Tranchyspermum Ammi : Natural pesticides

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ABSTRACT

The objective of this research work was to investigate the chemical composition and the insecticidal activity of *Tranchyspermum ammi* (Carom oil) against grain bugs originated in stored grains. In this study, essential oil of *Tranchyspermum ammi* is obtained by hydro-distillation using Clevenger apparatus. Thymol is a major chemical constituent isolated from Carom oil. Presence of thymol in Carom oil was determined and confirmed by GC, GC-MS and FT-IR analysis. Organoleptic and Physio-chemical properties of Carom oil were also analyzed. Apart from thymol, other compounds present in Carom oil were p-cymene, γ-terpinene and β-pinene. 67.73 % of thymol was quantified in Carom oil.

Keywords: Insecticidal activity, grain bugs, thymol, physio-chemical, β-pinene, γ-terpinene.

MS History: 11.10.2017 (Received)-15.11.2017(Revised)-20.11.17 (Accepted)

Citation: Sadanand S. Kadam, Snehal B. More and Jyotsna S. Waghmare. 2017. *Tranchyspermum Ammi*: Natural pesticides. *Journal of Biopesticides*, **10**(2): 90-98.

INTRODUCTION

Food damage caused by insects is a matter of serious concern for scientists in recent years. Grain bug alone brings about majority of reduction in the yield of stored grain products. Thus to against grain bugs, pesticides are used that kill or destroy pests (including Bacteria, Fungi, Insects, Weeds, Termites, etc), that damage fruits, vegetables, grains, etc. Greater part of country utilized synthetic pesticides for inhibiting growth of insects in stored grains. Synthetic pesticides do protect products (vegetables, fruits, grains, etc) but at the same time their excessive usage results in damage to the same product. Thus synthetic pesticides play a dual role of a protector and a destroyer. As synthetic pesticides are produced from harmful chemicals, they are hazardous and highly toxic to human health. Synthetic pesticides are not biodegradable in nature forming residues that remain in soil, water and air for months together affecting public health and environment. Moreover, synthetic pesticides are less resistant to pests due to which they take a long time to inhibit insects and so there is a need for an alternative to replace them. *Sitophilus granaries* is a pest of wheat, oats, rye, barley, rice and corn. It is of two types depending on the type of grains. In small grains like rye they are smaller, whereas in lager grains like corn they are larger in size. The adult bug is dark black in colour. The larvae feed inside the grain until pupation, after which they bore a hole out of the grain and emerge. They are rarely seen outside the grain kernel. The lifecycle takes about 5 weeks in summer, but may take upto 20 weeks in cooler temperatures. Adults can live upto 8 months after emerging (Woodbury, 2008). The bugs have the ability to attack whole grains. They can't be detected easily and usually all grain in an infested storage facility gets destroyed. The different methods used to prevent grains damage is usage of chemical pesticides, different methods of masking the odour of the grain with unpleasant scents. Farmers and growers rely on chemical pesticides for pest management, but the use of these agents is becoming more difficult due to the evolution of resistance in pest populations and product withdrawals, both of which are reducing the availability of effective compounds. There are also an increasing number of new threats from non-indigenous pest species (Pimentel *et al.,* 2005). Nowadays farmers are trying to reduce or alternatives the usage of conventional chemical pesticides to

meet demands from the retailers. There is an urgent need therefore, for developing environmentally sustainable systems for controlling pests that are less reliant on chemical pesticides as the primary management tool (Chandler *et al.,* 2009).

