

2.0 MATERIALS AND METHODS

2.1 Collection and maintenance of Test Organisms

2.1.1. Stored Product Pest *Tribolium castaneum* (Herbst)

The pest, *T. castaneum* was procured from the local market and godowns from the infested blackgram and have been continuously cultured on the same food source until the end of the experiments. The stock culture was maintained in the laboratory in dark atmosphere at $28 \pm 2^{\circ}\text{C}$ and 70 - 80% relative humidity. The eggs were collected on black filter papers by placing *T. castaneum* adults on the filter papers after 48 h (Ho *et al.*, 1996).

2.1.2 Polyphagous Field Pest *Spodoptera litura*

The army worm, *Spodoptera litura* was collected from field, cultured and maintained in the laboratory on castor leaves (*Ricinus communis* L.). Rearing conditions were 12 h photo regime at $28 \pm 2^{\circ}\text{C}$ and $75 \pm 5\%$ relative humidity. An insect culture was continuously refreshed with wild moths captures by a light trap in the vicinity of the agricultural fields near Bommidi village, Dharmapuri District, Tamil Nadu. Generally hale and healthy and uniform sized fourth instar larvae were used in the experiments.

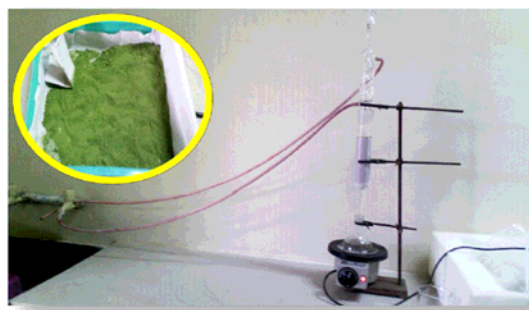
2.2. Collection and extraction of plant material

Fresh leaves of the selected plants were collected in and around Bommidi Village, Dharmapuri District, Tamil Nadu, India. Then the plant materials were thoroughly washed to remove particles and shade dried at room temperature ($27 \pm 2^{\circ}\text{C}$; R.H. $75 \pm 5\%$). After shade drying,

the leaves were powdered by using mechanical grinder. 100 grams of the dry powder were extracted in 100 ml of hexane, ethyl acetate and ethanol using soxhlet apparatus. After 48 hrs the crude extracts were condensed in vacuum by using rotary vacuum evaporator. The condensed crude extract was stored in refrigerator at 4°C for further studies. The extracts obtained were dissolved in the corresponding pure solvent for further experiments.



Photograph showing rotary vacuum evaporator (Model: Superfit, PBU -6) used for the condensation of the crude extracts.



Photograph showing the powdered plant material (encircled) and Soxhlet apparatus

2.3. Bioassays for *Tribolium castaneum*

2.3.1 Ovicidal activity of different solvent extracts of selected plants against the eggs (24 h old) of *Tribolium castaneum*

Ovicidal activity of selected plant extracts against the eggs (24 h old) of *T. castaneum* was performed. The eggs (50nos) were placed in a 9 cm petridish and kept in 650 ml jars with screwed lids. Different concentrations (12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml) of the plant extracts were applied on filter paper (Whatman No. 1) separately, cut into 9 cm diameter and were attached to the inner side of the lid of the jar. After evaporation of the solvent in about three minutes, the lids were closed tightly and the eggs were exposure for a period of 96 h. After exposure period, the petridishes were taken out of the jars and

kept in the incubator at $28 \pm 2^\circ\text{C}$. The final mortality counts were made after 11 days with the help of a hand lens. Unhatched eggs with black spots inside the jar were considered and counted as dead. The data obtained from the present experiment was subjected to the Abbott (1925) method to derive the ovicidal activity of the selected plant extracts. Percentage mortality was calculated and data were corrected for natural mortality in controls (distilled water alone was used as the controls using) the following formula:

$$\%OA = [\%EHC - \%EHT / \%EHC] \times 100$$

Where

$\%OA$ = % ovicidal activity

$\%EHC$ = % of eggs hatched in control

$\%EHT$ = % of eggs hatched in treatment

2.3.2 Oviposition deterrent activity of different solvent extracts of selected plants against the adults of *Tribolium castaneum*

