CHAPTER 3

3.0 MATERIALS AND METHODS

Insect procurement and rearing was done in the Humidity cum environment chamber maintained in the Department of Zoology, The Maharaja Sayajirao University of Baroda. The plant material was obtained from the Department of Botany, The Maharaja Sayajirao University of Baroda. Extraction and analysis of essential oils was performed using standard protocols with some modification depending on the plant species. GC-HRMS for the identification of chemical constituents was done from the IIT- SAIF (Sophisticated Analytical Instrument Facility), Bombay. Bioassays and biochemical estimations were performed in the Department of Zoology, The Maharaja Sayajirao University of Baroda. Moreover, SEM analysis of the grains for quality assessment was done from the IIT- SAIF as well. The work was carried out during January 2016- June 2019.

3.1 Insect Procurement and Rearing

3.1.1 Procurement

Tribolium casteneum was collected from the laboratory cultures maintained in the division of Entomology of the Department of Zoology, The Maharaja Sayajirao University of Baroda, for the last 3 years. The model insects were sustained in the humidity cum environment chamber (Forma Environmental chamber; Thermo Fisher Scientific, Asheville; Model no: 601B-X-LT-H-NH-RFNR-C-C-1982741) at their suitable temperature and humidity ranges without any exposure to pesticides. This ensures low rates of resistant individuals in the culture. Twenty unsexed adults were then separated from this setup to initiate a fresh culture in the laboratory conditions for acute toxicity assessment.

3.1.2 Rearing

Pests were reared in the defined culture media which consisted of wheat flour, wheat grains and Baker's yeast in the ratio of 6:3:1 (Figure 1). The separated adults were then transferred to the plastic jars containing defined media. 2

adults in the ratio of 1:1 male & female were released per 250 grams of flour. The jars were covered with the muslin cloth for the ease of aeration. Cultures were then transferred to the humidity chamber and maintained at 27±2˚C, 70±5 RH.

Figure 1: Rearing of red flour beetle in defined media

3.2 Biology- Red flour beetle

Biology of the model beetle was studied to have an insight into the different life stages, their characteristics along with their morphometric features unique to each stage. The cultures were observed regularly for oviposition to record the temporal data. A 250 µm sieve of 60 mesh size was used to separate the microscopic eggs from the media. Eggs were then isolated and kept in separate glass petriplate to study their incubation period. Newly emerged larvae were immediately transferred in the plastic container containing the media as they are feeding stage. When the pupae were noticed in the media they were separated using a paint brush and transferred in to glass petriplate. Time required to metamorphose into adult was recorded. Adults were then transferred to the food resources.

Size and age composition of the population were manually counted and the raw data was incorporated in an electronic spread sheet to measure the mean,

standard deviation and standard error to find out the degree of variation in the data. Morphometric analyses and photography of all the stages were done using computer assisted 13.1 mega pixel Catcam stereomicroscope. Distinguished morphological features unique to different stages were also noted.

3.3 Plant material

The plant material was procured from Dr. Alka Dangash and Dr. Bhavna Sharma working under the guidance of Prof. Neeta Pandya, Department of Botany, The Maharaja Sayajirao University of Baroda. The dried, finely grounded leaves of the *Artemisia annua* was procured in 2017 from the lab of Professor Neeta Pandya of Botany Department. Plant powder was then stored in Sterile Polyethylene Sampling Bags (5 kg capacity, Fisherbrand™; free from impurities) in refrigerator at 4˚C until it is used for the extraction of EOs.

3.3.1 Extraction and analysis of chemical composition of essential oils

Plants Essential oils (EOs) was extracted using different solvents to investigate the characteristic compounds unique to each solvent. Four different solvents viz. Methanol, Chloroform, Petroleum ether (40-60˚C) and n- Hexane were selected for the study in the order of decreasing polarity. Given to that analysing EOs using GC-MS, one of the most sophisticated and hyphenated technique; to unveil the chemical composition was a necessary step.

3.3.2 Extraction and analysis of essential oils

25 grams of plant powder was subjected to hydro-distillation for the extraction of EOs. The process was carried out in a round bottom flask standing on a heating mantle and connected to the Clevenger-type apparatus (Clevenger, 1928). To carry out Hydrodistillation, 300 mL of solvent was added in the extraction chamber. Among Polar solvents, methanol, & chloroform and in non-polar solvents, petroleum ether (40-60˚C), & n- hexane was chosen for the extraction process. Boiling of the flask marks the beginning of distillation. Conditions employed for Hydrodistillation process were as follows:

For polar solvents

Methanol: Process continued for 16 hours at 65˚C

Chloroform: Process continued for 9 hours at 60˚C

For non-polar solvents

Petroleum ether: Process continued 1 hour at 55˚C

n-Hexane: Process continued 2 hours at 65˚C

Distillation process continued till the solvent becomes transparent in the extraction chamber. The oil layer was then separated from the aqueous phase using a separating funnel. EOs were then collected and dried over anhydrous sodium sulphate (Na_2SO_4) to remove extra water. Crude extracts were further processed in rotary evaporator to remove extra solvents at their boiling ranges (Figure 2).