To overcome this scientist thought about replacing synthetic pesticides with natural pesticides originating from plant source. Natural pesticides have the ability to overcome all the limitation of synthetic pesticides. Tripathi *et al.,* 2009 reported potential use of essential oils as biopesticides. They are neither toxic nor hazardous to mankind but they are highly toxic to the pest. Natural pesticides do not leave any of its trace in soil, water or air as they are bio degradable in nature. These pesticides are not even toxic to kids as they have very less immunity to fight against harmful chemical**.** Koul *et al.* (2008) reported about the use of essential oils as green pesticides. In the present work fair efforts have been made to show a Carom oil derived from plant extracts i.e. essential oil possessing pesticidal (insecticidal) property. Carom seeds are old spices grown mostly in many states of India. Carom seeds are categorized under family Apiaceae (Kamal *et al.,* 2012). Carom seed's botonical name is *Tranchyspermum ammi* (Kamal *et al.,* 2012) where *Trachyspermum is genus and ammi species.* Carom seeds come under kingdom plantae. The Apiaceae family consists of almost 347 genera and 12816 species. Essential oil extracted from carom seeds is a volatile oil having distinguishable organoleptic and physio-chemical characteristics. Carom oil possesses many medicinal properties followed by a wide range of compounds (Nowak *et al.,* 2013) useful in perfume, flavor and fragrance industry. Carom oil is mainly used in mouthwash due to its good antimicrobial properties compared to other spices. Apart from medicinal usage the seeds of carom are used as spices and herbs in preparing various edible dishes. Traditionally smoke of heated carom seeds is inhaled for treatment of cough. Essential oil derived from carom oil possesses high antimicrobial (Hassanshahian *et al.,* 2014), anti fungal (Saad and Soad, 2005) and insecticidal (Chaubey *et al.,* 2007; Szczepanik *et al*., 2012) activity. These pesticide properties are basically because of thymol the chief constituent of oil. (Riccioni and Orzali *et al.,* 2011). Moreover this oil can also be used as natural antioxidant due to the presence of phenolic compound.

MATERIALS AND METHODS

Test insects: Insects were collected from local market, found adhering in stored grains. It was known that these insects remain alive in closed system for months together. They were stored in a container under observation at room temperature until applying for insecticidal tests.

Extraction materials

The fresh seeds of Carom trees were collected from latitude and longitude 19.0269° N, 72.8553° E, Maharashtra, Mumbai. India. Seeds were sunlight dried prior to extraction for a few hours. After drying, seeds were stored away from moisture until extraction of oil. The essential oil of different plants possesses high pesticides property. Similar is the case with Carom oil. Hence, these essential oils were selected as natural pesticides for further research work.

Isolation of essential oil

The isolation of Carom oil was done by using hydro distillation method. Cleaning of seeds was done to remove the external foreign material such as soil particles, dust, etc. Raw materials were ground to ease the operation of hydro-distillation and to increase the yield. Further 100 grams of ground raw materials were subjected to distillation i.e. hydro distillation using Clevenger apparatus. After completion of extraction, heating was stopped immediately to avoid excessive heating. Then sufficient cooling time was allowed to avoid the loss of essential oil in the form of vapors. Oil and water formed two separate layers in Clevenger tube based on density difference and accordingly separation of oil was carried out. Collected oil was further dried using anhydrous sodium sulphate to remove traces of moisture. Batches were carried out in atmospheric pressure. The ratio of sample to water was maintained at 1:10. The total

volatile oil was determined by clevenger type apparatus. Storage of essential oil was done in refrigeration until analysis.

Analysis of Carom oil

Physio-chemical analysis: Oil obtained from hydro-distillation was subjected to physio-chemical analysis such as refractive index, specific gravity, acid value, and moisture.

Moisture: Moisture content of all the samples were carried out by constant oven drying method.

Refractive index: The abbes refractometer is convenient for measurement of refractive index. To achieve accuracy apparatus should be calibrated against distilled water, which has refractive index of 1.3325 at 25 °C. After calibration samples refractive index was measured.

Specific gravity: Weight per milligram of a liquid is weight in gram of 1 mL of a liquid when weighed in air at 25 °C, unless otherwise specified. Procedure: A completely clean and dry pycnometer was selected. Specific gravity of liquid was obtained by dividing the weight of liquid contained in the pycnometer by the weight of water contained, both determined at 25 °C.

Acid value: Procedure: 2.5 g. of the oil was taken into a100 mL saponification flask. Then Addition of 15 mL of neutral 95 per cent alcohol and 3 drops of a 1% phenolphthalein solution was done. Titration of the free acids with a standardized 0.1 N NaOH solution was carried out by adding the alkali drop wise at a uniform rate of about 30 drops per min. The first appearance of a red coloration that does not fade within 10 sec is considered the end point.

Organoleptic evaluation: Organoleptic properties of oil such as color, physical appearance, odor and solubility were determined.