Ground nut seeds were cleaned and sterilized at 45°C for 6 h in order to kill the eggs and developing larvae. For each concentration (12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$) of the plant extract, 250 ground nut seeds were taken in a conical flask and mixed properly. After thorough mixing the seeds were air dried and separated into five lots each containing 50 seeds. The lots were then transferred into plastic containers (8 x 6.5 cm) separately and 5 pairs of newly emerged adult *T. castaneum* were introduced. Seeds treated with water alone were used as controls. The method was based on an area preference test described by Jayakumar (2010). Five replicates were maintained for each

concentration and controls. After 15 days, the number of eggs laid on treated seeds (Ts) and control seeds (Cs) were recorded and the percentage of oviposition deterrence (POD) was calculated using the formula:

$$\text{POD} = [(Ts-Cs)/Cs] \times 100.$$

2.3.3 Repellent activity of different solvent extracts of selected plants against the adults of *Tribolium castaneum*

Repellent activity of different solvent extract of selected plant extracts against the adults of *T. castaneum* was assessed in a choice bioassay system at $27 \pm 2^\circ\text{C}$ and 60 - 65% room heat previously reported by Bekele *et al.* (1996). It consisted of two 1 L glass jars connected together at their rims by means of a 30 x 10 cm nylon mesh tube. A 5.0 cm diameter circular hole was cut at the middle of the mesh for the introduction of test insects. 250 g of maize were put into each glass jars. Grains in one jar was treated with varying concentrations (12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$) of the plant extracts while untreated grains in the other jar acted as control. Twenty-five adult insects were introduced into the nylon mesh tube through the circular hole by means of a 5 cm diameter funnel. The number of insects present at the control (NC) and treated (NT) jars were recorded after 1- hour exposure. All repellency assays were replicated five times. A percent repellency (PR) value was calculated from the formula:

$$\text{PR} = [\text{NC} - \text{NT} / \text{NC} + \text{NT}] \times 100.$$

2.3.4 Insecticidal activity (Fumigant toxicity) of different solvent extracts of selected plants against the adults of *Tribolium castaneum*

The fumigant toxicity of plant extract was tested as described by Huang *et al.* (1997). To determine the fumigant toxicity of selected plant extracts, 2 cm diameter filter papers (Whatman No.1) were impregnated with the test doses (25, 50, 75, 100 μ g/ml) of the extracts. The impregnated filter paper was then attached to the screw caps of 44 ml Plexiglass bottle each of which contained separately 20 adult (1-5 days old) insects and screwed tightly. Mortality was recorded after 24 h of exposure. When no leg or antennal movements were observed, insects were considered dead. The mortality was calculated using the Abbott correction formula (Abbott, 1925). The filterpaper impregnated with distilled water alone were used as the controls. The experiment was performed in five replicates for both control and treated groups. After 24 h, the insects were transferred to clean vials with culture media and kept in the incubators for end-point mortality determination.

A second experiment was designed to assess 50% and 95% lethal doses. A series of dilutions was prepared to evaluate mortality of insects after an initial dose-setting experiment and each dose was replicated five times. Ten adult insects were put into 44 ml Plexiglass bottles with screw lids. Control insects were kept under the same conditions without any extract. The number of dead and alive insects in each bottle was counted 24 h after initial exposure. The mortality was evaluated by direct observation of the insects every hour till total mortality. Probit analysis (Finney, 1971) was used to estimate LC₅₀ and LC₉₀ values.

