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Evaluating phytochemical, antibacterial, antifungal and insecticidal properties of *zingiber zerumbet* (L.) roscoe ex sm rhizome extracts

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Abstract
The present investigation was focused on the study of the chemical composition variability and biological activities of the rhizome extracts from *Zingiber zerumbet* (L.) Roscoe ex Sm. The separation of the constituents was carried out by gas chromatography coupled with mass spectrometry (GC-MS). The *Z. zerumbet* rhizome extract was characterized by a high content of sesquiterpenes such as zerumbone and ginsenol. An antibacterial activity of rhizome extract from *Z. zerumbet* was tested against *Xanthomonas spp*, *Erwinia spp* and *Pseudomonas syringae*. We also examined the antifungal activities against *Rhizoctonia solani*, *Fusarium oxysporum* and *Colletotrichum capsici*. Further, we evaluated the pesticidal efficacy of *Z. zerumbet* rhizome extract against *Aphis gossypii*. All tested samples displayed noteworthy antibacterial antifungal properties, with the highest activity observed on *Xanthomonas spp* and *Rhizoctonia solani*. While, the pesticidal action was remarkably observed on *A. gossypii* with 87.0% mortality after 72 h of exposure. In conclusion, this study showed pronounced role of the *Z. zerumbet* rhizome extract as antibacterial, antifungal and insecticidal activity.

Keywords: Antibacterial, antifungal, insecticidal, medicinal plant, rhizome, *z. zerumbet*

Introduction
Environmental issues including contamination of food, water and soil pollution, and other issues are getting worse all over the world and causing a rising number of natural disasters and human tragedies. The overuse and indiscriminate application of pesticides to manage agricultural losses causing disturbances to the ecological balance (Boyer *et al*. 2012; Pavela, 2015) [10, 22]. Furthermore, the use of synthetic fungicides and fungal medications to treat mold in the environment and on animals is not any less important when discussing the drawbacks and emergence of infections resistant to fungicides (Abdel-Kawy *et al*. 2019; Rezgui *et al*. 2020) [1]. In order to feed the expanding human population in a healthy environment, there is growing concern about the quest for novel, highly selective, and sustainable alternatives of beneficial pest control agents.

A plethora of chemical compounds produced by medicinal plants aid to heal and restore human health. The study of plant extracts has received a lot attention because of its crucial significance as a source of agrochemicals, which are widely utilized to control plant diseases (Benelli *et al*. 2019; Pavela, 2019) [6]. With their potential to replace synthetic organic pesticides and prevent environmental contamination, natural herbicides, fungicides, and insecticides can be deployed as a substitute. As a result, numerous uses of essential oils have been documented, serving as safe fungicidal agents against a wide range of molds and as ecofriendly insecticides (Benelli *et al*. 2019; Benelli and Pavela, 2019) [6]. The chemical composition of medicinal plants contains various secondary metabolites and essential oils that are generated impact the mechanisms of action and establish the economic and medical value of different plants. Keeping in view of this, we aimed at evaluating the Zingiber zerumbet rhizome extract components to combat a variety of plant diseases, including pests, fungi, and bacteria.

Zingiber zerumbet (L.) Roscoe ex Sm is a perennial, aromatic rhizomatous plant species in the Zingiberaceae family, it is primarily found in Asia's tropical and subtropical regions (Anon, 1976). Traditional uses of the *Z. zerumbet* rhizomes include food and medicine (Yob *et al.* 011) [32]. Numerous pharmacological effects of plants have been documented, including anti-inflammatory, anti-tumor, anti-allergic, antioxidant, antibacterial, anti-viral, and treatments for sore throat, stomachaches, and worms (Rahman *et al.* 2014; Nhareet and Nur, 2003; Huang *et al.* 2005; Tewtrakul and Subhadhirasakul, 2007) [17, 3, 25, 30].

Till date, there was little data available on *Z. zerumbet* rhizome extracts applications in controlling plant diseases. Hence our given is first of its kind which focused on the role of *Z. zerumbet* extracts as antifungal, antibacterial and pesticidal action. This would be definetely helpful for the agrochemical industry's precise function of this medicinal plant in conserving human health as well as ecosystem.