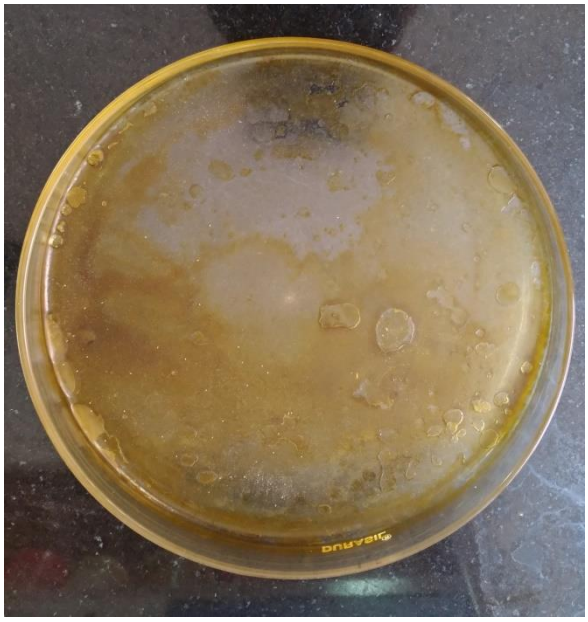

Figure 2: Essential oil extracted with Clevenger type apparatus

Oil yield was calculated on a dry weight basis employing the Yield (%) formula.

$$\text{Yield (\%)} = \frac{W_{EO}}{W_I} \times 100$$

Where, W_{EO} is the weight of dry essential oil and W_I is the weight of fresh plant powder taken for extraction. Extract were then stored in airtight plastic containers in a refrigerator at 4 ˚C until it is used for further experimentation.

3.3.3 Gas Chromatography-mass spectroscopy (GC- HRMS)

All chemicals used in the experimentation were of GC-MS grade. Conditions for GC-MS study were set according to the method of (Li et al., 2010). Gas chromatographic analysis was performed on an Agilent 7890N instrument equipped with a flame ionization detector and HP-5MS (30m × 0.25mm × 0.25μm) capillary column, while the EO components were identified on an Agilent Technologies Jeol mass spectrometer. The GC settings were as follows: the initial oven temperature was held at 60 °C for 1 min and ramped at 10 °C min^{-1} to 180 °C for 1 min, and then ramped at 210 °C min^{-1} to 280 °C for 15 min. The injector temperature was maintained at 270 °C. The samples (1 μL) were injected neat, with a split ratio of 1:10. The carrier gas was helium at flow rate of 1.0 mL min^{-1}. Spectra were scanned from 20 to 550 m/z at 2 scans s^{-1}. Most constituents were identified by gas chromatography by comparison of their retention indices with those of the literature or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of n-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in NIST 05 and Wiley 275 libraries or with mass spectra from literature. Component relative percentages were calculated based on GC peak areas without using correction factors.

3.4 Bioassays and Identification of Lethal doses

3.4.1 Stage specification for Bioassay studies

Newly emerged adults of 1-10 days old were used for the acute toxicity assays. Final larval stages i.e. 14 days old larvae were selected for the experiments of contact and fumigant toxicity. All the experiments were conducted in the dark under the same temperature and humidity ranges. However, only adults of pre specified ages (Newly emerged) were used for Behavioural bioassays.

3.4.2 Repellency test

3.4.2.1. Filter paper arena test

Repellency in insects was evaluated according to (Cosimi et al., 2009) where pests were exposed to solvent extracted EOs of *Artemisia annua.* In the behavioural bioassay, EOs derived from the selected solvents were dissolved in acetone to make desired concentrations. Four different sets of EOs eluted with methanol, chloroform, petroleum ether and n- hexane were evaluated separately in filter paper arena test (Figure 3).

Filter papers measuring about 7 cm in diameter were cut into two equal halves where one half was treated with the desired concentration of EO and other half with acetone. The half treated with acetone was considered as control. Concentrations used were in the range of 0.54- 0.90 mg cm^{-2}. After drying for two minutes, both the halves of filter paper were attached underside with the cellotape and fixed to the petriplate. 10 unsexed adults were released into the centre of the plate and their activities were noted. Five replicates were maintained for each concentration. Readings were taken at the interval of 1, 2, 3, 4, 5, 6, 12 and 24 hours. Insects were then transferred to the plastic vials containing defined media and checked regularly for 3 days if any mortality is recorded.