2.4 Bioassays for *Spodoptera litura*

2.4.1 Ovicidal activity of the plant extracts against *Spodoptera litura*

The egg mass was collected from the insectary and used for ovicidal activity. The method was based on an area preference test described by Jeyasankar 2013. The individual eggs were collected by carefully removing the scales from the egg masses by using a fine camel brush. 100 eggs were dipped in each concentration (25, 50, 75 and 100 µg/ml) of the plant extracts and air-dried. Eggs treated with distilled water alone were used as controls. Treated and control eggs were placed in petriplates containing wet filter paper and monitored for hatching. Number of eggs hatched in control and treatments were recorded and the percentage of ovicidal activity was calculated using Abbott's formula (Abbott 1925):

$$\%OA = \left[\frac{\%EHC - \%EHT}{\%EHC} \right] \times 100$$

Where

%OA = % ovicidal activity

%EHC = % of eggs hatched in control

%EHT = % of eggs hatched in treatment

2.4.2 Oviposition deterrent activity of the plant extracts against *Spodoptera litura*

For oviposition deterrent activity, the individual plant extract at varying concentrations (25, 50, 75 and 100 µg/ml) were sprayed on the leaves of the respective fresh host plant. Similarly unsprayed leaves of the host plant acted as the controls. The petioles of the treated and control leaves were inserted into a conical flask (cotton plugged) containing dechlorinated water to avoid early drying and placed inside the cage

(60cm x 45cm x 45cm). Ten pairs of *S.litura*, moths were introduced in to individual cages and 10% (w/v) sucrose solution with multivitamin drops was provided for adult feeding to increasing fecundity. The control and treatment groups were maintained in five replicates. After 48h, the numbers of eggs masses (*S.litura*) laid on treated and control leaves were recorded and the percentage of oviposition deterrence was calculated (Williams *et al.*, 1986).

$$\%ODI = [ELC-ELT/ELC] \times 100$$

Where

%ODI = % Oviposition Deterrence Index

ELC = No. of eggs laid in control

ELT = No. of eggs laid in treatment

2.4.3 Pupicidal activity of the plant extracts against *Spodoptera litura*

The pupicidal activity was assessed by feeding the fifth instar larvae (50nos) of *S. litura* with host plant leaves sprayed with varying concentrations (25, 50, 75 and 100 µg/ml) of the plant extracts and allowing them to pupate. Control groups were maintained by feeding the larvae with leaves sprayed with distilled water alone. Pupicidal activity was calculated by counting dead pupae from the total larvae (Baskar *et al* 2011). LC₅₀ and LC₉₀ values were calculated using probit analysis (SPSS Version 17, 2010).

2.4.4 Antifeedant activity of the plant extracts against *Spodoptera litura*

Antifeedant activities of the selected plant extracts were studied using leaf disc in no-choice method (Isman *et al.*, 1990). Fresh castor leaf discs of 3-cm diameter were used for the experiments. Solvent crude extracts with 25, 50, 75 and 100 µg/ml concentrations were treated individually on the fresh leaf discs. The treatment of castor leaf with acetone alone was used as positive control and the one without solvent was considered as negative control (0 ppm). In each Petri disc (1.5 cm x 9cm) wet filler paper was placed to avoid early drying of the leaf disc. Single fourth instar larva of *S.litura*, were introduced individually and the progressive consumption of the leaf area by the larvae after 24h was recorded in control and treated discs (Systronics India Limited, Model 211: Plate 2.3). Five replicates were maintained for each concentration.

$$AFI = [C-T/C+T] \times 100$$

Where

AFI = Antifeedant Index

C = Area protected in control leaf disc

T = Area protected in treated leaf disc

2.5 Purification of the Fractions from the ethanol extract of *Ocimum sanctum*

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase.

2.5.1 Thin Layer Chromatography

Thin layer chromatography appears to have been first developed and utilized by Schraiber in 1939. Unfortunately, Schraiber's work does not seem to have been heeded and the technique appears to have been rediscovered by Kirchner in 1951 (Scott, 2003). The technique was exerted using aluminium sheets coated with silica gel (TLC Silicagel 60 F₂₅₄) supplied by Merck, Germany. A small sample spot (1 to 2 mm diameters) was applied using a capillary tube, 1 cm above on one end of the plate, and was placed in the respective solvent chamber to obtain separation of mixtures. The adsorbent (silica gel) is known as the stationary phase and the solvent or solvent mixture is known as the mobile phase, which is drawn up the plate via capillary action due to which the separation of components in the sample mixture occurs. Various solvent mixtures (ethyl acetate, and ethanol) from non-polar to polar were employed to achieve maximum separation of the components. The chromatogram obtained was used as a reference for collecting fractions through column chromatography. The chromatogram was visualized through the following staining methods.