Materials and methods
Preparation of plant extracts
Z. zerumbet was collected and the harvested plants were identified and rhizomes were seperated. Matured rhizomes were air-dried for 72 h, and plant extracts were obtained by magnetic stirring of 2.5 g of powdered dry matter with 25 mL of solvent for 30 min at room temperature (25◦C). Extracts were obtained with solvents of increasing polarity: hexane/water (8:2, v/v), acetone/water (8:2, v/v) ethanol/water (9:1, v/v), methanol/water (8:2, v/v) and water. The extracts were kept for 24 h at 4◦C, filtered through Whatman No. 4 filter paper, evaporated to dryness under vacuum and stored at 4◦C until analysis.

Estimation of total phenolics content
Total phenols were assayed according to Dewanto *et al*. (2002) [11]. An aliquot of diluted extract was added to 0.5 mL of distilled water and 0.125 mL of Folin–Ciocalteu

reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 mL of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 3 mL and mixed thoroughly. After incubation in the dark, absorbance at 760 nm was read versus a prepared blank. The total phenol content of plant parts was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) from a calibration curve with gallic acid. All samples were analyzed in three replicates.

Estimation of total flavonoids

Estimation of total flavonoid content was carried out with the help of aluminium chloride method given by Dewanto *et al*. (2002) [11]. Total 250 mg of powdered sample was suspended in 10 ml of 80% methanol for 24 h at room temperature followed by centrifugation at 3000 rpm for 20 min. 1.0 ml of supernatant extract was taken out in the test tubes. To this, 0.3 ml of 5% $NaNO_2$ solution was added and was left it for 3 min. After 3 min, 0.3 ml of 10% $AlCl_3$ was added and was left the solution for 2 min. 2 ml of 1.0 M NaOH was added and mixed well. After that 2.4 ml distilled water was added to it and mixed well. Optical density of the yellowish brown color was read at 510 nm. Total flavonoid content was calculated from the standard curve prepared from the querctine and the results were expressed in mg/g dry weight.

Total condensed tannins

Condensed tannins (proanthocyanidins) were determined according to the method of Sun *et al*. (1998) [27]. To 50 µL of diluted sample, 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated HCl were added. The mixture was allowed to stand for 15 min, and absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin/g DW. All samples were analyzed in triplicate.

DPPH assay

The electron donating ability of the obtained extracts was measured by bleaching a purple solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to the method of Hanato *et al*. (1988). Extracts (0.1 mL, 5, 10, 50 and 100 □g/mL) were added to 0.5 mL of 0.2 m mol/L DPPH–methanol solution. After incubation for 30 min at room temperature, the absorbance was determined against a blank at 517 nm. The percentage inhibition of free radical DPPH was calculated from (A blank−A sample/A blank) × 100, where *A* blank is the absorbance of the control reaction and *A* sample is the absorbance in the presence of plant extract. The concentration of extract that caused 50% inhibition (IC50) was calculated from the regression equation for the concentration of extract and percentage inhibition. Samples were analyzed in triplicate.

Total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of a green phosphate–Mo(V) complex at acid pH Prieto *et al*, (1999) [24]. An aliquot (0.1 mL) of plant extract was added to 1 mL of reagent solution (0.6 mol/L H_2SO_4, 28 mmol/L Na_3PO_4 and 4 mmol/L ammonium molybdate). The tubes were incubated in a thermal block at 95◦C for 90 min. Once the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. Antioxidant capacity was expressed was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0–500 □g/mL. All samples were analyzed in triplicate.

Gas chromatography/mass spectrometry (GC/MS) analysis

The composition of *Z. zerumbet* rhizome was analyzed using SHIMADZU GCMSQP2010. The column used was 30 m × 0.25 mm inner diameter with a thickness of 0.25 µm. The carrier gas used was Helium gas (flow rate of 1 mL/min). The injector temperature was set at 280 ◦C and the oven temperature was raised to 300 ◦C at a rate of 4 ◦C per minute. The identification was carried out using the NIST library according to the standard methods described in the previous reports of Padalia *et al*. (2013) [21].

Collection of microorganisms

Antibacterial activity was determined against two Gram (-) ve bacteria (*Xanthomonas spp* and *Pseudomonas syringae*). The antifungal screening was carried out against fungi (*Rhizoctonia solani, Fusarium oxysporum* and *Colletotrichum capsici*). All these organisms were collected from the Crop Protection and Biological Control Laboratory, SLN Biologicals LLP, Nizamabad, Telangana, India.

Collection of test insect

For insecticidal screening the insect Aphids *(Aphis gossypii)* used in the experiment was provided from the stock cultures of the Crop Protection and Biological Control Laboratory, SLN Biologicals LLP, Nizamabad, Telangana, India.

