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Allomones confer resistance to *Musa paradisiaca* L. cultivar Pisanglilin against infestation by *Odoiporus longicollis* [Oliver] and characterization of Allomones

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ABSTRACT

In bioassay guided extraction of pseudostem powder of Pisanglilin by organic solvents we found the larvicidal activity in acetone extract, whose column chromatography by methanol-chloroform mixture separated the extract into 9-fractions, of which the 8th fraction showed larvicidal activity. Subfractionation of the active fraction by column chromatography resulted in the isolation of two larvicidal molecules [Stigmasterol-3-O-glucoside (SOG) and Sulfoquinovosyl diacylglycerol (SQDG)]. Yield of SOG was 0.002 % and SQDG was 0.005 % and both were highly toxic to *O. longicollis* larvae with LD_{50} of 0.40 and 0.378 ppm, respectively. Larvae fed these compounds stopped feeding on third day and died within one week. SOG inhibited the amylase and protease activity of gut and induced histolysis in the mid gut. While SQDG inhibited the leucine amino peptidase and trypsin like serine protease activities, which decreased the content of total free amino acids. Imbalance in the activities of aspartate amino transferase and alanine amino transferase disrupted the aminoacid metabolism and the compound inhibited the activity of tyrosinase (an enzyme involved in cuticle development). SQDG toxicity caused accumulation of 20-hydroxyecdysone, the active moulting hormone in the hemolymph. Simultaneous action of two allomones present in Pisanglilin effectively resisted the attack of endophytic larvae in the pseudostem and thereby conferred resistance against infestation by *O.longicollis.* Preliminary study by intrapseudostem injection of Pisanglilin extract in susceptible *M.paradisiaca* cultivar *Kappa,* gave complete protection to it from attack by this pest, under field condition.

Key words: Pisanglilin, Allomones, Pest resistance, *Odoiporus longicollis,* Stigmasterol-3-O-glucoside, Sulfoquinovosyl diacylglycerol, 20- hydroxyecdysone, *Musa paradisiaca*

INTRODUCTION

Banana and plantains are monocotyledonous plants which are unable to set viable seeds. Hence, they are propagated vegetatively through suckers (9). The Indonesia is the centre of origin of banana (38,39). Cultivated bananas are hybrids between two diploid plants *Musa acuminata* Colla and *M. balbisiana.* (44). There is extreme diversity in banana due to polyploidisation, somatic mutation, domestication and parthenocarpy (43,45,48). The first cultivated banana and plantains were diploids but the present banana are triploids. These triploids have rapid vegetative growth and large bunch of fruits than their diploid ancestors (38). Anthropogenic factors such as (cultivation, transportation) and establishment in new sites with new climatic conditions also increased the diversity (39).

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Modern, edible, diploid AA *M. paradisiaca* cultivars are small plants with small bunch of fruits, hence, not preferred by subsistence farmers, who prefer the cultivars with large fruit bunch. Triploid AAA cultivars are cross between edible diploid AA cultivars with wild *M. acuminata*.

Photo plate 1. Adult and larvae of *Odoiporus longicollis* L

Kerala, has rich diversity of *M. paradisiaca* cultivars, but these are infested by *Odoiporus longicollis* (Olivier) (1,2,18). Pisanglilin, a pest resistant, diploid AA *Musa paradisiaca* cultivar, indigenous to Pathanamthitta district of Kerala, India is a commercially non-viable cultivar but Red banana, a commercially viable triploid AAA *M. paradisiaca* cultivar is aggressively attacked by *Odoiporus longicollis* [Oliver]. Mother weevils (Photo plate1) specifically select the *M. paradisiaca* cultivars because these have least amount of phenols and flavonoids to keep their eggs in the pseudostem (3,21). The larvae developed from these eggs cut and feed on the internal soft tissues. Excretions and secretions of internal pest and chewed debris of plant materials invite decaying bacteria and finally caused premature death of host plant (18,19). To control this insect farmers use the banned systemic insecticide carbofuran, hence, most of the banana in the market are contaminated with this very harmful insecticide (19,20). Hence, there is need to find a safe systemic larvicide to kill this internal pest. The agroecosystems of Kerala possessed diversity of *M. paradisiaca* cultivars and sharing boundary with forests hence in such sites banana plants are free from *O.longicollis* infestation (18,21).The AAA *M. paradisiaca* cultivars have stout robust pseudostem, broad leaves and large fruit bunch upto 40 Kg, but are susceptible to *O.longicollis* infestation (3,21).

Photo plate 2. Susceptible and resistant *Musa paradisiaca* cultivars

The *O. longicollis* larvae live in the pseudostem of banana and are killed by allelopathic potential of *M. paradisiaca* cultivars in field condition killed in one week (21,20,22). One of the highly resistant *Musa* cultivars among them was identified as Pisanglilin, a diploid AA. *M. paradisiaca* cultivar (39). Pisanglilin pseudostem juice is used as herbal medicine in Chittar village of Pathanamthitta, Kerala, to treat diabetes, hyperacidity and urolithiasis (18). Another test cultivar in this study was Red banana AAA locally called as *Kappa,* which is highly susceptible to infestation by this pest (Photo plate 2). Bioassay guided extraction and separation by chromatography isolated two active larvicidal compounds from the dry pseudostem powder of AA cultivar Pisanglilin. This study aimed to find allelopathic chemical compounds present in Pisanglilin, which killed the endophytic *O.longicollis* larvae within one week in live pseudostem (22). The discovery of safe systemic insecticide to control this pest is very essential.

