Chemical Profiling and Biological Activity of leaf Essential Oil of *Eugenia rottleriana* Wt. & Arn.

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Abstract:

The pantropical genus *Eugenia* belonging to the family Myrtaceae has about 1,000 species distributed mainly in the New World and 13 species of *Eugenia* reported from Peninsular India. *Eugenia rottleriana* is a small tree and endemic to South Western Ghats and found in evergreen forests at low altitude. The essential oils from several species of this genus have medicinal properties. Many medicinal plants are frequently used in traditional medicine because of their essential oils have been known to possess biological activity and used for the treatment of microbial infections due to the presence of certain compounds. In the present study we focused on GC/MS chemical profiling, antimicrobial and anti-malarial screening of leaf essential oil of *Eugenia rottleriana*. In GC/MS analysis, *E. rottleriana* showed six major and 25 minor compounds. The antimicrobial potential of the essential oil were evaluated by disk diffusion method against selected microorganisms such as *Bacillus cereus, Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Candida albicans* and *Candida glabrata*. The oil at high concentration (200µg/ml) showed high inhibitory activity against all the test microorganisms and higher zone of inhibition recorded against *Candida glabrata.* In addition, the different concentrations of essential oil were assayed for larvicidal activity against *Anopheles stephensi*. All the concentrations showed larvicidal activity after 24hrs exposure of larvae. The current study found that the *E. rottleriana* essential oil has a commercial potential for antimicrobial and anti-malarial products.

Keywords: ***Anopheles stephensi, Candida glabrata,*** **Essential oil, GC/MS, Larvicidal activity**

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Introduction:

Globally 41 species of *Anopheles* species are documented to be the main vectors of malaria infection (Hay *et al*., 2020), with two of the major tropical species being *Anopheles gambiae* (prevalent in Africa) and *Anopheles stephensi* (*An. stephensi*) (prevalent in Asia) (Wells and Andrew, 2015). Malaria is a one of the major life-threatening tropical disease caused by mosquito. Chloroquine resistance has been reported in most of the malaria-endemic regions, while resistance to artemisinin has been detected in Cambodia, Thailand, Myanmar, and Vietnam. These facts highlight the need for the discovery of new drugs and new strategies for combating the disease (Moore and Lanier, 1961).

Historically natural products, such as aromatic plants, have been a source of new drugs and pharmacological alternatives for treating several diseases. The bioactive compounds responsible for pharmacological activity range from alkaloids to phenolics and essential oils. Essential oils are complex mixtures of terpenes and to a lesser extent of non-terpenoid compounds. Extensive research has been done on chemical characterization and determination of the antibacterial, antifungal, antileishmanial, and antiplasmodial activities of the essential oils extracted from a wide variety of plants (Oji and Shafaghat, 2012; Marwah 2007; Waikedre *et al*., 2012; Ahmed *et al*., 2011).

Aromatic plants are frequently used in traditional medicine because of their essential oils and volatile constituents. In last few years, there has been an increase in the use of aromatic medicinal plants and their essential oils in scientific research and industrial applications including nutritional, pharmaceutical, and cosmetic uses (Tsai *et al*., 2011; Zuzarte *et al*., 2009).

We set out to investigate the biological activities of essential oils extracted from *Eugenia rottleriana*. The pantropical genus *Eugenia* belonging to the family Myrtaceae has about 1,000 species distributed mainly in the New World (Mabberley, 1990). This genus is taxonomically related to the genus *Syzygium* and *Schimd* differentiated those convincingly providing adequate morphological and anatomical characters (Schimd, 1972). There are 13 species of *Eugenia* reported from Peninsular India. *Eugenia rottleriana* Wight & Arn. is a

small tree with leaves linear to oblong lanceolate, long acuminate at apex, endemic to South Western Ghats and found in evergreen forests at low altitude (Nayar, 1996). Hence, the present study was focused on GC/MS chemical profiling, antimicrobial and anti-malarial activity of leaf essential oil of *Eugenia rottleriana* Wt. & Arn. (Myrtaceae).