Color of all the four essential oil was done by visual observation. Physical appearance of all the four isolated essential oil was done by visual observation. Odor of oil was determined by sensory evaluation. Solubility of all isolated essential oil was checked in water as well in solvents.

Phyto-chemical analysis

This analysis does not give any brief information about compounds of oil but it does show group of phyto-chemical compound present in oil. Phyto-chemical screenings were performed using standard procedures. (Nagpal *et al.*, 2013).

This test confirms the presence of organic compound in isolated oil.

Test for aldehyde: 0.05 g or 1-2 drop of compounds + 3 cm^3 of 2, 4 – dinitrophenyl hydrazine solution, shake well.

Test for ketone: 0.01 g or 2-3 drops of compound + 1 cm^3 of sodium nitroprusside solution + 2 drop of NaOH solution.

Test for phenol: Phthalein test: 0.01 g of compound + 0.01 g of phthalic anhydride + 2 drops of conc. H_2SO_4. Heat it gently until the mixture fuses, cool and pour it in 20 cm^3 of very dilute NaOH solution. Liebermann test: 0.01 g of compound + 1 cm^3 of conc. H_2SO_4 + 2 crystals of $NaNO_2$. Heat it gently. Dilute it with water, add 20% NaOH solution.

Test for ester: Dissolve 0.01 g or 0.5 cm^3 of compound in 1 cm^3 of ethyl alcohol + a drop of phenolphthalein + 2 drops of very dilute NaOH solution and boil on a water bath.

Test for alcohol: Take a small piece of dry Na metal in a fusion tube and add a few drops of compound.

GC analysis

Gas Chromatography (GC) analysis of the essential oil was carried out on capillary column (HP-5 Agilent 19091J-413; 325 C 30 m X 320 µm X 0.25 µm). Temperature programming was 90 -190°C with 10 °C ramp/min and 190–290 °C with 5 °C ramp/min. Inlet temperature and detector temperature was 180 °C and 210 °C. Nitrogen was used as carrier gas at 1mL/min. FID section was used. The sample injection volume was 1.0 µl/min diluted in n-hexane, with split ratio of 100

GC-MS analysis

GCMS analysis of essential oil shows the presence of some important constituents in it. The GC-MS analysis of essential oil was carried out by electron impact ionization (EI+) method on gas chromatograph coupled to a

JMS-T100 Mass Spectrometer on fused HP-5 column (30 m x 0.25 mm; 0.25 µm film thickness). The components were identified by matching their mass spectra in the Indian Institute of Technology, Bombay, India and their retention indices were compared with literature value (All web references) and (Ameur *et al.*, 2012).

For GC-MS, the carrier gas was helium at constant pressure of 90 Kpa. Column temperature programmed as 100°C to 220°C at the rate of 10°C/min; temperature was maintained at 220 °C for 5 min; gradually increase column temperature from 220 °C to 260 °C at the rate of 20 °C/min and hold for 10 min; again increase temperature from 260 °C to 280 °C at the rate of 20 °C/min. Helium was used as carrier gas at a flow rate of 1 mL/min; Injector temperature 100°C, detector temperature 280 °C. The split ratio was 100:1 and 0.2 µL of sample was injected, the mass range was 40-300 mz^{-1}. Acquisition parameters full scan; scan range 40-500amu.

Condition for GC-MS analysis:

1. Solvent used: Hexane.
2. Column specification: Fused HP-5 column (30 m x 0.25 mm; 0.25 µm film thickness).
3. GC program

I. Carrier gas flow rate: 1 mL/min
II. Injector temperature: 100 °C
III. Detector temperature: 280 °C

FTIR spectrometry analysis

FTIR analysis does not determine quantitative determinations of different compounds present in the extracted sample. But it helps to known the functional group of different constituents present in samples. Infrared Spectra of different samples were recorded in a spectrophotometer shimadzu FTIR model happ-genzel in a frequency range from 4500 cm^{-1} to 500 cm^{-1}. The specimens having exposure area of 1 cm^2 per sample were prepared as mentioned above. The liquid sample was directly placed on a platform provided for sampling and sampling was done. Before and after sampling the specimen was properly cleaned by using n- acetone and again washed with distilled water and then dried. Subsequently, part of the surface of material was corresponding to obtain the infrared spectrum of specimen.

