Antibacterial Potential of *Luprops tristis* - the Nuisance Rubber Plantation Pest from Western Ghats of India

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***Abstract*- Insects hold potential for deriving novel bioactive substances against multiple drug resistant bacteria that pose serious challenges for the clinical management of several microbial mediated diseases. In this context, *Luprops tristis,* a rubber plantation litter pest with a high population in the moist South Western Ghats of India was explored for possible bioactive substances. *In-vitro* studies showed that all the Gram positive (*Staphylococcus aureus* NCIM No. 2079; *Bacillus cereus* NCIM No. 2217; and *Lysinibacillus* BTTS10) and Gram negative species (*E.coli* NCIM No. 2574; *Salmonella typhi* BTC12; *Klebsiella pneumonia* NCIM No.2957) tested were sensitive to this compound. The minimum inhibitory concentration was 0.067mg mL^{-1} for *S. aureus, L. fusiformis* and 6.7mg mL^{-1} for *E.coli, S. typhi, K. pneumoniae*. The extract was partially purified by column chromatography. FT-IR showed the presence of alkenyl (C=C) & carbonyl (=CO) groups. GC MS indicated the presence of [(hexadecyloxy) methyl]-, oleic acid, butylated hydroxytoluene and hexadecanoic acid.**

***Key words* – *Luprops tristis*; antibacterial compounds; Bioautography; GC/MS.**

I. INTRODUCTION

The indiscriminate, excessive and extensive use of antimicrobial agents in human and animal disease treatment and as growth promoters in animal feed has created a continuous selective pressure, promoting the development of multiple drug resistant strains. As a consequence there is challenge for combating the drug resistant strains. On the other hand, pharmaceutical companies have almost discontinued the search for new antibiotic agents and started producing second and third generation antibiotics by chemical modification of existing antibiotics. Therefore, no new antibiotics are available against the new or re-emerging infectious diseases caused by multiple drug resistant strains [1]. In this context, the academic and scientific community has sustained their inquisitive search for novel bioactive molecules from plants, bacteria, and mostly animals. Their efforts have resulted in the isolation of numerous peptides and polypeptides with antimicrobial properties.

The world of insects deserves great significance and attention by virtue of their diversity, large population and their significant role in the balanced maintenance of ecosystem. They remain a curious component of the ecosystem since they survive in the competitive environment and safeguard themselves against microbial pathogens and infections. Their bioactivity against microbial pathogens may be attributed to their diverse secretions such as protein, fatty acids, hormone, chitin, vitamins, and minerals to name a few. In fact knowledge on the bioactive potentials of such secretions could contain wide applications as active pharmaceuticals, in food and feed industry and for other applications. When compared to the quantum of literature on plant based bioactive substances, insect based literature on bioactive substances is limited. Consequently, insects are unexplored resource for the prospecting of novel bioactive substances.

Almost 50% of the reported antimicrobial substances were identified in invertebrates, predominantly in insects [1]. Insect body extracts have been used widely in folk medicine and Chinese traditional medicine for the treatment of throat and ear infections, tuberculosis, influenza, cancer and many other diseases and ailments [2]. Insects from 77 species, 14 families and 8 orders are known to be used in Chinese traditional medicine to treat tumors and cancer [3]. Considering some of the challenging and successful lifestyles of the insects that occupy microbe-infested niches, it is not surprising that they should possess very effective immune systems producing powerful antimicrobial and cytotoxic factors [4].

Luprops beetles (Tenebrionidae: Lagriinae: Lupropini) originated from tropical Africa and extended to Asia and the East Indies are generally regarded as an inconspicuous litter-dwelling detritivore. Huge aggregations of this litter dwelling beetle, *Luprops tristis*, numbering about 0.5 to over 4 million per residential building, is a regular event in rubber tree tracts along the western slopes of the southern region of the Western Ghats. They are usually found after summer showers, and stay in a state of dormancy for a prolonged period [5]. Their high abundance of about 20 million per hectare in rubber plantations illustrates the range of *L. tristis* infestation [6]. The abundance of *Luprops* beetles along with

extensive incidence of such aggregations in the region makes this organism a suitable candidate for the large scale extraction of the antibacterial compound. In this context, the present study was undertaken to explore *Luprops* beetles as a source of novel bioactive substances.