Materials and Methods:

Collection of plant material and Preparation of Essential Oil

The fresh mature leaves of *E. rottleriana* (1 kg) were collected from Sathuragiri hills, Part of Western Ghats, Tamil Nadu, India (9°44'19.9"N 77°39'09.4"E). Fresh leaves (200g) of *E. rottleriana* were cut into small pieces and transferred into 3 litre round- bottomed flask to which were added 2000 ml of distilled water. The mixture was distilled for four hours using a Clevenger apparatus at 80°C. The collected oil was stored in a refrigerator with temperature 4°C in a sealed vial prior to analysis (Anjana *et al.*, 2020; Manikandan *et al.*, 2020; Ramasubbu *et al*., 2020; Manikandan *et al*., 2021).

GC/MS Chemical Profiling

E. rottleriana essential oil was analyzed in a Shimadzu Gas Chromatograph (Model GC.MS-QP2010 Ultra) coupled with a non-polar Rtx-MS capillary column 30meter in length, diameter (0.25mm) and thickness (0.25µl) using a mass spectrometer detector and helium were used as a mobile phase (carrier gas) and an injection volume of 1 µl. The instruments were connected to a computer coupled with special software (Shimadzu Lab solutions GCMS solution) that was used to analyze the data. The percent of each compound was based on the peak area divided by the total area of component peaks. The temperature range were from 50-300°C, with a temperature programme rate of 10°C/min, starting at three minutes and finishing at thirty minutes. The pressure applied in this experiment was 100kpa with a total flow of 50ml/min and 1.69ml/min of column flow. The injection, ion source and the interface temperatures were 300°C, 200°C and 250°C respectively.

Identification of bioactive components

The identity of the components in the extracts was assigned by the comparison of their retention time, area percentage and mass spectra fragmentation patterns with those stored on the computer library and also with published literature. NIST08s.LIB, WILEY8. LIB library sources were used for matching the identified components from the essential oil.

Determination of antimicrobial activities

The antimicrobial potential test was carried out on essential oil of *E. rottleriana* using the disk diffusion method against selected microorganisms Table 1 (Packiyalakshmi *et al*., 2017, Manikandan & Ramasubbu, 2020 & 2021). The 6 mm diameter discs were prepared 3 different concentrations viz. 100µg/ml, 150µg/ml and 200µg/ml of the essential oil. Standard antibiotic, Ampicillin was (10 mcg) is a positive control and the loaded plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 hours for fungal pathogens. The diameter of the inhibition zone (mm) was measured by zone scale and the activity index was also calculated for triplicates of each experiment.

Collection of *Anopheles stephensi* larvae

The larvae of *Anopheles stephensi* were collected from the ICMR-Vector Control Research Centre Field Unit, Madurai, Tamilnadu, India. They were kept in a tray containing water in which the larvae had grown (culture medium) at laboratory conditions (29°C). All the instar larvae were collected and the 4th instar larvae were used in this study. Each sample larva will be individually mounted in Berlese's medium on a microscope slide and identified to species extent by the morphological characters.

Larvicidal bioassay

Thirty (30) larvae per plate were taken for the test and treated essential oil of *E. rottleriana* at various concentrations (50µl, 100µl and 150µl). Corresponding control Chloroquine 250 mg/ml were maintained. The larval mortality of fourth instar of *Anopheles stephensi* was

observed. The number of larvae surviving at the end of 24 and 48 hours were recorded. The percentage of mortality was calculated using this formula.

Percentage of mortality = No. of larva dead/Total No. of larvae×100

Results and Discussions:

GC/MS Chemical Profiling

The essential oils from several species of *Eugenia* have been the subject of previous studies (Apel *et al*., 2005; Pino *et al*., 2005; Vila *et al*., 2004; Apel *et al*., 2004; Martins *et al*., 1999; Weyerstahl *et al*., 1988). It is reported that the leaf essential oils from the species *E. brasiliensis*, *E. nudticostata*, *E. sulcata* and *E. xiriricana* were rich in monoterpene hydrocarbons. α-Pinene and limonene have been reported as the major constituents in the leaf oil of *E.speciosa* from South Brazil (Apel *et al*., 2004a), while α-pinene and α-terpineol have been reported as the major constituents from the same species in an earlier study (Alves *et al*., 2000).