Growth media and conditions

Nutrient agar media (HiMedia Laboratories) pH 7.2 and Potato dextrose agar media (HiMedia Laboratories) pH 5.6 were used for antibacterial and antifungal screening respectively.

Antibacterial screening

The *in vitro* antibacterial activity of the extract was determined by disc diffusion method (Bauer *et al.* 1996) [5]. Sample discs were prepared by allowing each sterile disc (6 mm in diameter) of filter paper to absorb 20 µl of a test solution in aseptic condition. The discs were allowed to dry until complete evaporation of solvent. Dried and sterilized filter paper discs, each containing a test sample of 500 µg of the test agent was placed on nutrient agar medium uniformly seeded with the test microorganisms. Kanamycin disc (30 µg/disc) and blank disc were used as the positive and negative control respectively. The plates were incubated at 37°C for 24 hours for optimum growth of the organisms. The antibacterial activity of the extract was determined by measuring the diameter of the zone of inhibition expressed in millimeter.

Antifungal screening

The extract was screened for its antifungal activity by disc diffusion method at the concentration of 500µg/disc. Potato dextrose (20 ml) plates were prepared and incubated by spread plate method under aseptic conditions. The sterile impregnated discs with plant extracts were placed on the agar surface with framed forceps and gently pressed down to ensure complete contact of the disc with agar surface.

Control discs of carbendezim were prepared and placed on the agar surface. All the plates were incubated at 37°C for 72 hours and the size of the inhibition zones were measured. The mean zone of inhibition of the three replicated tests (triplicate analysis) of the plant extract is expressed in millimeter.

Insecticidal screening

For the conduction of surface film activity test of the plant extract, test tubes were taken. The plant extract (500 mg) was dissolved into 1 ml methanol added 10 ml of sterile water containing 5% sucrose. This was poured into the lower part of the petridish, control experiment applying only the water into the test tube slants was also set at the same time under the standard conditions at 28ºC, 70 % RH at a photoperiod (12h light: 12h dark) (Bousquet 1990) [9]. After completing all the arrangements, treated petridishes were placed in a secured place at room temperature. The whole experiment was observed from time to time and mortality was observed after 24 h, 48 h and finally after 72 h of exposure and data were recorded. A simple microscope was used to observe each and every insects by tracing natural movements of each organism. In some cases hot needle was taken closer to the bodies (without movement) to confirm death.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), and differences among treatments were assessed using Duncan's multiple range test (DMRT) at the $P = 0.05$ level using SigmaPlot version 12.0. Probability analysis was performed for the calculation of LC50 values using the EPA Probit analysis program version 1.5. Inhibition ratio and EC50 values were obtained using Log-Probit analysis.

Results

Total phenolic, flavonoid and tannin contents

In the given study the Fig. 1 illustrates the extracts' total phenol contents. When extracted in ethanolic solvents (48 mg GAE/g DW), Z. zerumbet rhizome extract had a greater total polyphenol content than when extracted in other solvents (Fig. 1a). The highest quantity was found in the ethanol extract, which was followed by the extracts of acetone, methanol, water, and hexane; similar pattern was observed for the levels of flavonoids (except water exhbiting least) and condensed tannins (Fig. 1b & 1c).

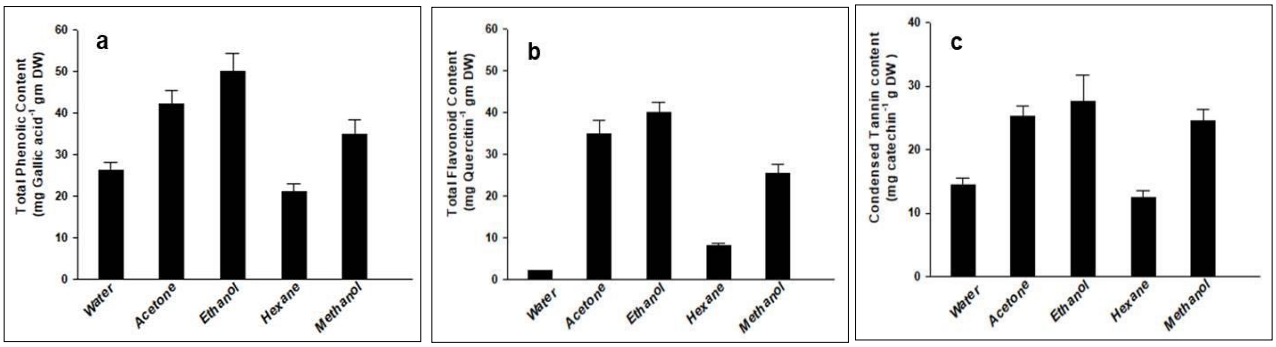

Fig 1: The total contents of **(a)** polyphenol (expressed as mg gallic acid equivalents/g dry weight), **(b)** flavonoids and **(c)** condensed tannin (expressed as mg catechin/g dry weight) in rhizome extracts of Zingiber zerumbet in different solvents