II. MATERIALS AND METHODS

A. Extraction and sample preparation

Luprops tristis insects (10 g) were washed with 70% ethanol, followed by thorough rinsing with sterile deionized water. Later, excess water was blot dried with sterile filter paper. The whole insects were then homogenized aseptically in physiological saline using mortar and pestle. The homogenate was then centrifuged at 12,000 rpm for 10 min at 4^{o} C, and the supernatant (referred to as extract) was used. For preparation of methanolic extract, the above procedure was followed except that the extraction solvent used was methanol:water:acetic acid (90:9:1); and n-hexane extract was prepared as per the protocol of Baulmer et al.[7]

B. Bacterial strains

Both Gram negative (*E. coli* NCIM No. 2574; *Salmonella typhi* BTC12; *Klebsiella pneumoniae* NCIM No.2957) and Gram positive bacterial strains (*Staphylococcus aureus* NCIM No. 2079; *Bacillus cereus* NCIM No. 2217; and *Lysinibacillus fusiformis* BTTS10) obtained from stock culture collection center, NCIM, Pune and DBT, CUSAT were used for the conduct of antibacterial activity studies.

C. Evaluation of antibacterial activity

Evaluation of antimicrobial activity of the prepared insect extract against known bacteria was performed by agar well diffusion assay [8, 9]. All the strains were grown in Mueller-Hinton broth (HiMedia, Mumbai, India) at 37^{o} C for 24 hours in a shaker incubator at 120 rpm. For inoculum preparation, culture with OD_{600} = 1 was used throughout the study.

D. Bioautographic overlay assay

Antibacterial activities of the prepared insect extracts were determined by bioautographic assay [10]. On a thin layer of MH (Mueller- Hinton) Agar (Himedia, India) Thin layer chromatographic (TLC) plate, developed as detailed below was placed. This was overlaid with molten MH agar (0.8%) containing 1 mL of the test organism. The plates were incubated at 37^{o}C for 24h. Zones of inhibition were then visualized by adding 2, 3, 5- Triphenyl tetrazolium chloride (TTC 1mg per 6mL), a dehydrogenase- activity- detecting reagent onto the plates [11]. Metabolically active bacteria convert the colourless tetrazolium salt into the corresponding intensely coloured formazan. The area where metabolically active bacteria were not present appeared as a clear zone against red background. TLC plate submerged in the mobile phase was used as control.

E. Thin Layer Chromatography

About 20 µL of the extract was loaded on a previously coated Silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany). The TLC plates were developed with hexane: diethyl ether: acetic acid in the ratio 60:40:1 for about 30 minutes and visualized using iodine chamber.

F. Column Chromatography

Silica gel G (60 – 120 mesh size from Merck), suspended in chloroform was packed to a height of 20 cm in a 45 x 1 cm column, supported and covered by small glass wool plugs. Then the column was washed with 20 mL chloroform to eliminate potential impurities. The sample was applied in hexane solution and eluted with hexane: diethyl ether (1:1) [12]. Solvent was evaporated from the active fraction, weighed and used to find the MIC. All the solvents used were of HPLC grade from Merck.

G. Minimum Inhibitory Concentration (MIC)

The MIC of the partially purified sample was detected by serial dilution method [13]. 67 mg of sample was dissolved in 1 mL of Mueller –Hinton broth. This was serially diluted to get concentrations of 67, 6.7, 0.067 mg mL^{-1}. 10µL culture of the test organisms with OD_{600} = 1 was added to the dilutions. The tubes were incubated at 37^{o}C at 120 rpm. The procedure was also repeated using the reference antibiotic ampicillin (5mg mL^{-1}). After incubation, the bacterial growth was determined as optical density (OD) at 600 nm in a UV visible spectrophotometer. The lowest concentrations without visible growth (OD_{600} zero) were defined as concentrations that completely inhibited bacterial growth (MICs).

H. Identification of bioactive compounds

Compounds that showed antibacterial activity were identified based on structure elucidated by FT-IR Spectroscopy and GC- MS analysis.

I. FT-IR Spectroscopy

The hexane extract was used for FT-IR analysis. Attenuated total reflectance (ATR) method was used for the analysis.

J. GC- MS analysis

The active fraction obtained from column chromatography was evaporated and analyzed using high resolution GC/MS - Varian 1200L. The column used was 30m x 25mm x 0.25µm.The temperature of the programme was 40 ^{0}C isothermal, heating up to 250 ^{0}C with a heating rate of 4 ^{0}C /min. Nitrogen was used as carrier gas with a flow rate of 1.2 mL /min and the molecules were scanned from 50-550(m/z). The most relevant peaks were identified by MS Single quadrapole, EI of Mass range: 10-800 amu and analysed by mass spectra databases (NIST).