- Iodine: Iodine vapor chamber was made by adding crystals of iodine with dry silica gel in a TLC jar. The TLC plates were introduced into the chamber for 1 min to develop brown spots respective to the number of compounds.
- Ultraviolet Light: The TLC plates were irradiated in UV-transilluminator to visualize UV-active compounds.

Each component separated in the chromatogram is known by its R_f value, which is calculated as mentioned below:

$$R_f \text{ value} = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent front}}$$

2.5.2 Column Chromatography

Column chromatography is typically subjecting the samples with mixture of components through a physio-chemical separation process to obtain purified individual components.

2.5.2.1 Preparation of column and sample loading

Currently, the study employs the use of a glass column with 450 mm length and 30 mm bore. The glass column was thoroughly washed with hexane and dried completely. The column was clamped vertically in a stand and was plugged with a piece of cotton at the bottom to prevent running out of silica gel. The column was then packed by wet method as follows. Hexane was filled till 3/4th of the height of the column. Activated silica gel (60-120 mesh size; kept at 120 °C for 60 min) was mixed with hexane to prepare a fine slurry and was carefully added into the column by gentle tap on the sides with simultaneous draining of the column. Care was taken to prevent formation of any air bubbles while packing the column. The sample was prepared by mixing 1 g of extract (dissolved in methanol) with 10 g of silica gel (60-120 mesh) until the mixture turned powdery. The sample mixture was then carefully added on top of the silica gel column to form a uniform sample layer. From this point, the column was never allowed to dry and the level of solvent was marked and maintained constantly.

2.5.2.2 Chromatography of the sample

The column run was performed using ethyl acetate and ethanol with varying polarity range (from non-polar to polar). Elution was performed with a flow rate of 1 ml/min. Eluate collected was then analysed in TLC for the confirmation of single spot, with ethyl acetate and ethanol at 1: 9 ratios as reference solvent system. Fractions with same R_f values were pooled together to get a homogeneous solution of the same compound.

2.6 GC-MS analysis for volatile compounds

2.6.1 Instrument specifications

The analysis was performed using Thermo GC – Trace Ultra ver 5.0 coupled with Thermo MS DSQ II, fixed with a DB5 - MS Capillary Standard column (30 m, i.d.: 0.25 mm, and film thickness: 0.25 μm).

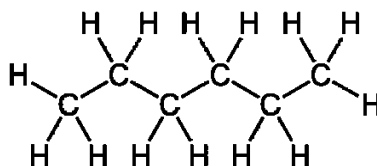
2.6.2 Analytical conditions

Helium was used as a carrier gas (flow-1.0 ml/min). Injector temperature was 260 °C and oven temperature gradually raised from 80 °C to 260 °C (5 °C/min).

One μl of sample was injected through injection port and the individual compounds were identified based on their retention times and standard matching spectral peaks available in Wiley mass spectral library.

2.7. Physical and chemical properties of solvents:

2.7.1. Hexane (C_6H_{14})

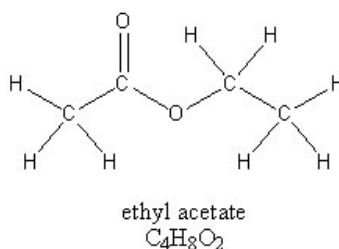


Structure of Hexane

Hexane is an alkane of six carbon atoms, with the chemical formula C_6H_{14} . The term may refer to any of the five structural isomers with that formula, or to a mixture of them (Scott, 2007).<http://en.wikipedia.org/wiki/Hexane> - cite_note-2 In IUPAC nomenclature, however, hexane is the unbranched isomer (*n*-hexane); the other four structures are named as methylated derivatives of pentane and butane. IUPAC also uses the term as the root of many compounds with a linear six-carbon backbone, such as 2-methylhexane (C_7H_{16}), which is also called "isoheptane".