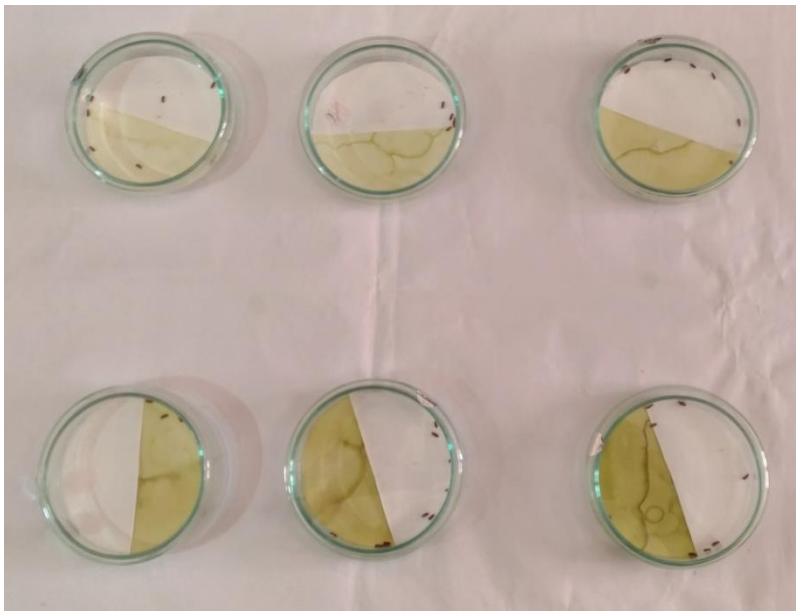

Figure 3: Filter paper arena test with different concentrations of EOs

Data recorded at the end of the 24 hours was considered as final and converted to percentile to calculate Percentage Repellency (PR) using the following formula:

$$PR = 2(C - 50)$$

Where, C is the percentage of insects recorded on the untreated half of the disc. Positive values expressed repellency and negative values attractancy. Results of PR were analysed using ANOVA and Tukey's honest significance test.

3.4.2.2. Multi-arm olfactometer test

Behavioural bioassay was also appraised through the multi arm olfactometer. The idea of olfactometer was taken from Jayakumar et al., (2017) with certain modification for the ease in the conduct of experiment in the present lab conditions. Fabrication of the set up was done by Durga Scientific Pvt. Ltd., Chhani Road, Vadodara, Gujarat.

Figure 4: Multi arm olfactometer for behavioural bioassay in response to EOs

Technical details of the olfactometer are as follows: middle glass chamber: 7 cm in diameter; arms: 1.5 cm in diameter & 14.5 cm in length. In the bioassay, filter papers were cut into two equal halves (Figure 4). Paper halves measuring about 3.5 cm were then treated with the different concentrations of EOs and

fixed in the inner side of the cork of the arms. The half treated with the acetone was used as control. 20 unsexed adults were released in the centre and then upper lid is covered with the muslin cloth for the ease of aeration. Movement of insects in different arms were recorded at 1, 2, 3, 4, 5, 6, 12 and 24 hours. After 24 hours insects were transferred in plastic vials containing food media and checked regularly for 3 days if any mortality is recoded.

Data recorded at the end of the 24 hours was considered as final and employed to calculate and Excess Proportion Index (EPI). EPI was assessed using the following formula: (Sakuma & Fukumi, 1985).

$$(\text{EPI}) = \frac{\text{Nt} - \text{Nc}}{\text{Nt} + \text{Nc}}$$

Where, Nt is the number of insects present in the treated arms, Nc is the number of insects present in the control arm. Negative values expressed repellency and positive values attractancy.

3.4.3 Contact toxicity

To evaluate contact toxicity following the method of (Huang & Ho, 1998), adults and larvae were treated with the solvent derived EOs. Concentrations used were in the range of 0.33- 3.33 mg adult^{-1}for polar solvents and 0.17 to 1.67 mg adult^{-1} for non-polar solvents. Concentrations were finalised after standardisation of the method hence differential concentration ranges between polar and non-polar solvents derived EOs were seen. 10 unsexed adults were taken in plastic vials and kept in the freezer for one minute. This made them least active and hence their handling became easy. An aliquot of 5µl of EO was then topically applied using a micro-syringe on the mesothoracic region. After 2 minutes they were transferred in the plastic vials containing media. The mortality was recoded till 3 days at the interval of 12 hours.

3.4.4 Fumigant toxicity

Fumigant toxicity was assessed according to (López et al., 2008). Filter papers (Whatman No.1 measuring 7 cm in diameter) were impregnated with the desired concentrations of solvent derived EOs.