III. RESULTS AND DISCUSSION

The aqueous, methanolic and hexane extracts showed antibacterial activity against the Gram positive and Gram negative organisms tested (Fig 1). Maximum activity was given by hexane extract. Hence it was used for further studies. MIC assay gave a complete inhibition at a concentration of 0.067mgmL^{-1} against *S. aureus* and 6.7mgmL^{-1} for *E. coli* while it was 0.01µg mL^{-1} and 0.1µg

mL^{-1} respectively. Detailed results are as given in table 1. TLC and bioautographic assay showed that a compound having an R_f value of 0.73 was the active compound (Fig 2). Column chromatography showed the presence of coloured bands. The first band gave maximum antimicrobial activity. The FT-IR of the purified sample showed the presence of three characteristic peaks (Fig 3). They indicated the presence of carbonyl groups and ester groups [14].

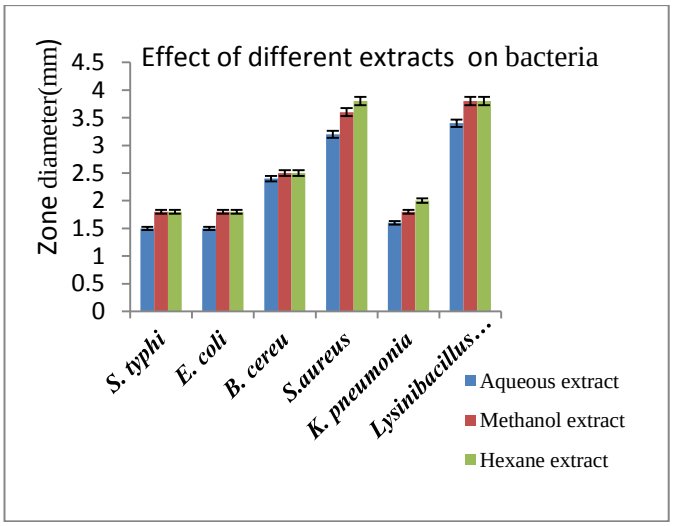


Fig 1. Antimicrobial activity of different extracts by agar diffusion method

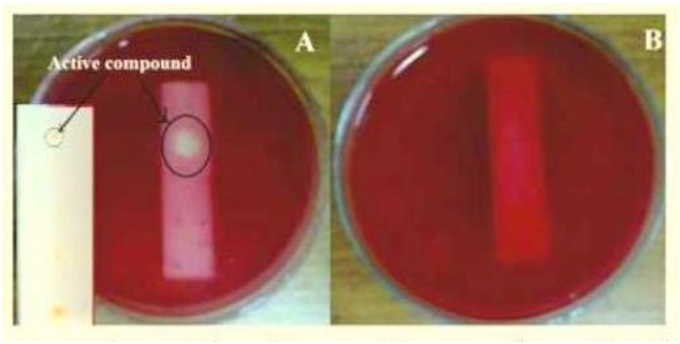
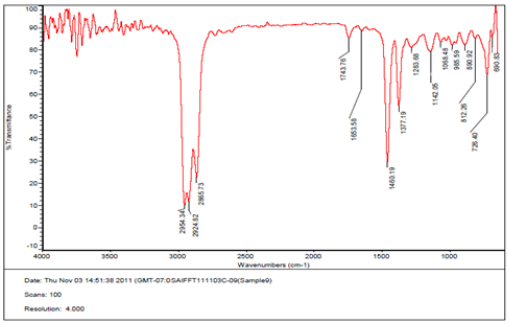


Fig 2. Bioautographic overlay assay and the corresponding TLC plate. The circled regions on the TLC plate indicate the active compound and clearing zone indicating the absence of bacterial growth due to inhibition by active compound. Red colour on the plate is due to metabolically active bacterial lawn.

GC MS showed four peaks at 12.798min, 20.158min, 20.375min and 22.490min, indicating the presence of four components in the hexane extract (Fig 4 & 5). The major peaks were obtained at 20.375min and 22.490min (Fig 4). The mass spectrum of these peaks show the presence of Oxirane, [(hexadecyloxy) methyl]- and oleic acid respectively (Fig 6 a & d). The minor peaks show more similarity to butylated hydroxytoluene and hexadecanoic acid (Fig 6 b & c). [15]



Wave number	Functional group*
1743.46	Ester
1283.68	C=O stretch

* All the other groups correspond to the functional groups in hexane.

Fig 3. F T IR spectrum of the active compound in hexane.