The GC/MS identification of the chemical constituents was based on comparison of their mass spectra with NIST and WILEY libraries. The essential oil of the leaves of ***E.*** *rottleriana* by GC/MS analysis clearly showed the presence of 31 compounds (see Table 2). The GC/MS analysis showed 06 major and 25 minor compounds. The major compounds were caryophyllene (34.49%), isocaryophyllene (34.49%), D-Limonene (14.72%), caryophyllene oxide (10.28%), Z-3-Hexadecen-7-yne (10.28%) and Humulene (9.54%). The 25 minor compounds such as Alpha-Terpineol (4.84%), 4-Carene (4.84%), Cis-Calamenene (3.65%), (E)-Calamene (3.65%), trans-calamenene (3.65%), Caryophylla-3(4),8-dien-5-ol (3.19%), Adamantane (3.19%), 2-Naphthalenol, decahydro- (3.19%), Copaene (2.74%), Alpha.-Copaene (2.74%), Cadina-1,4-diene (2.43%), (-)-Alpha-Cedrene (2.43%), Alpha-Pinene Oxide (2.26%), Limonene oxide, cis- (2.26%), Farnesyl acetate (2.08%), Nerolidol (2.08%), cis-p-mentha-1(7),8-dien-2-ol (1.88%), Bicyclo[6.1.0]nonane, 9-(1-methylethylidene) (1.88%), Alloaromadendrene (1.48%), (-)-Spathulenol (1.48%), .Alpha.-Longipinene (1.46%), Linalool

(1.36%), 2-Carene (1.13%), Terpinolene (1.13%) and 3-Carene (1.13%), were also reported from essential oil leaves of *E. rottleriana.*

The primary components of the oil of the fresh leaves were caryophyllene, a bicyclic sesquiterpene followed by isocaryophyllene, is the Sesquiterpenoids which occupied 34.49% respectively in the total composition of the fresh leaf essential oil. Sesquiterpenes were the predominant compound group of the fresh leaf essential oil with 31 identified compounds (see Table 2).

Gopan Raj *et al*. (2007) reported that, the *Eugenia rottleriana* was analyzed by GC and GC/MS. Twenty-eight compounds constituting 99.1% of the analyzed sample were identified. Caryophyllene (34.49%), Bicyclo [7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]- (34.49%), D-Limonene (14.72%), Caryophyllene oxide (10.28%), Z-3-Hexadecen-7-yne (10.28%) and Humulene (9.54%), were reported as 3 major compounds in the essential oil of the leaves of *E. rottleriana*.

The essential oils from several species of this genus have been the subject of previous studies. It is reported that the leaf oils from the species *E. brasiliensis, E. nudticostata, E. sulcata* and *E. xiriricana* were rich in monoterpene hydrocarbons. α−Pinene and limonene have been reported as the major constituents in the leaf oil of *E. speciosa* from South Brazil, while α−pinene and α−terpineol have been reported as the major constituents from the same species (Gopan *et al*., 2007).

Antimicrobial activity

In the present study, antimicrobial potential of essential oil of *E. rottleriana* against selected microorganisms were comparable to the standard antibiotic ampicillin (10 mcg) by disk diffusion method. Among these different concentration (100µg/ml, 150µg/ml and 200µg/ml) of the essential oil of *E. rottleriana* exhibited antimicrobial activity against all the selected microorganisms. Among these three concentrations (100µg/ml, 150µg/ml and 200µg/ml) of the essential oil of *E. rottleriana* higher concentration (200µg/ml) showed higher inhibitory activity against all the tested microorganisms. The essential oil of *E. rottleriana* (200 µg/ml)

showed higher zone of inhibition against *Candida glabrata* followed by, *Candida albicans, Klebsiella pneumonia, Staphylococcus aureus, Bacillus cereus and Escherichia coli* (Figure 1).