MATERIALS AND METHODS

Suckers of banana cultivars were collected from Chittar village (Longitude 09 ° 18´ - 09´ 20´ N Latitude 076° 57, Altitude :159 to 334 m above sea level, annual rain fall: 2822 to 3300 mm, mean temperature 18 to 35°C. Pathanamthitta district, Kerala, India. This place shares boundary with tropical ever green forest (Konni division, Kerala). They were planted in our campus of University College. No synthetic fertilizers were applied and maintained with 10 kg cow dung, once in two months and watered once in two days. These plants were used to collect the samples for phytochemical analysis and for getting pieces of pseudostem for toxicological studies.

Study period: Phytochemical analysis and bioassay guided fractionation of extracts and sub fractions were carried out between January 2017 and December 2018.

***Musa* cultivars:** Ten suckers, each of highly pest resistant and commercially non-viable AA *Musa* cultivar Pisanglilin and commercially viable and highly pest susceptible AAA *Musa* cultivar Red Banana(*Kappa*) were collected from the fields of Pathanamthitta district and planted in College Campus. Four to six months old plants were used for the study and were provided with cow dung 10 Kg once in two months and no synthetic fertilizers were used and watered every two days.

Experimental Organism: *O. longicollis* larvae were collected by dissecting the infested pseudostem of susceptible *Musa* cultivars. Actively feeding third instar larvae were maintained in plastic containers with 100 g fresh piece of pseudostem possessing single larvae. Fresh pseudostem of Red banana was provided every two days. Hemolymph of third instar larvae was used for biochemical analysis.

SECTION A: PHYTOCHEMICAL ANALYSIS

(a). Extraction and isolation of larvicidal molecules

Fifty kg pseudostem of both test *Musa paradisiaca* cultivars either susceptible *Kappa* or resistant *Pisanglilin* were cut vertically into small chips and dried in shade (ambient temperature 28-33°C) for two weeks. It was powdered in an electric pulveriser (mesh 0.5 mm), resulting in pseudostem powder.

(b). Soxhlet extraction of pseudostem powder

The pseudostem powder (1 Kg) was sequentially extracted with petroleum ether (2 L), acetone (2 L) and methanol (2 L) using soxhlet apparatus, 6.0 h for each solvent. Temperature was kept 45 ^{0}C for the first two solvents and 50 ^{0}C for methanol. The extracts were evaporated to dryness in a rotary vacuum evaporator and subjected to bioassay as described below.

(c). Bioactivity guided fractionation of acetone extract

Acetone extract (10.03 g) was dissolved in a minimum volume of chloroform and adsorbed with 30 g silica gel (60-120 mesh), which was loaded in a column packed with 300 g silica gel (60-120 mesh) and stabilized with petroleum ether. Column was eluted with step wise gradients started with Chloroform: Petroleum ether applied in relationships (v/v) of 0:100 (750 ml), 75:25 (750 ml), 50:50 (750 ml), 75:25(750 ml), 100:0 (750 ml), then followed with Chloroform: Methanol ion ratios (v/v) of 98:2 (900 ml), 95:5 (900 ml), 90:10 (900 ml), 80:20 (900 ml), 0:100 (900 ml). Fraction 8 eluted from this column was evaporated to dryness using a rotary vacuum evaporator. An amount of this fraction (1.2 g) was dissolved in a minimum volume of chloroform and adsorbed with 3 g of silica gel (100-200 mesh). Then, the silica gel was loaded on a column packed with 30 g silica gel (100-200 mesh) and stabilized with chloroform. Column was eluted with a stepwise gradient of chloroform-methanol provided in relationships (v/v) 95:5 (300 ml), 90:10 (300 ml), 85:15 (300 ml), 80:20 (300 ml), 75:25 (300 ml). The sub fractions 2nd and 3rd

exhibited pure compounds in HPTLC. Phytochemical analysis was done in the Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram.

(d). HPTLC analysis of extracts

HPTLC analysis of acetone extract, active fractions and sub fractions was done by CAMAG, HPTLC system (Switzerland). Samples were applied to a Silica gel 60, F254 TLC plate (Merck, Germany), (10 cm X 10 cm), using Linomat V sample applicator as 8 mm bands. It was then developed upto 80 mm in a twin trough glass chamber using the mobile phase ethyl Acetate: Formic Acid: Water (90:5:5, v/v). The plate was then sprayed with anisaldehyde-sulphuric acid reagent and heated at 110 ^{0}C for 10 min. Then, it was photo documented using CAMAG reprostar 3.

(e). Spectral analysis of larvicidal molecule

^{1}H NMR, ^{13}C NMR, HMBC, HSQC and LC-Q-T-MS of active compound were done, in School of Environmental Science, M.G. University, Kottayam, Kerala, India.

(f). GC-MS Analysis: GC-MS Varian CP 3800 connected to MS model Saturn 2200(Varian, USA), with Polyethylene glycol column, Helium as carrier gas and a well established. Data Base for fatty acid identification were utilized for the study. The facility is available in NIIST, (CSIR), Thiruvananthapuram.

SECTION B : BIOASSAY OF ALLOMONES IN *O. LONGICOLLIS* LARVAE

(i). Larvicidal tests

Freshly cut pseudostem of 100 g pieces of circular pieces were used for the study (19). Active compound or extract was dissolved in water containing 0.5 % Tween. The dissolved extract (1 ml) from susceptible or resistant pseudostem or active molecule in known concentration were injected into the 100 g piece of pseudostem evenly at 10 sites using insulin syringe. Each larvae was allowed to bore into the pseudostem through a hole (Photo plate 3) made on it by knife (19). Every day each piece of pseudostem containing a single larva inside was kept very close to the ear of investigator to hear the feeding sound, to know the health of larva inside (19). Each piece containing larva was kept in plastic container covered with cheese cloth and kept in dark, cool place. Fresh piece of pseudostem, treated with the test sample was provided once in two days. On 8th day the pseudostem piece was carefully dissected to observe the larvae. Minimum quantity of active compound required for mortality within 7 days was studied. Extract or fraction from the dry pseudostem of Red banana, injected into the pseudostem piece (100 g) served as control. Six larvae were used in each group and experiment was repeated thrice. Percentage of mortality and LD_{50} were calculated by Probit analysis (19).