Oleic acid also acts as a pheromone in insects. Its smell is recognized by insects as a sign of danger. It is usually emitted by dead insects. This unsaturated fatty acid is a constituent of essential oils in several plants [16]. The antibacterial activity of oleic acid is well known [17, 18]. Antibacterial extract isolated from *Helichrysum pedunculatum* and *Gongronema latifolium* Decne showed the presence of linoleic and oleic acids.

Oxiranes are the constituents of latex and other resins of plants that play an active role in the microbial defense against human and animal pathogens. They are found in the antibacterial and antioxidant extracts of many plants [19]. The terpenes identified in ethanolic extract of *Dregea volubilis* (Linn.) such as 1, 3-diazacyclooctane2-thione, Phen-1, 4,-diol, 2, 3-dimethyl-5-trifluoromethyl, Oxirane (hexadecyloxy) methyl exhibit antimicrobial activity [20]. Even though oxiranes are also soluble in water, their antibacterial action can be attributed to the lipophylic nature of the terpenes. Oxirane antibiotics are clinically important antibiotics produced as secondary metabolites by various *Streptomycetes* and *Pseudomonas* species [21]. Oxirane derivatives are of prime importance in antibiotic research since the heterocyclic oxirane rings are part of many natural products and they form intermediates for many chemical reactions for synthesis of antibiotics [22].

Hexadecanoic acid or palmitic acid is an aliphatic fatty acid present in many plants. It has been reported in extracts of various propolis samples collected from honeybees, which shows antibacterial activity [23]. The main constituents of propolis are beeswax, resin and volatiles. The biological activity of propolis is attributed to the plant derived substances like resin and volatiles [24 and 25]. Hexadecanoic acid was one of the main constituents of essential oils from fresh leaves of *Vitex negundo which showed antibacterial activity* against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* [26].

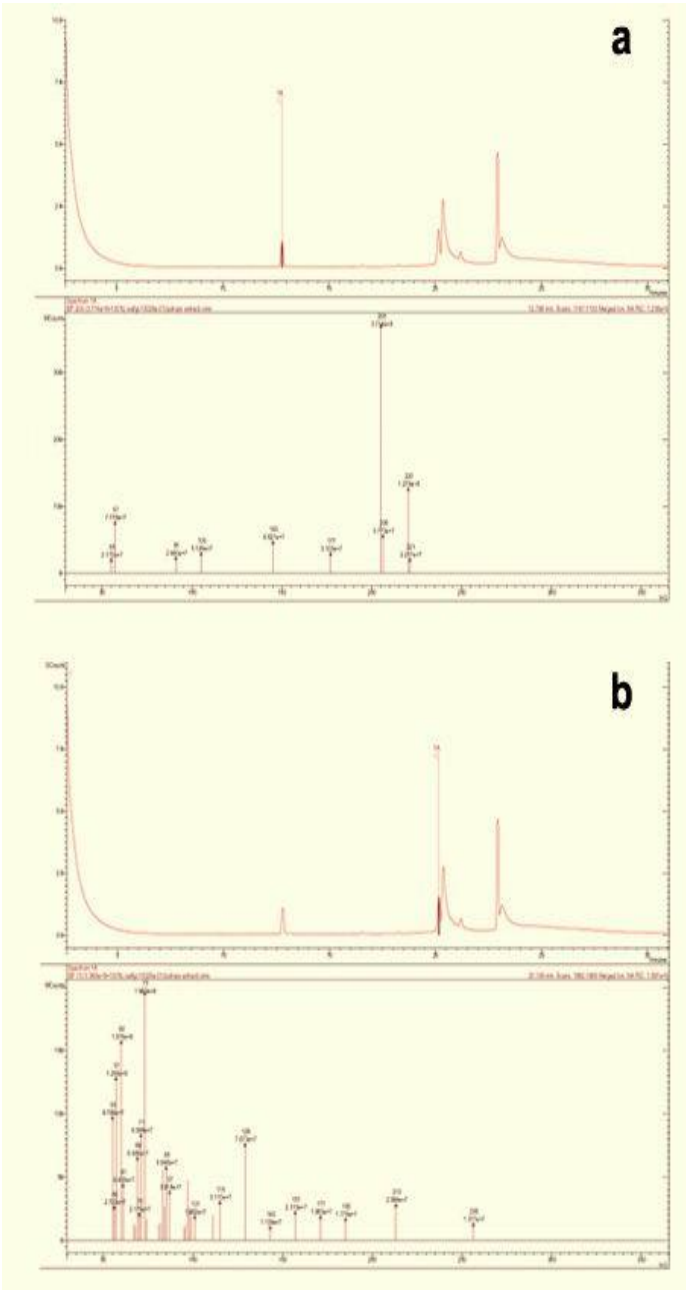


Fig 3. GC MS showing retention time and corresponding Mass spectrum. a) Peak of retention time of 12.798min and corresponding MS pattern. b) Peak of retention time 20.158min and corresponding MS pattern.