Identification of chemical constituents

Identification of thymol and other constituents of carom oil occurring in GC graph shown Fig. 1 were done by comparing both retention time and area of samples, of extracted oil and standard thymol (Fig. 2), p-cymene (Fig. 3) and β – pinene (Fig. 4). Calibration curve was used to quantify thymol in oil. Confirmation of thymol present in carom oil was done by GC-MS (Fig. 5-7) and FT-IR analysis (Fig. 8). In FT-IR analysis thymol was identified by confirming functional group of peaks adhering in FT-IR spectrum with standard peaks of thymol.

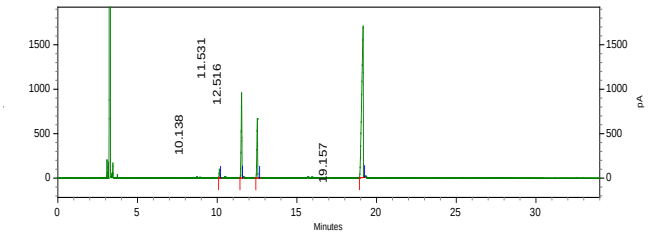

Fig. 1. GC graph of extracted Carom oil

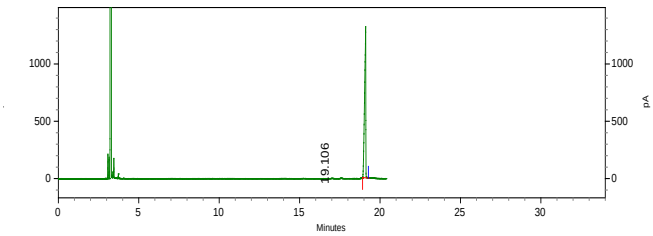

Fig. 2. GC graph of standard Thymol

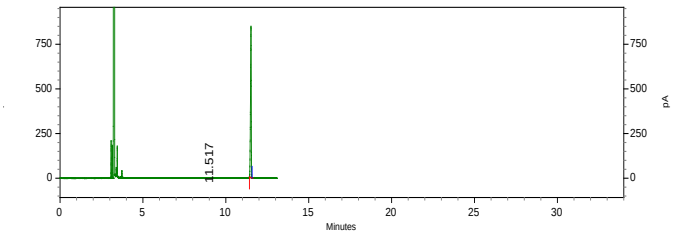