(ii). Collection of larval hemolymph for biochemical estimations

Third instar larvae on the 4th day of toxicity by active larvicidal molecules were immobilized by keeping them on glass plates placed over the ice cubes. A sharp cut was given on the ventral side of the neck region without breaking the gut. The hemolymph oozed out was collected in glass tubes and centrifuged in refrigerated centrifuge to collect cell free hemolymph.

Photo plate 3. Larva of *Odoiporus longicollis* allowed to bore into the pseudostem piece impregnated with test material.

SECTION C : ESTIMATION OF ENZYME ACTIVITIES AND BIOMOLECULES

(i). Quantification of 20-hydroxy ecdysone: The active moulting hormone was estimated from the larval hemolymph by Enzyme immunoassay (34) using an assay kit (A05120 96 wells supplied by Cayman Chemicals, France) and absorbance at 405 nm was read by Microplate reader. Six larvae were used in each dose and the expt was repeated thrice.

(ii). Estimation of total free amino acids: These were estimated quantitatively by Ninhydrin reaction (47). One hundred µl hemolymph was made upto 1 ml with water and protein was precipitated by adding 1 ml of 0.4M perchloric acid. The mixture was kept in ice bath for 10 min, centrifuged at 8000 g and supernatant 1 ml was neutralized by 4N KOH and made upto 2.5 ml using distilled water. 0.1 ml sample was mixed with 0.9 ml of ninhydrin, prepared by mixing equal volumes of 0.4 % ninhydrin in methyl cellosolve and 1.6 % $SnCl_2$ in 0.2 M citrate buffer pH 5. The mixture was kept in water bath at 100 ^{0}C for 30 min and optical dencity was measured at 570 nm. Amino acid leucine was used as standard. Values are expressed as mg quantity/g tissue.

(iii). Estimation of Aminotransferases: Aspartate aminotransferase (E.C.2.6.1.1) and Alanine aminotransferase (E.C.2.6.1.2) were estimated colorimetrically (37). Enzyme assay was conducted in pH 7.4 by using 0.1M phosphate buffer pH 7.4 and substrate of AsAT was prepared in buffer by dissolving 3.266 g of aspartate and 30 mg ∞ keto glutarate in 20 ml of 1N NaOH and made upto 100 ml in buffer. In a test tube 0.25 ml substrate and 0.05 ml of hemolymph were added, mixed well and kept at 37 ^{0}C for 1 h. The reaction was stopped by adding 0.25 ml of 0.2 % solution of 2,4-dinitrophenylhydrazine in 1N HCl. After 15 minutes 2.5 ml of 0.4 N NaOH solution was added and the colour was read at 540 nm. Various concentrations of oxaloacetate in buffer were used as standard. A sample from enzyme was subjected to protein estimation (26). Activity of enzyme is expressed as microgram of oxaloacetate liberated /min/mg protein.

Alanine amino transferase (AlAT) substrate was prepared by dissolving 1.78 g of alanine and 30 mg of ∞ keto glutarate in 100 ml of phosphate buffer. Pyruvic acid dissolved in buffer was used as standard. Activity of enzymes were expressed as microgram of pyruvic acid liberated /minute/mg protein.

(iv). Estimation of Tyrosinase: Activity of this enzyme, also called as phenol oxidase (E.C.1.14.18.1) was estimated colorimetrically (28), by using L-DOPA as substrate. L-DOPA (0.8 mg/ml) was dissolved in phosphate buffer of pH 6 and it (2.9 ml) was pre incubated at 30 ^{0}C for 5 min and 0.1 ml of hemolymph was added. The mixture was rapidly mixed and poured into the cuvette. Increasing intensity of color was read at 475 nm, at every 15 seconds for 1 minute. One unit of enzyme activity is defined as that amount of enzyme which catalyze the oxidation of one micromole of L-DOPA/minute at 30 ^{0}C. Under the assay condition given above, this corresponds to absorption change of 0.6 per minute. Specific activity was expressed as enzyme units / mg protein.

(v). Estimation of Leucine Aminopeptidase: Activity of this enzyme (E.C.3.4.11.1) was estimated colorimetrically (4) using the synthetic substrate L-leucyl β-naphthyl amide. Buffered substrate was made by dissolving 40 mg substrate in 0.1M phosphate buffer pH 7. Hemolymph was diluted in buffer and 1 ml enzyme solution and 1 ml of buffered substrate were mixed and incubated for 2 h at 37 ^{0}C. Enzyme activity was stopped by adding 1 ml of 40 % trichloroacetic acid. After centrifugation 1 ml of supernatant was mixed with 1 ml of 1 % sodium nitrite followed by 1ml of 0.05 % solution of N-1-Naphthyl ethyelene diamine tetraacetate. The color developed was read at 560 nm. Standard β naphthyl amine was used as standard. Activity was expressed as micrograms of naphthyl amine liberated / minute / mg protein.