Hexanes are significant constituents of gasoline. They are all colorless liquids at room temperature, odorless when pure, with boiling points between 50 and 70 °C. They are widely used as cheap, relatively safe, largely unreactive, and easily evaporated non-polar solvents. Hexanes are used in the formulation of glues for shoes, leather products, and roofing. They are also used to extract cooking oils (such as canola oil) from seeds, for cleansing and degreasing a variety of items, and in textile manufacturing. A typical laboratory use of hexanes is to extract oil and grease contaminants from water and soil for analysis. Since hexane cannot be easily deprotonated, it is used in the laboratory for reactions that involve very strong bases, such as the preparation of organolithiums. For example, butyllithiums are typically supplied as a hexane solution. Hexanes are chiefly obtained by the refining of crude oil. The exact composition of the fraction depends largely on the source of the oil (crude or reformed) and the constraints of the refining.

2.7.2. Ethyl acetate (CH₃-COO-CH₂-CH₃)

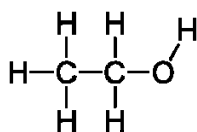


Structure of ethyl acetate

Ethyl acetate (systematically, ethyl ethanoate, commonly abbreviated EtOAc or EA) is the organic compound with the molecular formula CH₃-COO-CH₂-CH₃. This colorless liquid has a characteristic sweet smell (similar to pear drops) and is used in glues, nail polish removers, decaffeinating tea and coffee, and cigarettes. Ethyl acetate is the ester of ethanol and acetic acid; it is manufactured on a large scale for use as a solvent. Ethyl acetate is synthesized in industry mainly via the classic Fischer esterification reaction of ethanol and acetic acid (Wilhelm Riemenschneider *et al* 2005).

In the laboratory, mixtures containing ethyl acetate are commonly used in column chromatography and extractions. Ethyl acetate is rarely selected as a reaction solvent because it is prone to hydrolysis and transesterification. Ethyl acetate is fairly volatile at room temperature and has a boiling point of 77 °C. Due to these properties, it can be removed from a sample by heating in a hot water bath and providing ventilation with compressed air.

2.7.3. Ethanol (CH₃CH₂OH)



Structure of ethanol

Ethanol is the principal type of alcohol found in alcoholic beverages, produced by the fermentation of sugars by yeasts. It is a psychoactive drug and one of the oldest recreational drugs still used by humans. It can cause alcohol intoxication when consumed to excess. It is used in thermometers, as a solvent, as an antiseptic and as a fuel. The name ethanol was coined as a result of a resolution that was adopted at the International Conference on Chemical Nomenclature that was held in April 1892 in Geneva, Switzerland (Armstrong, Henry 1892).

Ethanol is a 2-carbon alcohol with the structural formula $\text{CH}_3\text{CH}_2\text{OH}$. An alternative notation is $\text{CH}_3\text{-CH}_2\text{-OH}$, which indicates that the carbon of a methyl group ($\text{CH}_3\text{-}$) is attached to the carbon of a methylene group ($\text{-CH}_2\text{-}$), which is attached to the oxygen of a hydroxyl group (-OH). It is a constitutional isomer of dimethyl ether. Ethanol is sometimes abbreviated as EtOH, using the common organic chemistry notation of representing the ethyl group (C_2H_5) with Et (Armstrong, Henry 1892).

Ethanol is a volatile, colorless liquid that has a slight odor. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of

similar molecular weight, such as propane. Ethanol is slightly more refractive than water, having a refractive index of 1.36242 (at $\lambda=589.3$ nm and 18.35 °C or 65.03 °F). <http://en.wikipedia.org/wiki/Ethanol> - cite_note-crc-62 The triple point for ethanol is 150 K at a pressure of 4.3×10^{-4} Pa (Armstrong and Henry, 1892).

2.8 Statistical analysis

Effective concentration (EC_{50}) was calculated using probit analysis (SPSS, 2010). Data from enzyme activity were analyzed with ANOVA of arcsine transformed percentages followed by Tukey's multiple range test ($P<0.05$) (SPSS, 2010). In Tukey's test, the lowest different values from average, detected by statistical testing, were marked with letter "a," the next text higher with "b" etc., (Snedecor and Cochran, 1989).