Fig.3. GC graph of standard p- cymene

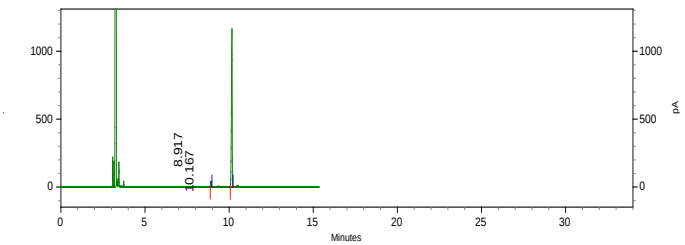

Fig. 4. GC graph of standard β – pinene

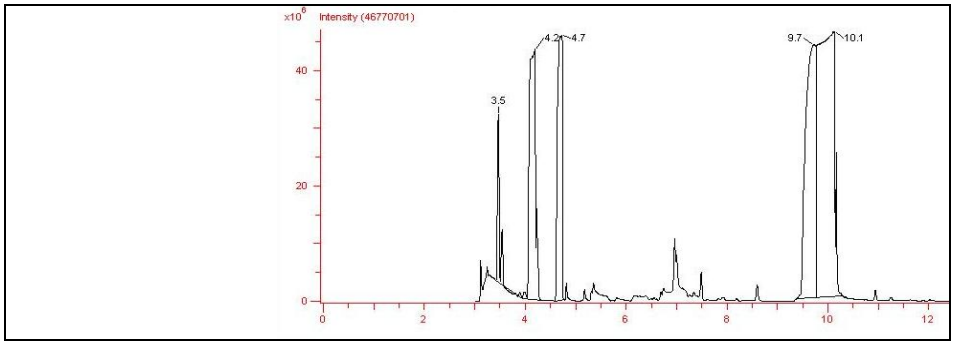

Figure 5. GC-MS graph of extracted carom oil

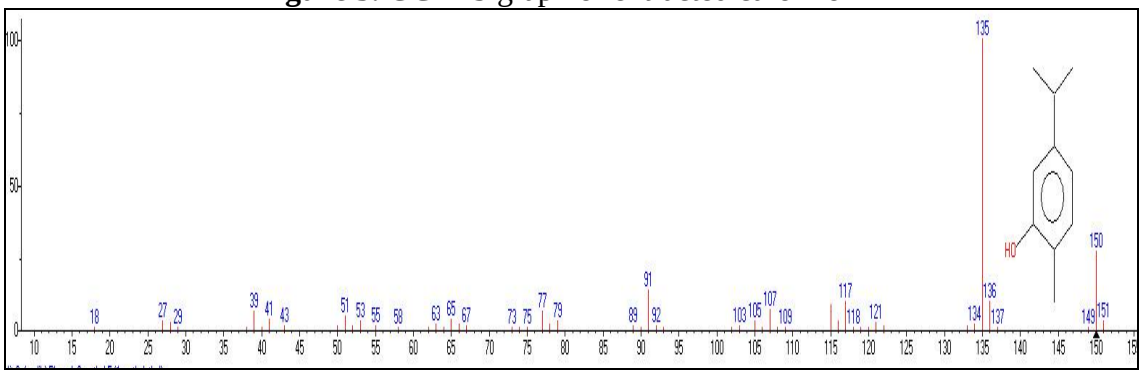

Figure 6. GC-MS graph of Thymol (present in carom oil)

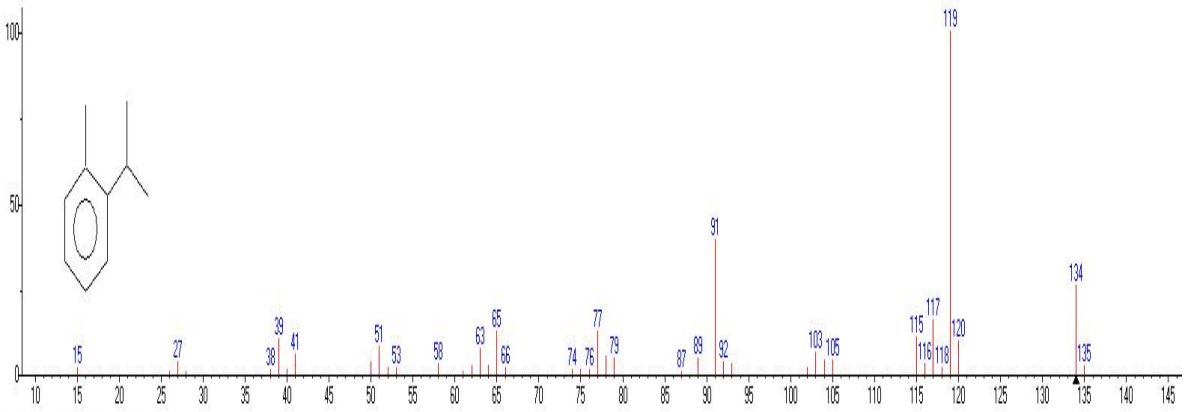

Figure 7. GC-MS graph of p-cymene (present in carom oil)

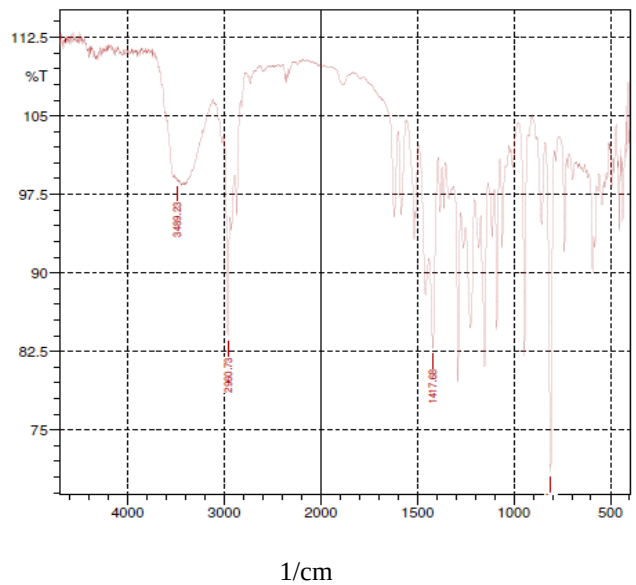

Figure 8. FT-IR spectrum of Carom oil

Insecticides test

Test insects: Insects were collected from local market, found adhering in stored grains. It was known that these insects remain alive in closed system for months together (almost 6 months). Thus, they were stored in a container under observation at room temperature until applying for insecticide tests.

