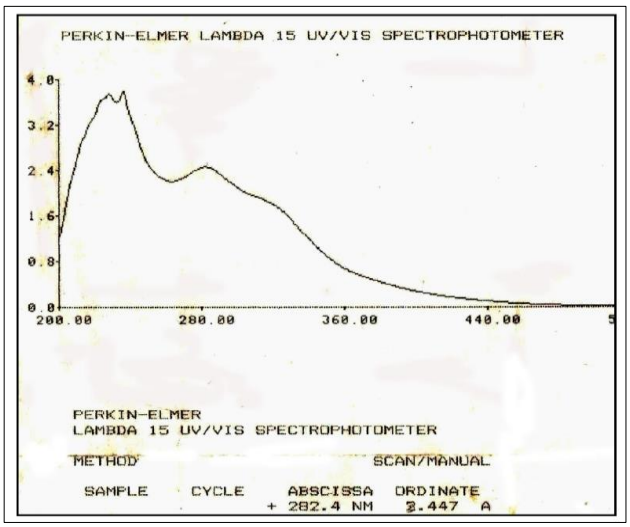

Fig 2 UV spectrum of spot I

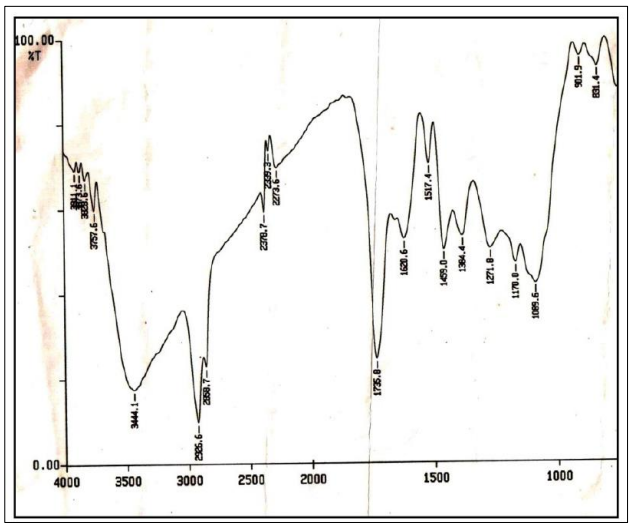

Fig 3 IR spectrum of compound I

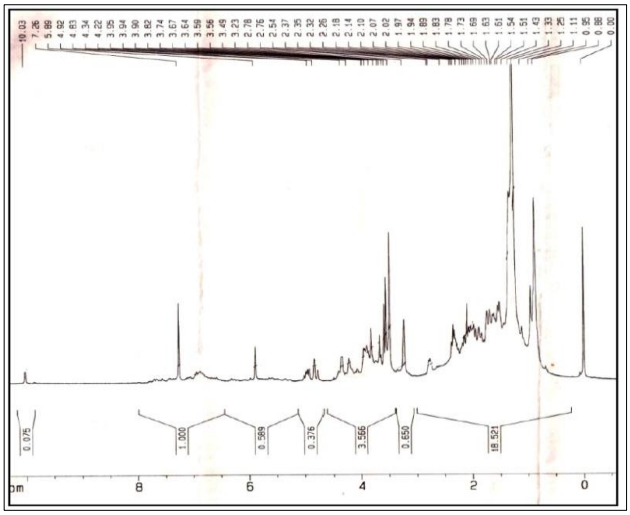

Fig 4 ^{1}H NMR spectrum of compound II

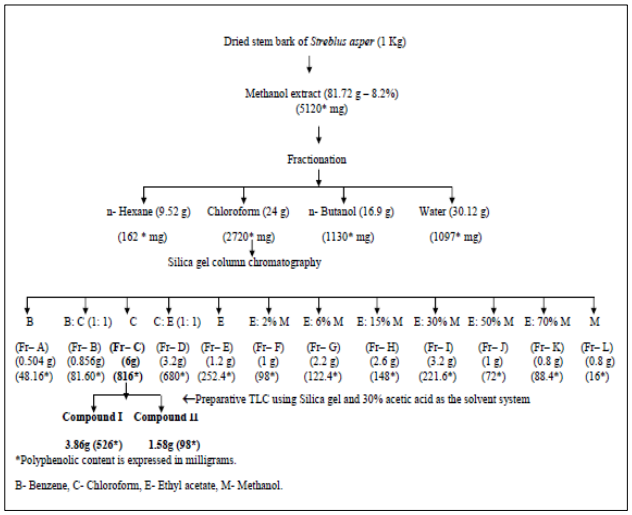

Fig 5 Yield and polyphenolic content of different fractions from *Streblus asper*

CONCLUSION

In conclusion, polyphenolic rich fractions and its partially purified compounds from *Streblus asper* had significant effects in the mortality of newly emerged fifth instar *Dysdercus cingulatus*. The possible reason for this mortality could be the inhibition of acetyl cholinesterase activity in red cotton bug (*Dysdercus cingulatus*). Therefore, the polyphenolic secondary metabolite-based insecticide may serve as an effective alternative to conventional pesticides in the control of

red cotton bugs. Further studies are now required to determine the exact mechanism of action of insecticidal activity exhibited by the purified compound from *Streblus asper*.

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