(vi). Estimation of trypsin like serine protease: The enzyme (E.C.3.4.21.4) was estimated (10) using N-benzoyl-DL-arginine-p-nitroanilide as substrate. 1 ml of substrate and 1 ml of diluted hemolymph were mixed and incubated at 37 ^{0}C for 1 h. Activity was terminated by adding 30 % acetic acid. The supernatant was monitored spectrophotometrically at 410 nm. Trypsin standards were run alongside and activity was expressed as units / mg protein. One trypsin unit was determined as the amount of homogenate trypsin required to hydrolyse 1 micromole of substrate / minute at 30 ^{0}C.

(vii). Gut histology of larvae: *O. longicollis* larvae were allowed to live and feed in the pseudostem administered with 3-concentrations of stigmasterol-3-O- glucoside for four days. Larval gut of control and intoxicated larvae were dissected out and done the histology (15,25). Midgut was dissected out in insect ringer and fixed in 10 % formalin. Fixed tissues were transferred to a tissue processing capsule. Dehydrated in 50, 70, 80 and 100 % alcohol for 1 h each. Cleared in xylene for 2 h. Impregnated in molten paraffin wax for 3 h, sections were taken using microtome (Leica 213 Rotary type) and stained in haematoxylin and eosin. Photographs were taken using digital camera attached to microscope. (Labomed, USA)

(viii). Assay of digestive enzymes of gut: Gut was dissected out, washed in buffer weighed and homogenized in appropriate buffers under ice cold condition, centrifuged in

refrigerated centrifuge (8000 g) and supernatant made up to 2 ml using buffer and used as enzyme. Three larval guts were put together and homogenized for each dose and repeated six times.

(ix). Amylase enzyme: Activity (E.C.3.2.1.1) was assayed (32) by mixing 0.2 ml of gut homogenate, 0.2 ml Tris-HCl buffer (pH 8.2) and 0.4 ml 1 % starch as substrate. Blank was run without enzyme and 0.1 % maltose was used as standard. Incubated at 37 ºC for 20 min and 1.2 ml Dinitro salicylic acid (DNSA) reagent was added. Heated at 100 ºC for 5 min, optical density of the solution was read at 550 nm.

(x). Protease activity: Activity (7) was studied by mixing 0.2 ml gut homogenate, 0.2 ml Glycine- NaOH buffer (pH 9) and 0.4 ml 1 % casein solution as substrate. Blank was maintained without enzyme and 0.005 % tyrosine was used as standard. Incubated at 37 ºC for 30 min and activity was stopped by adding 1.20 ml of 5 % trichloro acetic acid. Centrifuged at 13,000g for 15 minutes. OD of the supernatant was read at 280 nm.

Statistical Analysis of results

The results obtained are represented as Mean ± SEM. Comparison of results were performed by one way analysis of variance followed by Duncan multiple range test using SPSS21 software. The results are considered statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

Red banana, locally called as *Kappa* is a commercially viable triploid AAA *M. paradisaca* cultivar which is aggressively attacked by *Odoiporus longicollis* Olivier, under field condition. Fruit bunch of this cultivar is large and it may reach upto 40 Kg. Pisanglilin, a diploid AA *M.paradisaca* cultivar yields fruit bunch upto a size of 6 Kg is not preferred by farmers for commercial cultivation but it is totally free from infestation by *O. longicollis.* Adult weevils of *O. longicollis* exhibited high host specificity in selecting a particular *Musa* cultivar for depositing their eggs on the pseudostem, which form the food and shelter of their future progeny (1,2). The mother weevils preferred commercial cultivars, which have extremely low content of secondary metabolites (phenols and flavonoids) compared to pest resistant *M. paradisaca* cultivars (3,21). *M. paradisaca* a pest resistant cultivar AA 'Pisanglilin, ' was never attacked by this pest in field conditions (20) and in experimental rearing of *O. longicollis* larvae in the live pseudostem of this cultivar caused 100 % mortality of larvae within one week (22). Although this AA cultivar is non-viable for commercial cultivation, but is maintained in villages of Kerala due to its usefulness as folk-lore remedy for urolithiasis, diabetes and hypercholesterolemia (18). The crushed juice of fresh pseudostem of Pisanglilin is used for the above purposes.

Under field condition, *Kappa* (AAA) cultivar is highly susceptible to infestation by this pest and the mother weevils show high affinity to it, in depositing their eggs in the pseudostem of this cultivar (18,20). Study revealed that the cultivar possessed extremely low content of phenols and flavonoids. Activities of supporting enzymes [such as phenyl alanine ammonia lyase (PAL), peroxidase (PO) and poly phenol oxidase (PPO)] were minimum in the pest susceptible cultivar *Kappa* (Red banana) than their occurrence in the

resistant cultivar Pisanglilin (21). Another commercially viable *M. paradisaca* AAB cultivar is '*Nendran'* in Kerala, which has 12 ecotypes but all are highly susceptible to infestation by *O. longicollis*. They possessed phenol, flavonoids and activities of enzymes PAL, PPO and PO contents were only one fourth to highly pest resistant *M. paradisaca* cultivar '*Yangambi* AA'(1).

Bioactivity guided extraction of pseudostem

Dry pseudostem powder of resistant cultivar Pisanglilin on serial extraction with petroleum ether, acetone and methanol resulted localization of larvicidal activity in acetone extract (Table 1) with LD_{50} value of 3.88 ppm. Column chromatographic separation of acetone extract by column chromatography with a methanol-chloroform gradient resulted in 10 fractions. Fraction 8 corresponding to the solvent mixture of methanol-chloroform (90:10, v/v) gave larvicidal activity (Table 1) with LD_{50} of 1.79 ppm. Thin layer chromatography of fraction 8 showed only two prominent bands after spraying with anisaldehyde: sulfuric acid reagent (Photo plate 4), which are indicated as C1 and C2. Both C1 and C2 showed larvicidal activity against *Odoiporus longicollis* (Table 1). The HPTLC profile of acetone extract of susceptible and resistant cultivars showed that both contained the larvicidal compound C1, but in susceptible cultivar, its content was too low to kill of *O. longicollis* larvae. Interestingly, the presence of C1 in susceptible cultivar *Kappa* indicated the existence of its synthetic mechanism in both susceptible and resistant cultivars.