Insecticides test of pure essential oil was performed using two toxicity tests

Fumigant toxicity test and Contact toxicity test

Fumigant toxicity test: Sterilized Petri dish was used for performing this test to avoid any contamination of oil and insects. Carom oil being tested to check insecticidal activity was accurately weight and applied at exactly the

centre of the Petri dish. Surrounding the Carom oil placed at centre of Petri dish and away from their boundary; five samples of insects were introduced in Petri dish. This prevented direct contact of insects with the Carom oil and all the insects were surrounding the essential oil placed at centre. Sufficient care was taken to avoid direct contact of oil and insects in the Petri dish. The insects kept in the dish along with oil were covered from top with another dish. Before doing this it was checked that there insects remain alive for months in closed system without any contact of air. As soon as the Petri dish was covered with another Petri dish from top, a stop watch was started. Time was noted when all the insects were found dead. Bachir *et al.* (2012) and Maciel *et al.* (2010) reported similar study. Their mortality rates were noted in terms of time and concentration of oil required for their death. These insects were considered dead if their appendages did not move when prodded with a brush. This treatment was carried out three times to know exact parameter of insect mortalities. Also minimum inhibition concentration of oil required for insects mortalities in minimum time intervals were noted. Standard sample was prepared wherein ten insects sample were kept in empty Petri dish free of essential oil. The Petri dish containing insects were covered from top with another dish and their mortality time was noted. These standard samples were compared with one containing essential oil.

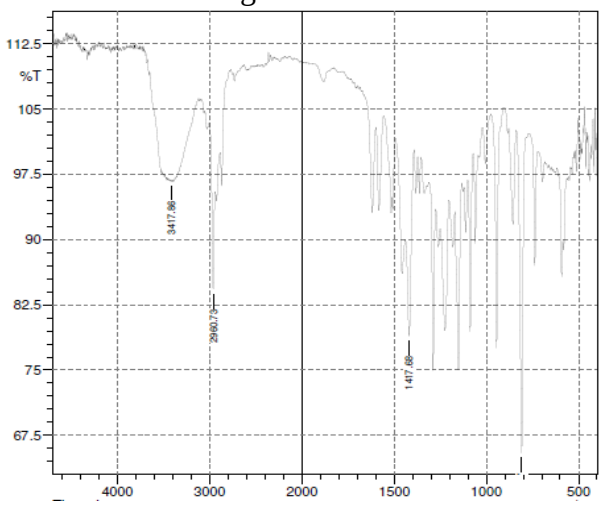

Fig 9. FT-IR Spectrum of Thymol

Contact toxicity test: Sterilized Petri dishes were used for performing this test to avoid contamination of oil and insects. This test was carried out in three different ways: 1) Carom oil being tested to check insecticidal activity was accurately weigh and spreaded on Petri dish using cotton. On this spreaded essential oil, insects were placed or made to walk to know the mortality rate of insects on oil in terms of time and 2) Insects were kept in Petri dish on which oil to be tested was poured. Their mortality rates were considered in terms of time.

RESULTS AND DISCUSSIONS

Analysis of essential oil composition: Physio-chemical and organoleptic analysis of Carom oil are very much similar to standard oil samples (Table 1).

Table 1. Physio chemical and organoleptic analysis of Carom oil

Test	Carom oil
Moisture	0.08%
Odour	Woody spicy
Solubility	Soluble in solvent
Physical state	Liquid
Colour	Slight yellow
Refractive index at 25 °C	1.491
Specific gravity at 25 °C	0.93
Acid value	1.312

Phyto-chemical test of Carom oil confirms presence of Terpenoids, Saponin (Table 2).