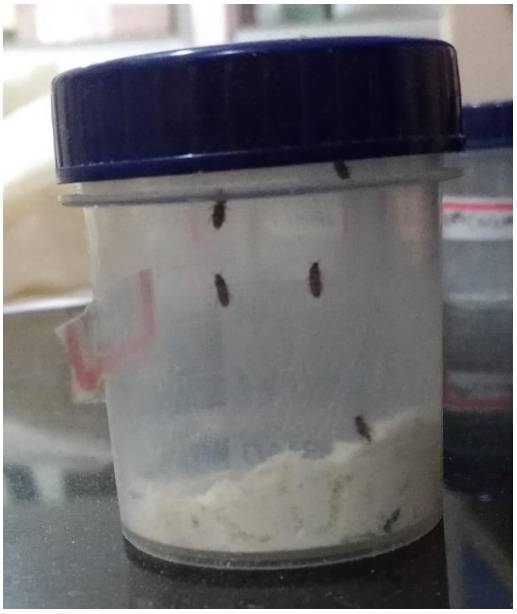

Figure 5: *T. casteneum* exposed to EOs for the evaluation of Fumigant toxicity

Concentrations were ranged from 0.24 to 2.37 mg L air^{-1} of methanol and chloroform EOs. On the other hand, for non-polar solvents 0.14 to 1.42 mg L air^{-1} was employed. Concentrations were decided after multiple experiments were conducted and the process was standardised as done in the case of contact toxicity.

The filter papers were allowed to air dry for 2 minutes to evaporate the solvent. Control sets were exposed to acetone only. Impregnated paper was then sealed on the screw cap of the plastic vials (25mL). 10 unsexed adults and larvae were released separately for each concentration (Figure 5). Five replicates were maintained for each concentration. Mortality was determined regularly for 3 days at the interval of 12 hours. Loss of antennal and leg movement was considered as an indication for mortality.

For the above explained acute toxicity assays, data obtained at the end of the third day was considered as final and processed further for statistical analysis. Probit analysis (Finney, 1971) using Medcalc software was employed in analysing the dosage- mortality response in both the acute toxicity assays. Percentage mortality would be calculated using Abbott's formula (Abbott, 1925) to correct natural mortality, if any, in control groups.

3.5 Understanding biomolecular pathways

Quantitative analyses of biomolecular constituents in viable (LC_{50}, LC_{90}) and control sets were assessed. After three days, the dead *T. casteneum* (both larvae and adults) were weighed in an analytical grade weighing balance (High Precision Balance, Scale – Tec, Model no: SAB203) to ensure the exact weight of the tissue. This confirms the precise quantification of the homogenate processed for the biomolecular assays. Protein profiling by Biuret method (Reckon Diagnostics Pvt. LTD.) and enzymatic activities of Acetylcholine Esterase (AChE), Glutathione S Transferase (GST), Reduced Glutathione (GSH) and Lipid Peroxidases (LPO) were performed following the methods of Ellman et al. (1961), Habig et al. (1974), Jollow et al. (1973) and Buege & Aust (1978) respectively.

3.5.1 Protein Profiling by Biuret kit method

Required reagents

i. Total protein reagent

Sodium Potassium tartarate	50 mmol L^{-1}
Copper Sulphate	15 mmol L^{-1}
Potassium iodide	4 mmol L^{-1}

ii. Total protein standard 5gm/dl

Principle

Biuret Reagent containing copper reacts readily with the protein in an alkaline condition. The reaction leads to the increase in absorption due to the formation of a coloured complex at 540 nm. Concentration of protein is directly marked in the absorption level.

Representation of chemical reaction:

$$\text{Protein} + Cu^{++} \rightarrow \text{Blue - Violet complex.}$$

Protocol

- Five cuvettes labelled as blank, standard, control, LC_{50} and LC_{90} were used.

- 2.5 mL of test reagent were added to all the five cuvettes.
- 0.05 mL of water and standard reagent was added to the blank and standard cuvette respectively.
- 0.05 mL of homogenised tissues of the pest exposed to different treatments were added to the control, LC_{50} and LC_{90} cuvettes sequentially.
- After mixing the contains thoroughly, the cuvettes were allowed to stand for ten minutes at room temperature.
- Absorbance of the standard, control, LC_{50} and LC_{90} were recorded against blank. Readings were taken within one hour as the coloured complex destabilizes after the period.
- Calculation of total protein in each set were evaluated employing the following formula:

$$\text{Total protein concentration (gm/dL)} = \frac{\text{Abs. of test}}{\text{Abs. of Standard}} \times 5$$

3.5.2 **Acetylcholine Esterase** (Ellman et al., 1961)

Required reagents

i. Triton X- 100
ii. Phosphate buffer (PB) 0.05M (pH 8.0)
iii. Ethylenediaminetetraacetic acid (EDTA)
iv. 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB)
v. Acetylthiocholine Iodide (AChI) 0.1M, 0.075M, 0.05M, 0.025M, 0.01M, 0.005M

Principle

AChI is a widely used synthetic substrate for the enzyme Acetylcholine esterase (AChE). The enzyme breaks down the substrate into thiocholine and acetate. The following method made use of DTNB more popularly known as Ellman reagent which readily reacts and accurately quantify the concentration of thiol group in a given sample. Only protein thiols are accessible to this water soluble reagent and forms chromophore 5-merapto-2-nitrobenzoic acid, a yellow colour complex. Intensity of colour development is a measure of the

enzymatic activity. Maximum absorption was recorded spectrophotometrically at 410 nm.