Previously *E. rottleriana* was analyzed by GC and GC/MS. Twenty-eight compounds constituting 99.1% of the analyzed sample were identified. The oil was characterized by the predominance of monoterpenes (61.2%) with limonene (50.3%) as the major constituent. α−Humulene (13.3 %), β−caryophyllene (8.8%) and linalool (3.2%) were the other major components. The oil displayed significant antibacterial activity against *Bacillus cereus* and moderate activity against *Serratia marcescens* and *Escherichia coli* (Gopalan *et al*., 2007).

In the present study, limonene found as a major compound. This compound has antimicrobial activity. Previously limonene demonstrated prominent antibiotic effects vs *S. aureus* and *Pseudomonas aeruginosa* (Onawunmi, Yisak, and Ogunlana, 1984). Recently, concentrations of 400 µg/mL inhibited biofilm formation of the pathogen *Streptococcus pyogenes* SF370 and *S. nutans*, which produces dental caries, down regulating various genes mediating surface-associated proteins (Subramenium *et al*., 2015).

Larvicidal Bioassay

The larval mortality of *An. stephensi* after the treatment of leaf essential oil of *E. rottleriana* was observed comparable to the standard antibiotic Chloroquine 250 mg/ml. The different concentrations of essential oil were assayed for larvicidal activity against *Anopheles stephensi*. All the concentrations showed larvicidal activity after 24hrs exposure of larvae. Figure 2 showed mortality at different concentrations for 24 hours of inhibition against *An. stephensi*.

Carene demonstrated larvicidal activity against *Anopholes stephensi*, vector of malaria (LC50 [lethal concentration] 16.37 µg/mL), *A. aegypti*, vector of dengue (LC50 17.91 µg/mL), and *C. quinquefaciatus*, vector of filariasis (LC50 19.5 µg/mL) (Govindarajan et al., 2016; Govindarajan et al., 2016a). In the present study we found 1.13% of 2-carene in leaf essential

oil of *E. rottleriana* and 31 other compounds. According to the literature most of these compounds have potential activity against mosquito larvae.

A large number of plant extracts have been reported to have mosquitocidal or repellent activities against mosquito vectors, but very few plant products have shown practical utility for mosquito control (Sun *et al*., 2006). Phytochemicals derived from plant sources act as larvicides, insect growth regulators, repellent, ovipositor attractant and have different activities which have been observed by many researchers (Venketachalam and Jebasan, 2010). Triterpenoids are generally credited with mosquito larvicidal activities (Gbolade, 2000). In the present study, the results clearly indicate that the leaf essential oil of *E. rottleriana* exhibited potent lethality against the *An. stephensi* mosquito species tested.

Conclusions:

Six major and 25 minor compounds were identified through GC/MS analysis of *E. rottleriana*. The major compounds were caryophyllene (34.49%), isocaryophyllene (34.49%), D-Limonene (14.72%), caryophyllene oxide (10.28%), Z-3-Hexadecen-7-yne (10.28%) and Humulene (9.54%). The essential oil of *E. rottleriana* showed good inhibitory activity against Gram negative, gram positive and fungi microorganisms with *Candida glabrata* being the most sensitive. *E. rottleriana* essential oil showed potent larvicidal activity against *Anopheles stephensi* with mortality rate expressed as percentage and all the concentration showed larvicidal activity after 24hrs exposure of larvae. The identified bioactive compounds effectively worked against pathogens and mosquito larva. We are now exploring these oils for use in antibacterial soaps and anti-mosquito sprays.