Structural features of Stigmasterol-3-O-glucoside

The larvicidal compound C1 on spectral analysis by ^{13}CNMR, ^{1}HNMR, HRMS and Mass spectroscopy resulted characteristic peaks specific to each group, which are elaborated.

ESI-MS: m/z 575.2375 $[M+H]^+$. ^{1}H NMR ($CDCl_3$-CD_3OD, 400 MHz): δ(ppm): 5.42 (1H, br.s, H-6), 5.09 – 5.21 (2H, m, H-22 & H-23), 4.47 (1H, br.s, H-1'), 3.28-3.93 (6H, m, H-2'- H-6'). ^{13}C NMR ($CDCl_3$-CD_3OD, 100 MHz): δ (ppm) 141.2, 139.2, 130.1, 122.7, 102.0, 79.7, 77.4, 77.0, 74.5, 71.1, 62.4, 57.7, 56.8, 51.1, 50.7, 43.1, 39.4, 38.1, 37.5, 36.9, 32.7, 32.6, 30.4, 30.1, 25.0, 23.4, 21.8, 21.5, 21.3, 19.8, 19.4, 12.7, 12.4.

^{13}C NMR spectrum showed a total of 33 peaks which included four olefinic carbon signals (δ 141.2, 139.2, 130.1 and 122.7) similar to those of stigmasterol. But there was a chemical shift value of 77.4 ppm observed instead of 71 ppm at C-3, indicating a different attachment at C-3 instead of hydroxyl group. The attachment at C-3 was found as glucose moiety from the peaks between δ 3.28-3.93 and 4.47 in ^{1}H NMR and carbon signals at δ 102.0, 62.4, 71.1, 74.5, 77.0 and 79.7 in ^{13}C NMR spectrum. The mass data (m/z 575.2375 $[M+H]^+$) further confirmed the presence of sugar attachment to stigmasterol skeleton. The ^{13}C NMR values of larvicidal compound C1 were perfectly identical with literature values and hence confirmed as stigmasterol-3-*O*-glucoside (Fig.1).

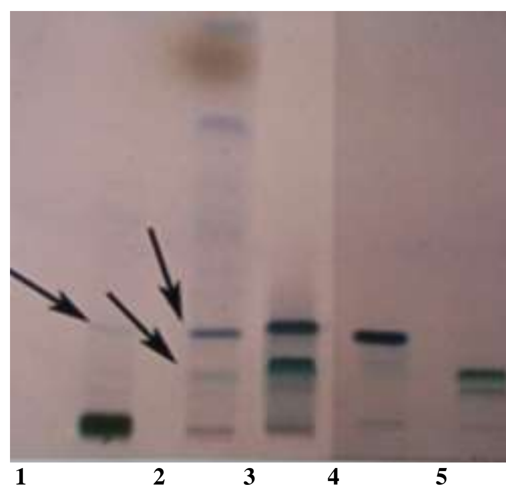

Photo plate 4. HPTLC profile of acetone extract and its subfractionfrom susceptible and resistant Musa cultivars. The HPTLC chromatogram is shown after spraying with anisaldehyde-sulphuric acid reagent and heated at 110ºC for 10 minutes.
1. Acetone extract of susceptible cultivar Red banana, **2**. Acetone extract of resistant cultivar Pisanglilin, **3**. Eighth sub fraction of Acetone extract of Pisanglilin, **4**. Larvicidal compound C1, **5**. Larvicidal compound C2

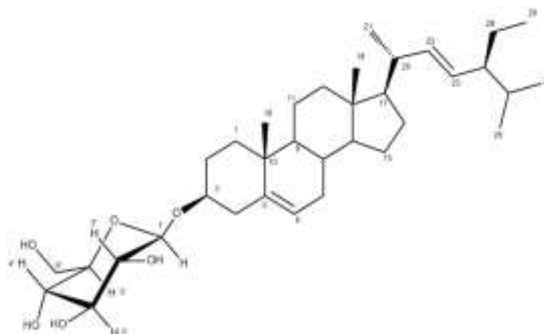

Figure 1. Structure of Stigmasterol-3-*O*-glucoside

The spectral characters of larvicidal compound C1 (which was subjected to ^{1}H NMR, ^{13}C NMR, HMBC, HSQC and LC-Q-T- MS), perfectly matched with Stigmasterol-3-0-glucoside (SOG) reported in *Prunella vulgaris* (24) and in the roots of *Melochia umbellate* (40). This compound is also present in many plants [*Emilia sounchifolia* (11)*, Eclipta alba* (50) and *Ficus hirta* (27)] and it possessed antihyperglycaemic (33), hypocholesterolemic (8) and hepatoprotective activities (17). Our studies revealed that SOG is present in another pest resistant triploid AAB *M. paradisaca* cultivar, and confers resistance against infestation of *O. longicollis* (19). The larvae reared in the live pseudostem of Pisanglilin showed greater cellular damage on hemocytes (21). The larvae reared in pseudostem administered with SOG also resulted in severe cytopathological changes in their hemocytes (19). The SOG is cytotoxic to various types

of cancer cells (29) and also on cells obtained from *Spodoptera mauritius* (14). The strong cytotoxic action of SOG may be the reason for the observed gut histolysis in SOG intoxicated larvae. The larval gut of the affected larvae showed disappearance of muscular layer, displacement of nucleus and excessive vacuolation in the cells of gut lumen. The larvae of all experimental groups stopped feeding on the third or fourth day of the experiment, due to SOG induced histolysis of gut. Inhibition of protease and amylase of midgut by SOG resulted in inhibition of feeding. Similar observation was reported in *Oryctes rhinoceros* larvae intoxicated with 22-hydroxy hopane, a larvicidal compound isolated from *Adiantum latifolium* (35).