Representation of chemical reaction:

AChI → thiocholine + acetate

thiocholine + DTNB → chromophore 5-merapto-2 nitrobenzoic acid (Yellow colour)

The enzymatic activity is expressed in terms of rate of the reaction i.e. the amount of enzyme required per unit time (min) to catabolise the substrate (moles). Whilst calculating the rate of the present molecular reaction, amount of AChI required to breakdown AChE per minute is taken under consideration

Protocol

- Whole tissue homogenate of the control and treated sets were prepared in 0.05M PB.
- 0.5% Triton X- 100, a surfactant, is added to the homogenate to lyse the cells in order to extract the internal components like proteins, enzymes etc.
- EDTA is poured followed by the centrifugation at 10,000g for 20min at 4°C.
- Supernatant is separated and used as the source of enzyme.
- 0.1mL of supernatant was taken in a cuvette followed by the addition of 2.86mL PB and incubated for 5 minutes at room temperature.
- 0.03 mL of AChI and 0.01 mL DTNB solution is added to the cuvette successively.
- Optical density (O.D.) was recorded for 30 minutes against the blank for continuous 30 minutes.

3.5.3 **Glutathione S Transferase** (Habig et al., 1974)

Required reagents

i. Phosphate buffered saline (PBS) pH 6.5, 0.1M
ii. Reduced Glutathione in PBS 50mM
iii. 1-Chloro-2,4-dinitrobenzene (CDNB)

Principle

Conjugation of 2, 4-Dinitrochlorobenzene (CDNB) with reduced glutathione (GSH) is mediated by the Glutathione S transferase enzyme (GST). This leads to the formation of a yellow coloured product which has maximum absorbance at the range of 340 - 360nm. The magnitude of colour production indicates enzymatic activity which is calculated spectrophotometrically by the increase in the absorbance at 340nm.

Protocol

- Whole tissue homogenate of the treated sets were prepared in PBS containing EDTA
- Processed homogenate was then kept for 5 minutes at room temperature
- Subsequent to this, tissue homogenate was centrifuge at 10,000 RPM for 10 min.
- Separation of 0.03 mL supernatant in a 4 mL cuvette followed by the successive addition of 2.77 mL of PBS, 0.15 mL reduced Glutathione and 0.05 mL CDNB
- O.D. of the final product was then recorded at 340nm.

3.5.4 **Reduced Glutathione** (Jollow et al., 1973)

Required reagents

i.	Phosphate buffer (PBS)	0.1M (pH 7.4)
ii.	4% Sulfosalicyclic acid	
iii.	bis-(3-carboxy'-4-nitropimenyl) disulphide	pH 8

Principle

Reduced glutathione is an important antioxidant and its concentration within the cells is an indicator of oxidative stress. The reaction of GSH with DNTB leads to the formation of TNB- chromophore that forms the basis of the reaction. The complex shows maximum absorbance at 412 nm. The rate of TNB formation is directly proportional to the GSH content in the given sample.

Protocol

- Tissue homogenate of the treated sets were made in PBS.
- After adding equal volume of salicylic acid to the homogenate, the protein content was precipitated
- Cuvettes were then subjected to centrifugation for 10 minutes.
- After centrifugation, 0.5 mL of supernatant was transferred in a new cuvette.
- Following to this 4.5 mL of bis-(3-carboxy'-4-nitropimenyl) disulfide reagent was added.
- OD was recorded at 412 nm.

3.5.5 **Lipid Peroxidases** (Buege & Aust, 1978)

Required reagents

i. Phosphate buffer (PBS) 0.1M (pH 7.4)

ii. Thiobarbituric Acid Reagent (TBA) (Note: Freshly prepared)

TBA	100 mg
EDTA	46mg
20% Trichloroacetic acid (TCA)	10mL
2.5 N HC1	5mL (Final volume 20 mL)

iii. Drabkin's Reagent

Principle

Lipid peroxidation, the process of oxidative degradation of lipid molecules, readily forms malondialdehyde (MDA). This endoperoxide reacts with Thiobarbituric acid (TBA) which leads to the formation of Thiobarbituric reactive substances (TBARS). Characteristic pink colour is marked in the cuvette due to the formation of TBARS. The colour intensity which is positively correlated with the enzymatic activity gives the maximum absorbance at 532 nm.

Protocol

- Whole tissue homogenate of the control and treated sets were prepared in PBS.