Total antioxidant activity and DPPH radical-scavenging activity

The total antioxidant capacity of *Z. zerumbet* rhizome extract varied according to the solvent. Our results depicted that total antioxidant capacity of *Z. zerumbet* rhizome extract was much higher in ethanol extract, followed by the acetone extract, the methanol extract, the extract in water and least was observed in the hexane extract (Fig 2a).

Even the DPPH radical-scavenging activity (Fig 2b) exhibited similar pattern as that of total antioxidant capacity. Surprisingly, the rhizome extracts of *Z. zerumbet* extracted in water exhibited significantly high DPPH radical scavenging activity than those collected in other solvents (Fig. 2b). Hexane extracts had the lowest anti-radical activity, while the most polar solvent (water) had potent activity followed by ethanol, methanol and acetone.

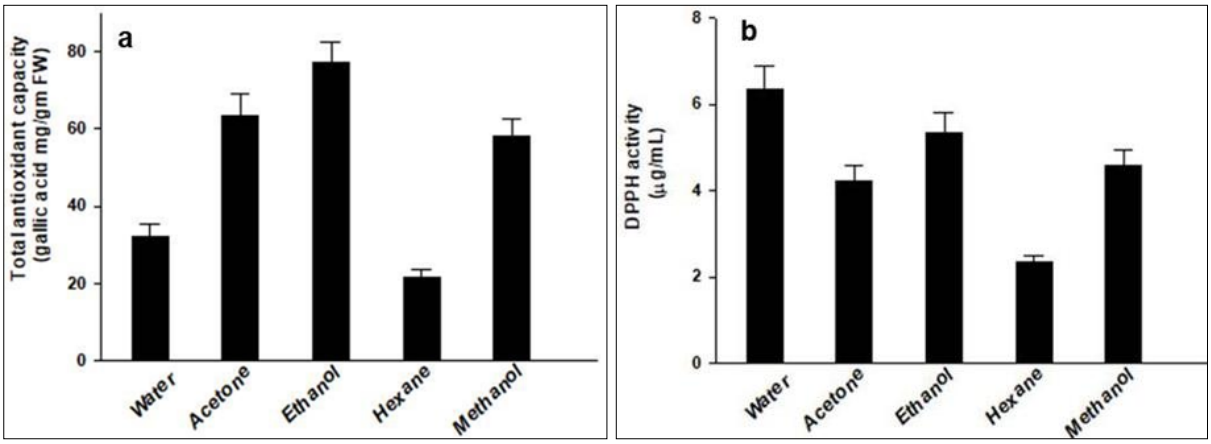

Fig 2: (a) Total antioxidant capacity (expressed as mg gallic acid equivalents/g dry weight and **(b)** Scavenging activity, expressed as median inhibitory concentration (□g/mL), in the DPPH test in rhizome extracts of Zingiber zerumbet in different solvents

Chemical Composition of Extract

The results of GC/MS analysis of rhizome extracts of Z. zerumbet was presented as Figure 1 highlighting the most important and abundant secondary metabolite compounds such as zerumbone and ginsenol. The identification of these compounds was carried out using the NIST library following to the standard methods.

Antibacterial activity

The extract of *Z. zerumbet* rhizome (500 µg/disc) was found to be effective against various plant pathogenic fungi as indicated by the zone of inhibition (Table 1). Maximum inhibition was obtained against *Xanthomonas spp* (18 mm), *Erwinia spp* (14 mm) and *Pseudomonas syringae* (8 mm) in comparison to reference standard kanamycin 30µg/disc (Table 1). The EC50 value for rhizome extracts of *Z. zerumbet* against *Xanthomonas spp* was 156.12 µg mL^{-1}, demonstrating the activity of the extract against this fungus. Its activity against *Erwinia spp* was moderate, with an EC50 value of 399.33 µg mL^{-1}, but it provided negligible control of *Pseudomonas syringae*, with an EC50 value of 986.17 µg mL^{-1}. Carbendazim (30 µg/disc**)** showed excellent results against *Xanthomonas spp* (<31 µg mL^{-1}), *Erwinia spp* (<67 µg mL^{-1}) and *Pseudomonas syringae* (<23 µg mL^{-1}).