Structural features of Sulfoquinovosyl diacylglycerol

The second larvicidal compound (C2) showed the following spectral characters. ^{1}H NMR (METHANOL-d_4, 400 MHz): δ (ppm) 5.60 - 5.70 (m, 1H), 5.40 (dd, J = 15.3, 7.3 Hz, 1H), 5.28 (t, J = 4.5 Hz, 1H), 4.53 (s, 1H), 4.18 (d, J = 7.8 Hz, 1H), 4.00 - 4.08 (m, 2H), 3.87 - 3.94 (m, 2H), 3.78 (d, J = 11.5 Hz, 1H), 3.54 - 3.65 (m, 2H), 3.07 - 3.14 (m, 1H), 1.91 - 2.07 (m, 5H), 1.57 - 1.67 (m, 1H), 1.40 - 1.52 (m, 2H), 1.20 (s, 39H), 0.81 (t, J = 6.5 Hz, 6H). ^{13}C NMR (METHANOL-d_4, 100 MHz): δ (ppm) 177.3, 134.4, 131.4, 130.0, 104.7, 78.0, 77.9, 75.0, 73.1, 72.8, 71.6, 69.8, 62.7, 54.6, 35.9, 33.7, 33.1, 30.9, 30.8, 30.5, 30.5, 30.5, 28.3, 27.9, 26.2, 23.8, 23.8, 14.5, 14.5.

The ^{13}C NMR spectrum showed two distinct set of peak regions corresponding to fatty acid (δ 14.5 – 35.9) and sugar (62.7 – 78.0). The attachment of sulfur to sugar was observed via a peak at δ 54.6 in the ^{13}C NMR spectrum. The fatty acid component must be unsaturated as it was evident by peaks at δ 130.0 – 134.4 in the ^{13}C NMR spectrum. The two methyl group protons at δ 0.81 in the ^{1}H NMR spectrum and δ 14.5 in the ^{13}C NMR spectrum revealed the molecule to be diacylated. The values at δ 62.7, 69.8 and 71.6 are characteristics of glycerol unit in the SQDG (sulfoquinovosyldiacylglycerol). The molecule was acid hydrolyzed to yield different fatty acids which were identified by GC-MS. The composition of identified fatty acids are also listed. Structure of Sulfoquinovosyl diacylglycerol (SQDG) is shown as Figure 2. GC-MS analysis revealed that the compound possessed six unsaturated fatty acids and two of them are attached with the compound at a time.

The second larvicidal molecule Sulfoquinovosyl diacyl glycerol (SQDG) has larvicidal activity against a potential pest of Musa cultivar is increasing its importance. SQDG was isolated first time as sulfur containing lipid of plants (6) and later identified as lipid of thylakoid membrane (16). This compound has antitumor activity against the cells of cervical carcinoma and He La cells, due to the inhibitory effects on DNA polymerase (31).

Toxicity and yield of allomones

The larvicidal molecule stigmasterol-3-O-glucoside (SOG) is highly toxic to the endophytic larvae of *O.longicollis*, which completed the whole lifecycle in *Musa* cultivars. Yield of the molecule was 0.002% from dry pseudostem powder and on calculating the yield in fresh pseudostem, it will be lower than 0.0005% because of high water content in fresh pseudostem which is above 70%. The second larvicidal molecule sulfoquinovosyl

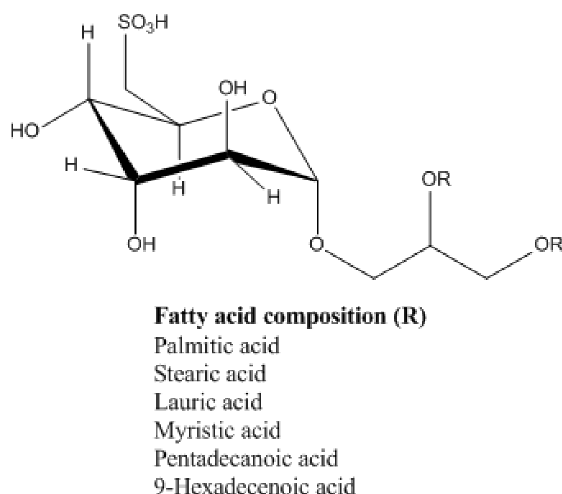

Figure 2. Structure of Sulfoquinovosyl diacylglycerol (SQDG)

Table 1. Larvicidal activity of pseudostem powder extract, its chromatographic fraction and active compounds isolated from Pisanglilin

Active extract/ Fraction/Active compound	LD_{50} (ppm)
Acetone extract	3.88
Eighth fraction of acetone extract	1.79
Stigmasterol-3-O-glucoside (C_1)	0.400
Sulfoquinovosyl diacylglycerol (C_2)	0.378

LD_{50} values are calculated through dose response study by Probit analysis

diacyl glycerol (SQDG) is also highly toxic to the larvae and the toxicity is almost equal to SOG, but the yield of SQDG is very high compared to SOG and is 0.005%. The larvicidal activity of SOG and SQDG are shown as Table 1. The LD_{50} of SOG is 0.40 ppm and LD_{50} of SQDG is 0.378 ppm.