- Blank which is devoid of tissue homogenate, 1mL of Double distilled water and TBA reagent of the same volume was added.
- On the other hand, same volume of Distilled water and TBA was added in the treated test tubes along with the respective tissue samples.
- Test tubes were kept in the water bath for 20 minutes at approximately 95 ˚C
- The test tubes were allowed to stand at room temperature for cooling down the contents.
- Following to this, centrifugation of the tubes at 3000 rpm for 15 min was done
- O.D. was recorded at 532 nm

Results of above mentioned estimations were further processed for statistical compatibility. Analysis of Variance (ANOVA) and Tukey's honest significance test were employed using PAST (Paleontological Statistics Software Package) to compare means. Graphical representations were fabricated using Sigma plot 13.0 statistical software package.

3.6 Quality Parameters

3.6.1 Feeding deterrence and % weight loss

The feeding deterrence test performed was adopted from (Owusu, 2001) with some modifications by (Iram et al., 2013). Wheat grains weighing 5 grams were transferred in plastic containers (25 mL). Three containers were supplemented with 0, 0.5 and 1 grams of dried, powdered leaves of *Artemisia annua* respectively. 10 unsexed insects were then introduced in each vial and allowed to infest the grains for three months. Five replicates were maintained for each set. After the completion of the assessment period, beetles were separated with the paint brush and the grains were reweighted. The data obtained was processed to calculate Feeding ratio (Fr) using the following formula:

$$Fr = 1 – FW/5$$

Where, FW represents the final weight grain after 90 days of feeding period.

For the assessment of % weight loss, following formula was employed:

$$\% \text{ Weight loss} = (U\ Nd) - (D\ Nu) / U\ (Nd + Nu) \times 100,$$

Where, U = weight of undamaged grains

D = weight of damaged grains

Nu = number of undamaged grains

Nd = number of damaged grains

3.6.2 Flour disc Bioassay

Flour discs were prepared according to the method of Xie et al. (1996) with some modifications by Huang et al. (1997). Aliquots of 200 µl of a stirred suspension of wheat flour in water (10 g in 50 mL) were decanted into a petri dish to form the disks. The discs were left in the fume hood overnight to dry and then they were transferred to incubators maintaining at 30±1˚C and 70-80% RH to equilibrate for 24 hours. Weight of the discs was ranged from 85 to 88 mg and the moisture content was recorded to be 11.3±0.1%.

Flour discs were treated with an aliquot of 5 µl of EOs of *Artemisia annua* derived with the four different solvents. Five different concentrations of the extracts were prepared for treatment and acetone was used as control. The solvent was allowed to evaporate for one hour and then individual discs were transferred in plastic vials (diameter 4.2 cm height 6.8 cm). Weight of each plastic vials used were recorded prior to the bioassay. Ten unsexed previously starved adults were weighed and added to individual vials. Each treatment and control sets were maintained in triplicates. Weight of the flour disks and insects were weighed separately again after 72 h. Nutritional indices were calculated according to Manuwoto & Mark Scriber (1982), Farrar et al., (1989) with slight modifications by Huang et al., (1997):

- Relative growth rate (RGR) = (A-B)/(B × day)

Where A is the weight of live insects on the third day (mg)/ number of live insects on the third day and B is the original weight of insects (mg)/original number of insects

- Relative consumption rate (RCR) = D/ (B × day)

Where D is the biomass ingested (mg)/number of live insects on the third day, and biomass ingested is the (original weight of flour disks 2 weight of flour disks on the third day

- Percentage efficiency of conversion of ingested food (ECI) = (RGR/RCR) × 100.
- Antifeedant effect [AE] (%) = [(C-T)/C] × 100

Where C is the consumption rate of control discs and T is the consumption rate of treated discs.

3.6.3 Scanning Electron microscopy analysis

Scanning electron microscopic analysis was performed to evaluate the topographical characters of damaged and undamaged grain samples. Dried damaged and undamaged wheat grains were used as a specimen for the analysis. SEM measurements were performed with JEOL JSM-7600F series. Details of the instrument are as follows: SEI resolution of 1.0nm at 15 kv 1.5nm at 1 kv, in GB mode; Magnification Low: 25X to 10,000X; High: 100X to 1,000,000X at 4x5 photo size; Accelerating voltage: 0.1 to 30 kv; Probe Current Range: 1 pA to ≥ 200 nA).

3.6.4 **Measure of PH** (AACC International Method, 02-52.01)

Objective

To determine the hydrogen ion activity (pH) of *T. casteneum* infested and infestation free flour.

Apparatus

pH meter (Micro Controller based; Chemi Line Technologies, Model no CL 180) equipment was used which has been calibrated against buffer solutions of known pH.