Table 1: Antibacterial activities of the *Zingiber zerumbet* (L.) rhizome extract

Test bacterial strains	Diameter of Zone of Inhibition(mm)			EC_{50}
	Extract 500 µg/disc	Std. Kanamycin 30 µg/disc	Extract 500 µg/disc	Std. Kanamycin 30 µg/disc
Xanthomonas spp	18	28	156.12	<31
Erwinia spp	14	25	399.33	<67
Pseudomonas syringae	08	26	986.17	< 23

Antifungal activity

Table 2 presents data on the fungicidal activity of rhizome extracts of *Z. zerumbet* as well as synthetic chemicals carbendazim, which served as positive controls. Our results demonstrated the high inhibition of *Z. zerumbet* rhizome extract (500 µg/disc) against *Rhizoctonia solani* (36 mm), *Fusarium oxysporum* (23 mm) and *Colletotrichum capsici* (11 mm) in comparison to reference standard kanamycin 30µg/disc (Table. 2). The EC50 value for rhizome extracts of *Z. zerumbet* against *R. solani* was 164.43 µg mL^{-1}, demonstrating the activity of the extract against this fungus. Its activity against *F. oxysporum* was moderate, with an EC50 value of 432.21 µg mL^{-1}, but it provided least control against *C. capsici*, with an EC50 value of 656.23 µg mL^{-1}. Carbendazim showed excellent results against three fungi; *R. solani* (< 22 µg mL^{-1}), *F. oxysporum* (< 34 µg mL^{-1}) and *C. capsici* (< 51 µg mL^{-1}).

Table 2: Antifungal activities of the *Zingiber zerumbet* (L.) rhizome extract.

Test fungal strains	Diameter of Zone of Inhibition(mm)			EC_{50}
	Extract 500 µg/disc	Std. Carbendezim 50 µg/disc	Extract 500 µg/disc	Std. Carbendezim 50 µg/disc
Rhizoctonia solani	36	42	164.43	<22
Fusarium oxysporum	23	45	432.21	<34
Colletotrichum capsici	11	46	656.23	< 51

Insecticidal activity

The data presented in Table 3 show the aphicidal activity of rhizome extracts of *Z. zerumbet* against the cotton aphid, *A. gossypii*. The results exhibited that after a prolonged exposure period of 72 h at a 500 µg mL^{-1} concentration, 87.0% mortality was observed

Table 3: Insecticidal activities of the *Zingiber zerumbet* (L.) rhizome extract

Extract	Concentration (□g/ml)	Insects (no's)	Number of insect dead				Mortality (%)
			0 h	24 h	48 h	72 h	
Z. zerumbet rhizome extract	500	15	0	6	9	13	87

Discussion

In the present investigation, we evaluated *Z. zerumbet's* phenolic content, antioxidant, antibacterial, antifungal, and insecticidal properties. Methanol extracts produced the largest output of total polyphenols, flavonoids and tannins with highest yield. The phenol content was dependent on the solvent used and its polarity. Phenols have been extracted in the past using methanol, ethanol, acetone, propanol, ethyl acetate, and dimethyl formamide (Tan *et al* 2012) [29]. The degree of polymerization of phenols, their solubility in the extraction solvent, the kind of solvent, the interaction of phenols with other plant elements, and the development of insoluble complexes significantly affect the recovery of polyphenols from plant materials (Galvez *et al* 2005) [13].

Variations in the antioxidant activity can be attributed to variations in their polarity of solvents used. Moreover, phenolic solubility is enhanced by solvent polarity (Naczk and Shahidi, 2006) [19]. For this reason, defining a consistent protocol for the extraction of plant phenols is challenging. Generally speaking, unless extremely high pressure is applied, the least polar solvents are thought to be appropriate for extracting lipophilic phenols, while polar solvents are employed for hydrophilic phenols (Alothman *et al* 2009) [2].