Inhibition of digestive enzymes and histolysis of gut

Larvae of *O.longicollis* intoxicated with either SOG or SQDG stopped feeding on the third day at a very low larvicidal concentration of 0.4ppm, which could be assessed by keeping the pseudostem containing larvae very close to the ear of the investigator. Feeding sounds inside the pseudostem indicated the health of larvae. On the third day onwards the live larvae were unable to bore themselves into the pseudostem through a hole made on it using a knife. Toxicity by SOG resulted sharp inhibition of gut amylase and protease (Table 2). SOG caused a dose dependent inhibition on the activities of both enzymes on the 4th day of toxicity. On this day amylase and protease activities of gut showed 50% reduction while SOG was present in the pseudostem at a concentration of 0.4 ppm. Histology of gut showed dose dependent elevation of histolysis (Photo plate 5), characterized by displacement of nucleus and vacuolation in gut epithelium and disappearance of muscular layer during toxicity by SOG.

Table 2. Activity of Gut amylase and protease in *O. longicollis* larvae intoxicated by Stigmasterol-3-O- glucoside on the fourth day of toxicity.

Concentration (ppm) of Stigmasterol-3-O- glucoside	Activity of Gut Enzymes. (toxicity* on 4th day)	
	Amylase	Protease
Control	14.5± 1.00	16.70±1.20
0.4	7.33 ± 0.42	9.34± 0.22
0.6	3.42±0.24	6.63 ± 0.25
0.8	3.16±0.20	3.73 ± 0.27

*Enzyme activity showed significant dose dependent inhibition compared to control.
Values are mean ± SEM, n = 6

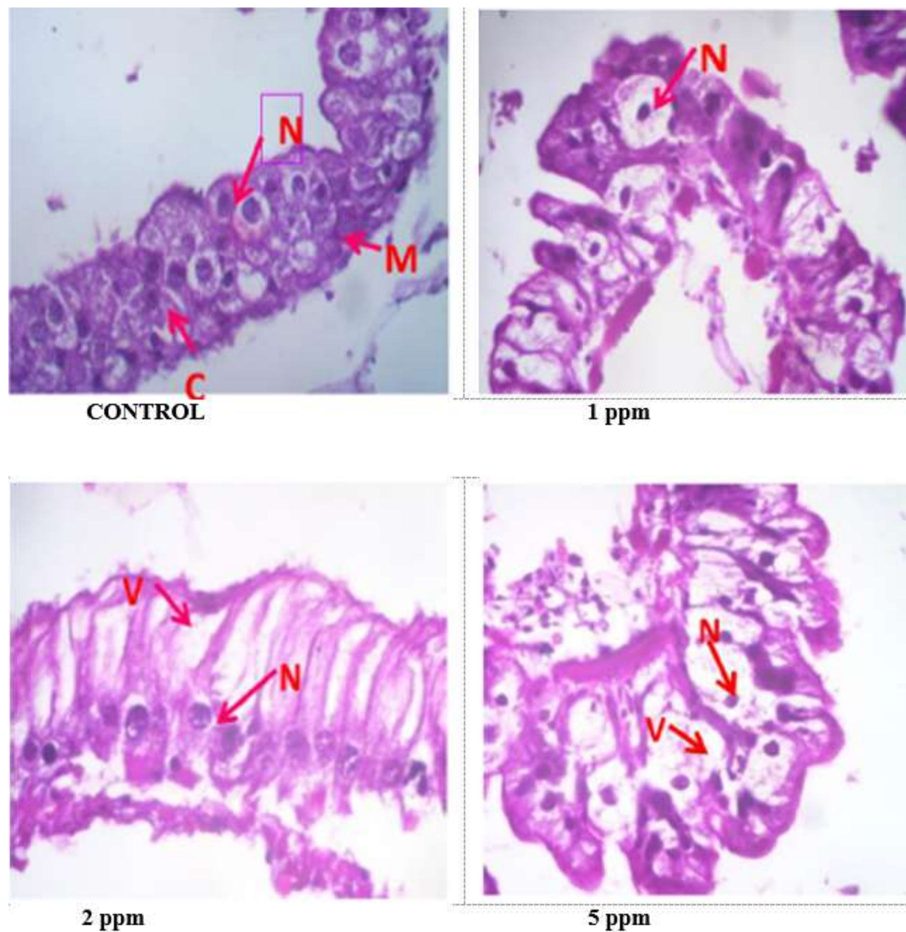

Photo plate 5. Histology of gut in healthy and intoxicated larvae at various doses of Stigmasterol-3-0 glucoside. N: Nucleus, M: Muscle layer, V: Vacuole

Allomones impede protein turn over and endocrine system of larva

Intoxication by Sulfoquinovosyl diacyl glycerol (SQDG) resulted sharp, dose dependent elevation on the content of active moulting hormone 20- hydroxyecdysone in the hemolymph on the fourth day of toxicity (Table 3). The affected larvae showed a sharp decrease on the content of total free amino acids. Intoxicated larvae showed a sharp increase on the activity of aspartate amino transferace (AsAT) but the activity of another related enzyme alanine amino transferace (AlAT) showed a sharp decrease. The ratio of AlAT/AsAT which was 0.33 in normal healthy state of larvae decreased to 0.17 during toxicity. Proteolytic enzymes such as leucine amino peptidase (LAP) and trypsin like serine protease (TLSP) showed inhibition of activity. Tyrosinase activity also became inhibited due to toxicity by the larvicidal compound SQDG (Table 4).