Declaration of competing interest

The authors declare no conflict of interest

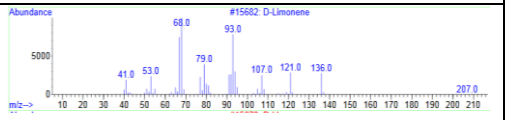
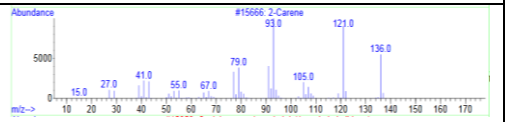
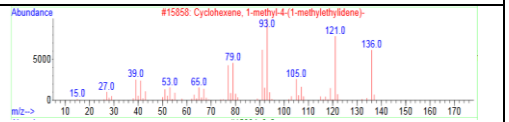
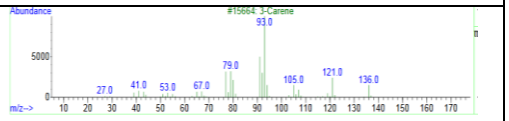
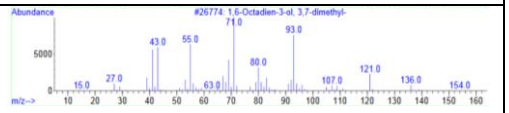
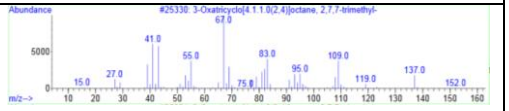
Acknowledgments

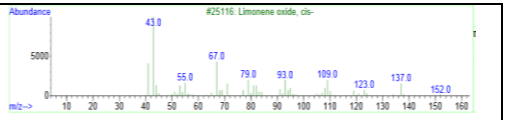
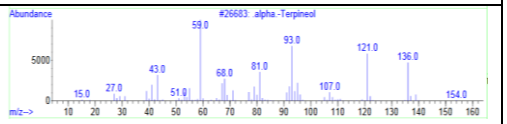
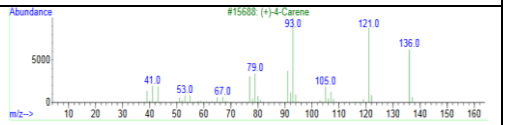
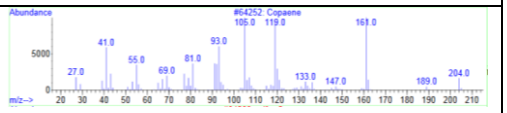
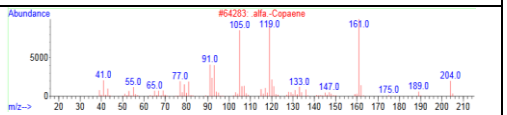
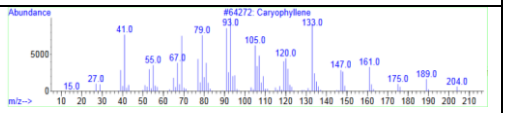
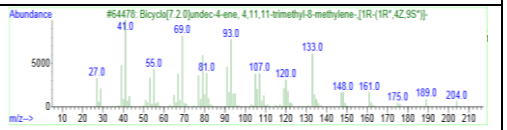
The authors are thankful to the ICMR-Vector Control Research Centre Field Unit, Madurai, TN, India for providing mosquito larva to completing this research work.

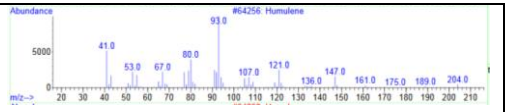
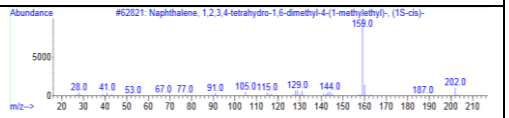
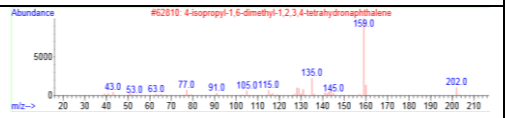
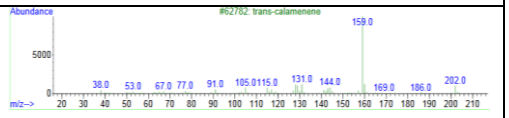
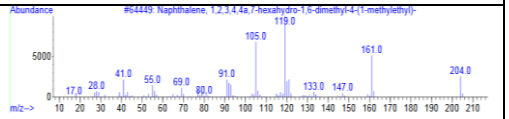
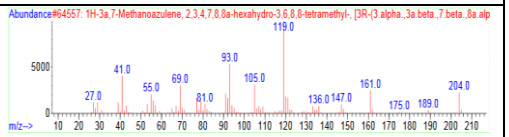
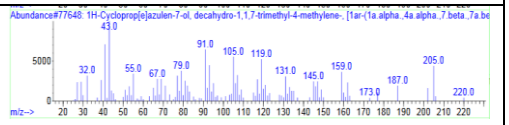
Table 1. Standard microbial strains used for screening of antimicrobial activities of *Eugenia rottleriana*