Table 2. Phyto-chemical analysis of carom oil

Phtyo-Chemical	Carom oil
Anthraquinones	Absent
Terpenoids	Present
Flavonoids	Absent
Saponin	Present
Tannins	Absent
Cardiac glycosides	Absent

Confirmatory test of Carom oil confirms presence of aldehyde, ketone, ether, alcohol and ester group (Table 3).

Table 3. Confirmatory test of Carom oil

Tests	Carom oil
Aldehyde	Present
Ketone	Absent
Phenol	Present
Ester	Absent
Alcohol	Absent

Table 4 and Table 5 indicate GC and GC-MS results of extracted carom oil.

Table 4. Chemical constituents of carom oil (analysed by GC)

Retention Time	Peak Area (%)	Constituents	Molecular Formula
10.138	2.2	β-pinene	$C_{10}H_{16}$
11.531	35.7	p-cymene	$C_{10}H_{14}$
12.516	13.8	γ-terpinene	$C_{10}H_{16}$
19.157	47.6	Thymol	$C_{10}H_{14}O$

GC result of Carom oil shows the presence of many different compounds out of which thymol is the major compound followed by β-pinene, p- cymene etc. Table 4 indicates different compounds present in Carom oil including thymol based on their retention time.

Table 5. Chemical constituents of carom oil (analysed by GC-MS)

Retention Time	Constituents
3.5	β-pinene
4.2	p-cymene
7	limonene
9.7	Thymol

Presence of β-pinene, p-cymene and thymol in carom oil is confirmed by GC-MS. Similar results were reported by Ameur et al. 2012.Also, presence of thymol in Carom was confirmed by FT-IR analysis (Table VI).

Table 6. FT-IR results of carom oil for thymol

Bond	Functional group	Frequency, cm^{-1}	Compound confirmed
O-H stretch	Phenol	3462.22	Thymol
C-H stretch	Aromatic ring	2960.73	Thymol
C-C stretch	Aromatic ring	1417.68	Thymol
C-H substituted	Meta disubstituted aromatic	808.17	Thymol

The entire bonds present in structure of thymol were confirmed with standard frequency and functional group adhering in their spectrum. **Table 7** shows the minimum inhibitory concentration of Carom oil against bugs. At 0.03 grams, Carom oil was 100% effective against bugs and its mortality time was 10 minutes. But as concentration increased from 0.03 to 0.1 grams mortality time of bugs was reduced to 6.5 minutes.

Table 7. Minimum inhibitory concentration of carom oil against grain bugs

MIC (grams)	Essential oil	Mortality time (mints)
0.01	Carom oil	Not effective
0.03	Carom oil	10
0.06	Carom oil	9-10
0.1	Carom oil	6.5

Table 8 indicates the fumigant effect of Carom oil against grain bugs. Five samples of insects were tested against carom oil at same concentration. This test was conducted thrice at same concentration to confirm their mortality time. Average time required for Carom oil to kill grain bugs was 10 minutes.

Table 8. Fumigant toxicity results of carom oil

Oil	MIC of oil (grams)	Mortality of grain bugs (min)	Blank sample (months)
Carom oil	0.03	10	4 months
Carom oil	0.03	10	4 months
Carom oil	0.03	12	4 months

Table 9 shows the contact toxicity effect of Carom oil against grain bugs. Five samples of insects were tested against Carom oil at same concentration. This test was conducted twice at same concentration to confirm their mortality time. Minimum inhibitory concentrations of individual essential oil were 0.05 grams. Average time taken by carom oil was 27 sec and 14 sec.

Table 9. Contact toxicity results of carom oil

Oil	MIC of oil (grams)	A	B
		Sample 1 Mortality time of grain bugs (In secs)	
Carom oil	0.05	30	15
Carom oil	0.05	25	13

Oil was applied at the centre of the Petri dish and insects were allowed to walk on it (A) and Oil was poured on insects (B).

From the above a work it can be concluded that carom oil is a good source natural pesticide. Moreover it is an efficient oil to isolate natural thymol considering yield, purity and aroma of oil

Acknowledgement

Authors are thankful to the Department of Oils, Oleo-chemicals and Surfactant Technology, Institute of Chemical Technology, Mumbai India for providing suitable analytical facilities to carry out the entire research work successfully.

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