Since the plants contain an extensive array of antioxidants, it is challenging to determine the individual antioxidant potential of each molecule. The majority of techniques created to gauge the antioxidant potential of various plant materials (Swapana *et al*., 2013) [28] assess a material's power to scavenge certain radicals by chelating metal or preventing lipid peroxidation. The *Z. zerumbet* extracts we investigated shown moderate ferrous iron lowering and carotene bleaching inhibitory powers along with significant total antioxidant and DPPH activity. Given that phenols

account for the majority of plant antioxidant activity, Mansouri *et al*. (2005) [18] suggested that a high phenol concentration may be linked to antioxidant capacity. Phenols are composed structurally of an aromatic ring with one or more hydroxyl substituents. The type of molecule's ability to bind metal cations, donate hydrogen atoms or electrons, and scavenge free radicals accounts for its antioxidant activity (Amarowicz *et al* 2004) [3].

In general, plants are prone to attacked by insects, microbes, and mammals typically develop self-defense mechanisms through the production of a range of secondary metabolites, including steroids, alkaloids, terpenoids, and aromatic compounds, which are likely repulsive or even poisonous to the adversary (Fisher *et al*. 1984, Gurney and Mantle 1993) [14, 12]. Yang and Tang (1998) [31] conducted a survey of plants used for insect control and discovered a close relationship between pesticidal and therapeutic plants. Insect-caused food grain storage losses are estimated to account for 10% of global food production annually, although losses of up to 25% may also arise in tropical nations due to insect attacks following harvest (Howe 1965) [16].

It was quite familiar that, *Z. zerumbet* plant has been the subject of prior phytochemical studies that have identified flavonoids, steroids, triterpenoids, and alkaloids. It is possible that the presence of these kinds of phyto-constituents is what causes the extract of *Z. zerumbet* rhizome to exhibit insecticidal activity. Drug resistance in phyto-pathogens can significantly reduce the efficacy of certain pesticides (Rosenberger and Meyer 1981) [26].

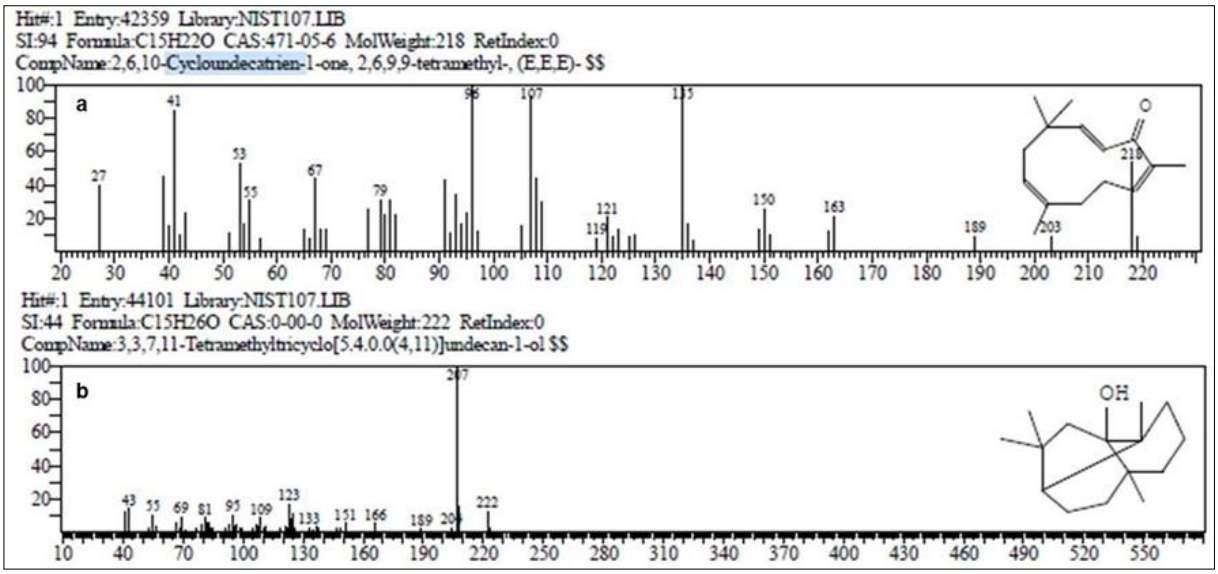

Fig 3: The GC-MS chromogram of methanolic extracts of Z. zerumbet rhizomes with peaks depicting a) Zerumbone and b) Ginsenol

Conclusion

This work outlines the possible role of medicinal plants especially *Z. zerumbet* containing the chemical constituents which may have a scope of acting as biopesticides and safer antimicrobial agents which are readily available, renewable, non-petrochemical, and naturally eco-friendly.

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