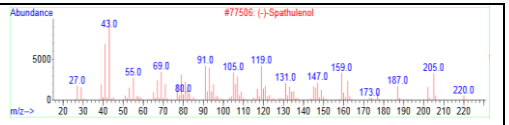
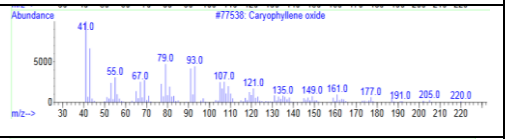
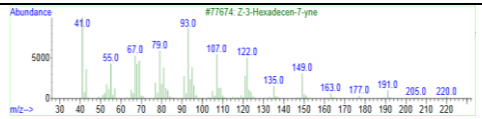
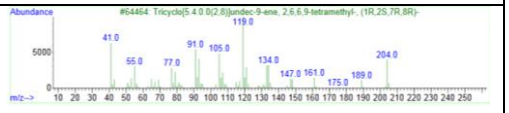
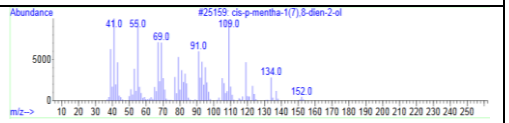
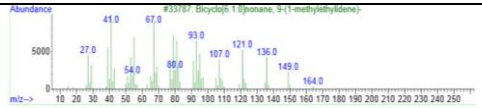
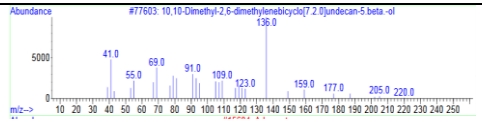
S.No	Name of the microbial strain	Grams nature	Strain No.
1	*Bacillus cereus*	Gram-positive bacteria	MTCC 430
2	*Staphylococcus aureus*	Gram-positive bacteria	MTCC 96
3	*Klebsiella pneumonia*	Gram-negative bacteria	MTCC 4030
4	*Escherichia coli*	Gram-negative bacteria	MTCC 443
5	*Candida albicans*	Fungi	MTCC 854
6	*Candida glabrata*	Fungi	MTCC 6507

Table 2. Bioactive compounds identified from leaf essential oil of *E. rottleriana*

S.No.	Name of the Compound	Compound Nature	Retention Time	Concentration %	Mass Spectrum
1.	D-Limonene	Cyclic monoterpene	2.36	14.72	
2.	2-Carene	Bicyclic monoterpene	2.749	1.13	
3.	Terpinolene	Monoterpenes	2.749	1.13	
4.	3-Carene	Bicyclic monoterpene	2.749	1.13	
5.	Linalool	Monoterpenoid	2.787	1.36	
6.	Alpha-Pinene Oxide	Bicyclic monoterpenoids	2.872	2.26	

7.	Limonene oxide, cis-	Oxepanes	2.872	2.26	
8.	Alpha-Terpineol	Prenol lipids	3.609	4.84	
9.	4-Carene	Prenol lipids	3.609	4.84	
10.	Copaene	Bicyclic monoterpene	5.548	2.74	
11.	Alpha.-Copaene	Sesquiterpenoids	5.548	2.74	
12.	Caryophyllene	Bicyclic sesquiterpene	6.077	34.49	
13.	isocaryophyllene	Sesquiterpenoids	6.077	34.49	