Table 3. Amount of 20 hydroxy ecdysone in the hemolymph of SQDG intoxicated larvae on the fourth day of toxicity.

Sulfoquinovosyl diacyl glycerol (ppm)	Hemolymph level of 20-hydroxyecdysone* (picogram)
Control	86.65±6.12
0.05	90.81±5.61
0.1	109.19±8.03
0.2	176.52±13.67
0.4	218.06±16.82
0.6	289.37±20.17

*Significant increase of 20-hydroxyecdysone was observed in treated groups compared to control. Values are mean ± SEM, n = 6

Table 4. Effect of Sulfoquinovosyl diacyl glycerol on the free amino acid pool and activity of related enzymes on the fourth day of toxicity.

Biochemical Parameters	Sulfoquinovosyl diacyl glycerol (ppm)			
	Control	0.5	1	2
Total free amino acids	0.507±0.04	0.486±0.03	0.410±0.03	0.387±0.02
Aspartate amino transferase	3.09±0.16	3.96±0.17	4.21±0.19	4.96±0.31
Alanine amino transferase	1.05±0.12	0.96±0.07	0.91±0.08	0.86±0.07
Ratio of AlAT/AsAT	0.33	0.242	0.216	0.173
Leucine amino peptidase	3.62±0.21	3.12±0.18	2.90±0.19	1.96±0.18
Trypsin like Seriene protease	0.32±0.02	0.30±0.02	0.20±0.01	0.18±0.011
Tyrosinase	23.31±1.95	19.21±1.81	16.67±1.20	12.08±1.12

Values are mean ± SEM and are significantly different ($p \leq 0.05$).

Hemolymph of SQDG intoxicated larvae showed exponential elevation of 20-hydroxy ecdysone (20 HE), the active moulting hormone of insects. Compared to 20 HE of normal healthy larvae, the concentration of it was elevated to double fold at 0.2 ppm SQDG and three-fold elevation at 0.6 ppm SQDG. Intoxication by SOG also resulted elevation of active moulting hormone in larval hemolymph of *O. longicollis* (19). It is well known that many phytocompounds are acting as phyto ecdysone and the presence of such molecules confer protection from insect attacks on such host plants. Stigmasterol is reported to be acting as antifeedent and interfering on the insect development through disrupting the insect endocrine system (17,14). Both the allomones present in Pisanglilin are effectively

disrupting the endocrine system of the endophytic larvae. Another study conducted on the larvicidal efficacy of *Adiantum latifolium* on the larvae of Oryctes *rhinoceros* proved that both larvicidal molecules such as *Adianto bischrysene* (36) and 22-hydroxy hopane (35) are effectively disrupting the endocrine system of *Oryctes rhinoceros* through accumulating 20-hydroxy ecdysone in the hemolymph.

Sulfoquinovosyl diacyl glycerol effectively disrupted the mechanism of maintenance of free amino acid pool in affected larvae. Insects are characterized by their ability to maintain very high content of free amino acids in their hemolymph and it is true in all the insects studied such as *Culex quinquefasciatus* (5), *Oryctes rhinoceros* (46), *Oecophylla smaragdina* (49), *O. longicollis* (19) and in *Periplaneta americana* (41). Activity of transmination is very high in insects and it has very important role in energy metabolism (5). In the present study SQDG toxicity resulted imbalance in transamination through elevation of AsAT and inhibition of AlAT. The imbalance in transamination led to steep decline on AsAT/AlAT ratio which is 0.33 at normal state, declined to 0.17 during SQDG toxicity. Affected larvae showed sharp decrease on the content of total free amino acids. Inhibition of proteolytic enzymes, LAP and TLSP during SQDG toxicity may be the reason for decline on free amino acid pool. Another characteristic action of SQDG on aminoacid metabolism is inhibition of tyrosinase activity. This enzyme is very important to insects in cuticle melanisation, sclerotisation and also in immune system (13) and is reported that compounds inhibiting tyrosinase activity can be effective pest control agents (12). Simultaneous action of both allomones present in a *Musa* cultivar which are effectively interfering on various vital activities of the endophytic larvae are successfully killing them and thereby conferring resistance to the host plant against its serious pest.

Intra pseudostem injection of freshly crushed pseudostem extract (20 ml) of Pisanglilin (23) or aqueous suspension of acetone extract (2.0 ml) from dry pseudostem powder of Pisanglilin (42) into *Kappa* the highly pest susceptible *M. paradisaca* gave complete protection from infestation by *O.longicollis*. Injection was given once in fifteen days for five months ,starting from 4th month of planting to 9th month (duration to set flower).As all the 10 control group of plantains became severely affected and toppled down through cracks developed at the middle of the pseudostem due to infestation by this pest , all the treated group of plantains (*Kappa*, 10 plants in each group)survived very well and gave good harvest (23,42).In the present study SOG and SQDG , the two active larvicidal molecules were isolated and characterized from the acetone extract of Pisanglilin.

CONCLUSIONS

Pisanglilin is a highly pest resistant, diploid AA plantain, crushed juice of the pseudostem is used as folk-lore remedy for various health problems. The plantain possessed two allomones such as Stigmasterol-3-O-glucoside and Sulfoquinovosyl diacylglycerol. These compounds can kill the endophytic larvae of banana pseudostem borer at extremely low dose. Attack by banana pseudostem borer is highly damaging and causes heavy economic loss to subsistence farmers doing commercial cultivation of banana. Now they are using highly toxic systemic insecticides meant for applying on fibre yielding crops such as cotton on edible fruit crops such as banana. The two allomones of

Pisanglilin can be used as safe systemic insecticides as intra pseudostem injection against the pest living as internal parasite in the pseudostem.

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