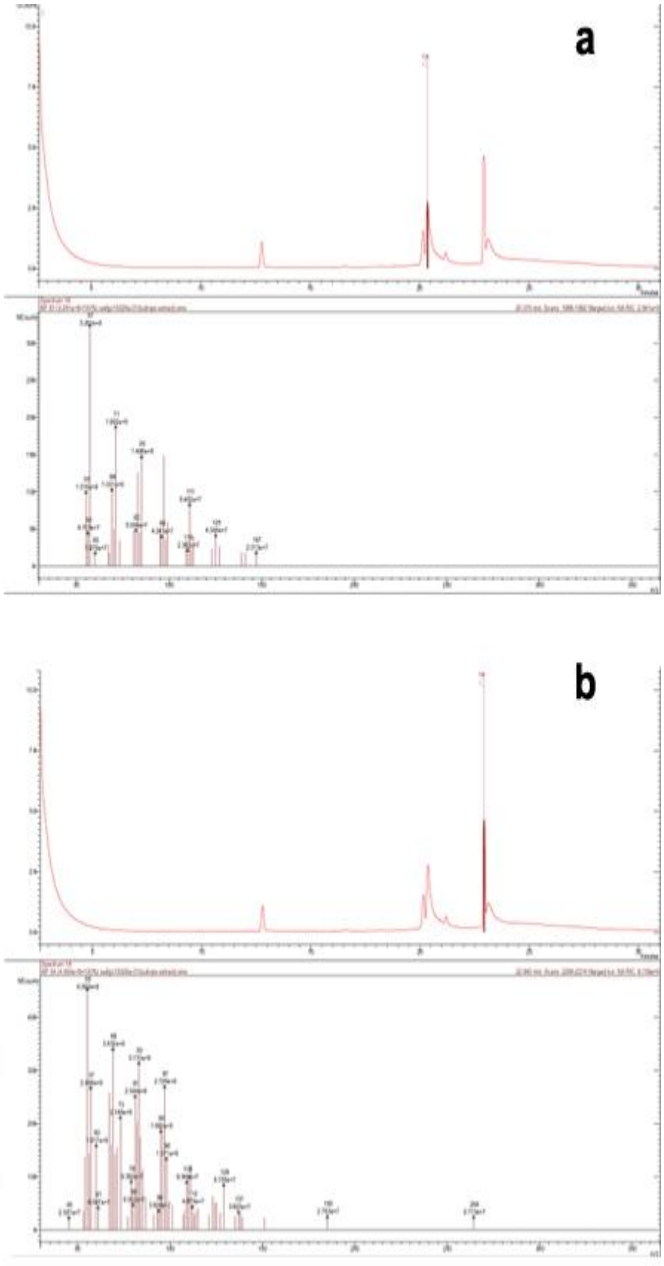


Fig 4. GC MS showing retention time and corresponding Mass spectrum. a) Peak of retention time of 20.375min and corresponding MS pattern. b) Peak of retention time 22.490min and corresponding MS pattern.

Butylated hydroxytoluene (BHT) is an antioxidant with antibiotic and antiviral properties. It acts on the lipid layer of the cells. *In vitro* assays showed that the addition of these antioxidants to the culture media at concentrations lower or equal to that used in nutrition inhibit or decrease the growth of certain microorganisms.[27]. Butylated hydroxytoluene (BHT) is one of the synthetic antioxidant commonly used as food additives. However, four freshwater phytoplanktons, including a green alga (*Botryococcus braunii*) and three cyanobacteria [*Cylindrospermopsis raciborskii* , *Microcystis aeruginosa* and *Oscillatoria* sp.] are capable of producing