14.	Humulene	Sesquiterpenoids	6.455	9.54	
15.	Cis-Calamenene	Sesquiterpenoids	7.193	3.65	
16.	(E)-Calamene	Sesquiterpenoids	7.193	3.65	
17.	trans-calamenene	Sesquiterpenoids	7.193	3.65	
18.	Cadina-1,4-diene	Sesquiterpenoids	7.306	2.43	
19.	(-)-Alpha-Cedrene	Sesquiterpenoids	7.306	2.43	
20.	Alloaromadendrene	Sesquiterpenoids	7.855	1.48	

21.	(-)-Spathulenol	Sesquiterpenoids	7.855	1.48	
22.	Caryophyllene oxide	Terpenoids	7.930	10.28	
23.	Z-3-Hexadecen-7-yne	Alkanes	7.930	10.28	
24.	.Alpha.-Longipinene	Sesquiterpene	8.394	1.46	
25.	cis-p-mentha-1(7),8-dien-2-ol	Monoterpenoids	8.469	1.88	
26.	Bicyclo[6.1.0]nonane, 9-(1-methylethylidene)	Sesquiterpenoids	8.469	1.88	
27.	Caryophylla-3(4),8-dien-5-ol	Sesquiterpenoids	8.507	3.19	

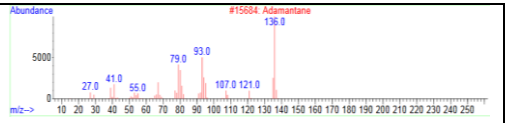
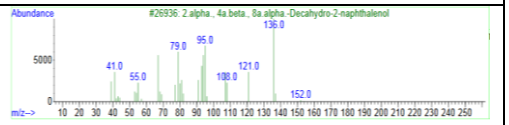
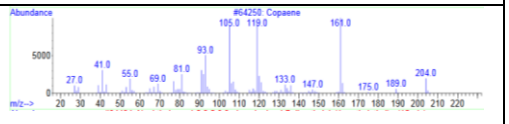
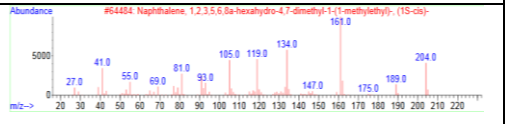
28.	Adamantane	Polycyclic aromatic hydrocarbon	8.507	3.19	
29.	2-Naphthalenol, decahydro-	Terpenoids	8.507	3.19	
30.	Farnesyl acetate	Sesquiterpenoids	9.301	2.08	
31.	Nerolidol	Sesquiterpenoids	9.301	2.08	

Figure 1. GC/MS Chromatogram of leaf essential oil of *E. rottleriana*

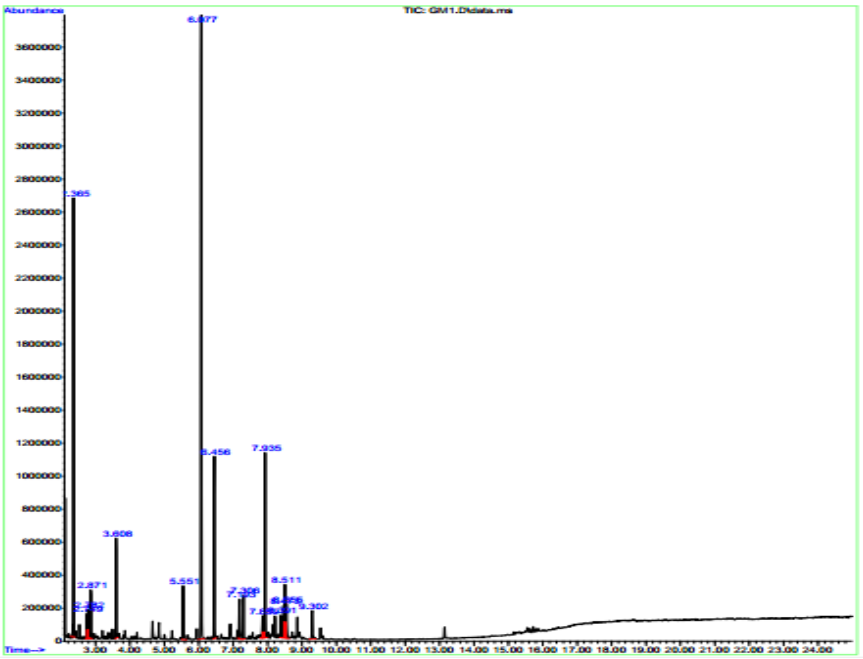
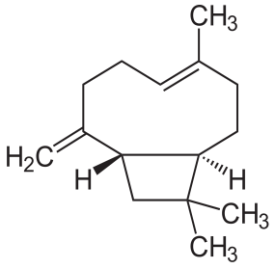
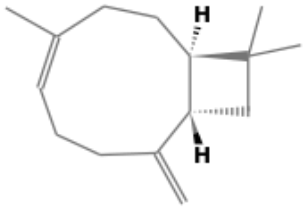