Required reagents

Buffer solutions pH 4.2, 7, 9.2

Procedure

- 10 g of infested and infestation free flour was transferred in two separate Erlenmeyer flask and labelled as I (Infested) and U (Uninfested).
- Flour samples were drenched in 100 mL of deionised water.
- Flasks were continuously agitated to ensure the contents are free of lumps
- Suspension was maintained for 15 minutes using continuous manual shaking
- Following to this, the suspension was allowed to stand for 10 minutes.
- Supernatant was separated and transferred to electrodes and there pH was determined using potentiometer which has been calibrated against known buffer solution.

3.6.5 Iodine method for insect eggs in flour (AACC International Method, 28-44.01)

Objective

To evaluate the presence of insect eggs and also to detect possible "carryover" infestation in infested and infestation free flour.

Apparatus

i. Sieve, no. 60 (Mesh size 250 microns)
ii. Hot plate.
iii. Beaker, 250-mL.
iv. Buchner funnels.
v. Filter paper
vi. Squeeze bottle.
vii. Petri dish.
viii. Stereoscopic microscope.
ix. Magnifier, 20x

Required reagents

i. H_2SO_4: 1 volume of concentrated sulphuric acid (H_2SO_4) was mixed thoroughly to 19 volumes of water.

ii. Iodine 0.1N.
iii. Isopropyl alcohol.

Procedure

- 50 grams of infested and uninfested flour were transferred through the no. 60 (250 micron) sieve separately.
- Residue of both the samples remaining on the sieve was then transferred to 250 mL beakers followed by their dilution with 3 mL of alcohol and 30 mL of H_2SO_4.
- Beakers were covered and heated for 10 minutes on steam bath.
- Contents were filtered through the whatman no 1 paper on suction funnel.
- Deionised water is used to rinse the processed samples whilst the suction force was turned off.
- 15–20 mL of iodine was added to the paper placed in the funnels and allowed to stand for 15 sec to ensure thorough staining of the contents by iodine.
- Gentle suction was applied to aid the easy movement of iodine through filter papers.
- Washed with 30 mL of 1% H_2SO_4 followed by several washing with deionised water.
- Filter paper was transferred to a glass petriplate and observations and determinations of number of eggs were made using 20x magnifier.

3.6.6 **Crude Fat** (AACC International Method, 30-10.01)

Objective

To determine crude fat content of infested and uninfested flour samples by acid hydrolysis. Lipid contents were extraction with ether followed by the continuous heating of lipid residue at 100˚ for 1o minutes. Weight of the residue is expressed as % crude fat.

Apparatus

i. Erlenmeyer flask 1 L.
ii. Water bath 70–80°

iii. Beaker 50 mL
iv. Fat extraction apparatus.
v. Steam bath.
vi. Oven.
vii. Centrifuge.

Required reagents

i. Ethyl alcohol 95%.
ii. HCl solution (Preparation: 110 of mL deionized water was transferred in a 1 L Erlenmeyer flask followed by the addition of 250 mL of concentrated HCl. Contents were mixed carefully and allowed to cool before using)
iii. Ethyl ether
iv. Petroleum ether (Boiling point below 60°)

Procedure

- 2 grams of infested and uninfested flour was added separately in two beakers of 50 mL capacity.
- 2 mL alcohol was added to the samples to moisten the flour particles which ensure no lumping would form while adding the acid.
- Addition of 10 mL HCl in the beaker followed by the heating in water bath at 70-80˚ C for 40 minutes confirms complete hydrolysis of the samples.
- 10 mL of alcohol was added, cooled and transferred to the extraction apparatus.
- Extraction was carried out with petroleum ether (25 mL).
- Crude extract was centrifuged at 6000 rpm for 20 minutes.
- Ether fat solution was drawn off through filter made up of pledget of cotton fixed firmLy in the stem of funnel which assured free passage of ether in a dried, previously weighed flask (Note: Drying of the flask was done in oven at 100° along with a counterpoise flask and then allowed to stand in air to achieve the persistent weight).

- Extraction process was repeated twice using 15 mL petroleum ether followed by the drawing off ether solution through filter in the same flask.
- Petroleum ether was evaporated on steam bath at its boiling range.
- Post evaporation the fat content was dried in oven at 100° for 90 minutes.
- Flask and counterpoise were allowed to stand in air for 30 minutes to obtain stable weight and then weighed.
- Weight of fat content was corrected using blank

Calculation:

% Fat = weight of fat (corrected for blank) /weight of sample × 100

3.6.7 **% Moisture content** (AACC International Method, 44-01.01)

Objective

To determine the moisture content, an integral characteristics of the wheat grains, of the infested and uninfested grain samples.

Apparatus

i. Oven

Procedure

- Oven temperature was set at 130.
- Both infested and uninfested samples were weighed and placed in oven for 16 hours
- Final weight of the samples were measured and computed employing the following formula

Calculation

Moisture content is calculated by employing the following formula:

% Moisture= loss in moisture (g)/ wet weight of sample (g) × 100

3.6.8 Total Protein: Biuret Kit

Objective

To analyse the protein content of the infested and non-infested wheat grains to have an insight into the quality differences in the selected samples.