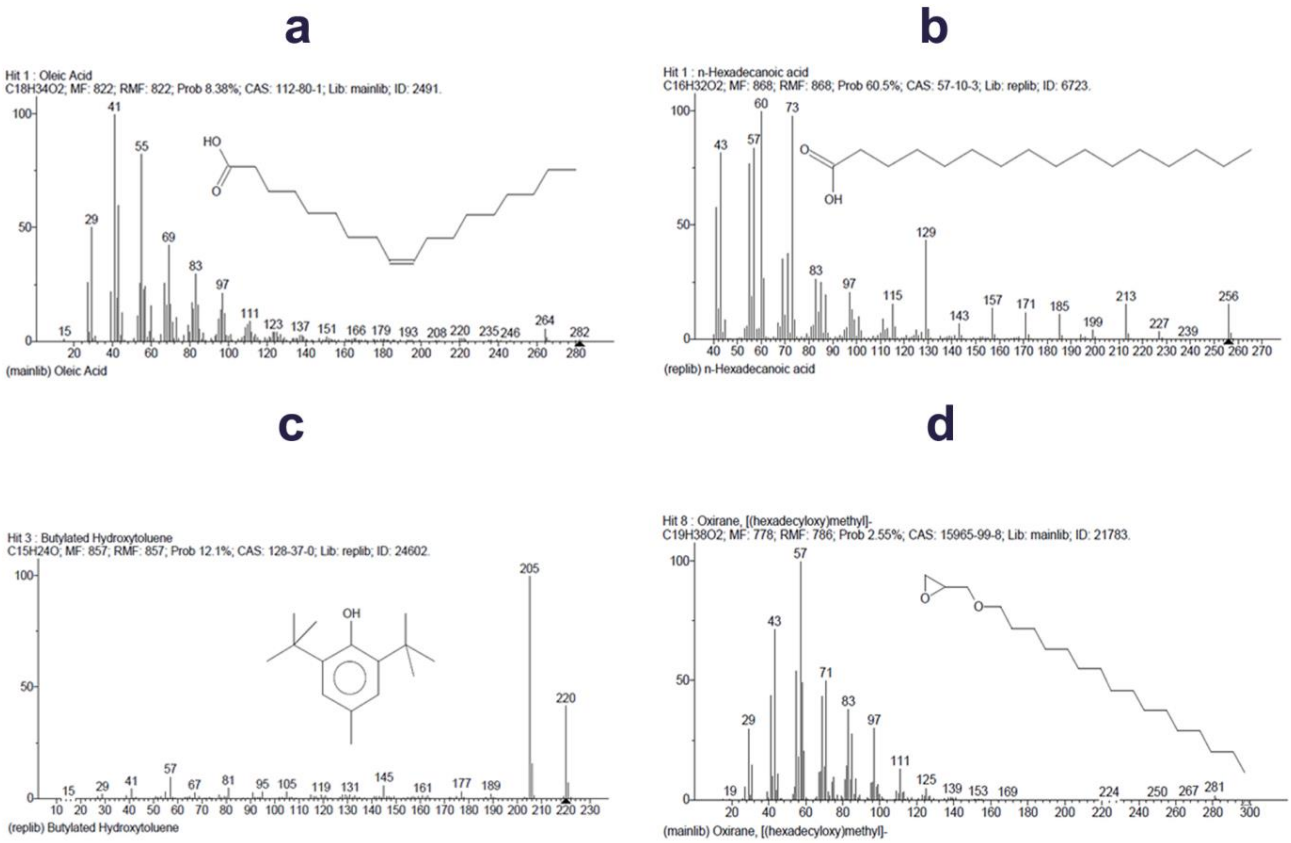


Fig 5. Mass spectrum of the compounds as identified from NIST library [15].

this compound. Gas chromatography/mass spectroscopy (GC/MS) analysis of the purified fractions revealed the similarity of the active compound to synthetic BHT [28].

Gram positive organisms are more susceptible to the above compounds than Gram negative organisms [29, 30, 31]. Consistent with the earlier reports, zone of inhibition was larger with Gram positive organisms. The interaction of the antioxidants with the essential oils of the extract, accounts for the antibacterial potential of the *Luprops* extract. The large zone sizes are explained by the combined effect of BHT and oxirane with fatty acids like oleic acid and palmitic acid that have a profound effect on the antibacterial activity [27].

From the above results, it can be concluded that a considerable proportion of the bioactive fraction of Luprops are plant-derived. The composition of the plant source determines the chemical composition of most of the insect exudates. Many studies proposed the presence of BHT and palmitic acid in the phospholipid component of natural rubber latex [32]. A study on the lipid composition of rubber latex proposes the presence of about 11% of oleic acid [33]. Thus the present study sheds new light on the potential of insect pests as a source of antimicrobial agents, which in fact, serves as a blessing in disguise.

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