Required reagents

i. Total protein reagent

Sodium Potassium tartarate	50 mmol L^{-1}
Copper Sulphate	15 mmol L^{-1}
Potassium iodide	4 mmol L^{-1}

ii. Total protein standard (5 mg/dl)

Protocol

- Four cuvettes which were labelled as blank, standard, undamaged, & damaged were used.
- 2.5 mL of test reagent were added to all the four cuvettes.
- 0.05 mL of water and standard reagent was added to the blank and standard cuvette respectively.
- 0.05 mL of undamaged & damaged flour samples were added to the respective cuvettes.
- After mixing the contains thoroughly, the cuvettes were allowed to stand for ten minutes at room temperature.
- Absorbance of standard, undamaged and damaged cuvettes was recorded against blank. Readings were taken within one hour as the coloured complex destabilizes after the period.
- Calculation of total protein in each set were evaluated employing the following formula:

$$\text{Total protein concentration (gm/dL)} = \frac{\text{Abs. of test}}{\text{Abs. of Standard}} \times 5$$

3.6.9 Carbohydrate by GOD-POD

Objective

To quantify the carbohydrate content of the damaged and undamaged wheat grains for nutritional analysis.

Required reagents

i. Reagent-1	pH 7.4±0.5 AT 25 ˚C
• Buffer	100 mmol/L
• Glucose oxidase (GOD)	≥ 20000 U/L
• Peroxidase (POD)	≥ 2500 U/L
• 4-Aminoantipyrine (4- AAP)	0.25mmol/L
ii. Reagent-2	
• Phenol	10 mmol/L
iii. Glucose Standard	100 mg/dl

Protocol

- Four cuvettes labelled as blank, standard, undamaged, & damaged were used.
- 1 mL of working reagent which was formed by the addition of equal volume of Reagent-1 and Reagent-2 (100 mL) was added in all the cuvettes.
- 0.01 mL of standard was added to the cuvette labelled as standard
- Undamaged and damaged (0.01 mL) were added to the undamaged & damaged cuvettes respectively.
- Contents were mixed well and incubated for 5 minutes at 37 ˚C.
- Absorbance was recorded at 505 nm.
- Calculation of glucose content in each set was analysed using the following formula:

$$\text{Glucose concentration (mg/dl)} = \frac{\text{Abs. of test}}{\text{Abs. of Standard}} \times 5$$

3.6.10 **Acid hydrolysis** (AACC International Method, 28-41.03)

Objective

To evaluate the presence of insect fragments in infested and non-infested flour samples. Reduction of starch and saturation of non-oleophilic food products were mediated through hydrolysing the samples without affecting insect fragments. Oil is added to the solution for coating the extraneous matter. Attraction of extraneous matter to the oil phase of oil- aqueous mixture formed the basis of separation. Aqueous phase was drained and oil phase containing the insect fragments were extracted in glass beaker. Draining of oil phase through whatman filter paper no.1 was done for microscopic examination.

Apparatus

i. Autoclave.
ii. Separatory funnel.
iii. Filtration funnel.
iv. Hot plate with magnetic stirrer.
v. Beaker, 1 L.
vi. Glass Petri dish
vii. Probe. See
viii. Stir bar.
ix. Squeeze bottle.
x. Watch glass.
xi. Whatman no. 1 Filter paper.
xii. Microscope, wide-field type stereoscopic.
xiii. Percolator, 2-liter.

Required Reagents

i. HCl 5% in water.
ii. Mineral oil.
iii. Detergent, 5% aqueous Na lauryl sulfate
iv. Glycerin-alcohol (50-50).

Procedure

- Samples weighing 50 g were transferred in glass beakers.
- 500 mL of 5% HCl were added to the beaker and stirred continuously until smooth slurry was obtained.
- Glass beakers are checked thoroughly to certain no dry flour left.
- Mineral oil (50 mL) was added and the beaker was heated for 10 minutes.
- Occasional stirring was ensured to prevent blazing
- Magnetic stir bar was added and stirred for 5 minutes, time required to form vortex.
- Contents of beaker minus stir bar were then transferred to the separatory funnel using warm water and allowed to stand for half an hour.
- Initial 10 minutes required gentle stirring with the glass rod.
- Aqueous phase was drained off to about 3 cm of interface
- Process was repeated twice in order to obtain a transparent lower phase
- Oil phase was then transferred to the beaker and 50 mL of HCl was added followed by boiling for 3 minutes.
- Heated solution was then filtered through the whatman filter paper no.1.
- Enough glycerine-alcohol solution was then added to wet paper and microscopically studied.
- Number of insects and insect fragments were counted.

"I believe that for every illness or ailment known to man, that God has a plant out here that will heal it. We just need to keep discovering the properties for natural healing."

- Vannoy Gentles Fite