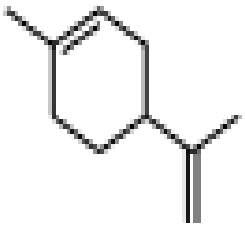
Figure 2. Structure of the major components of leaf essential oil of *Eugenia rottleriana*



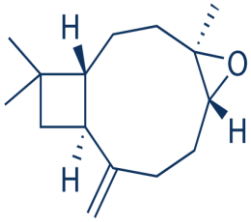
Caryophyllene



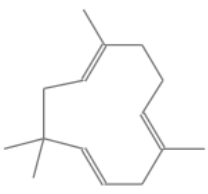
Isocaryophyllene



D-Limonene



Caryophyllene oxide



Humulene

Z-3-Hexadecen-7-yne

Figure 3. Antimicrobial activity of essential oil leaves of *E. rottleriana* against selected human pathogenic microorganisms

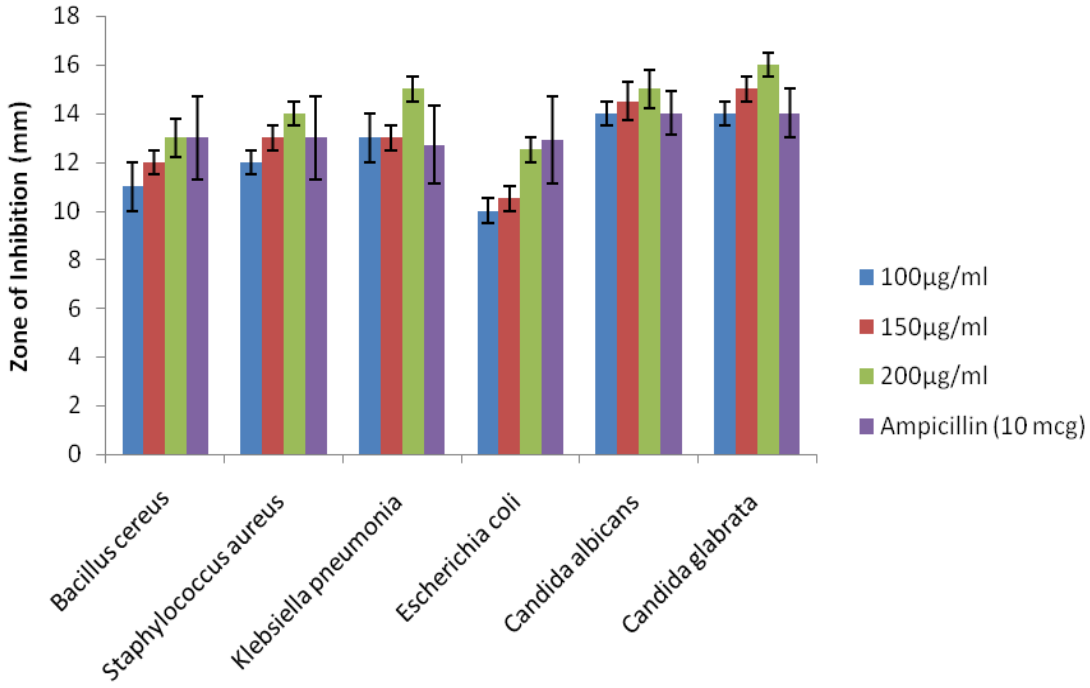
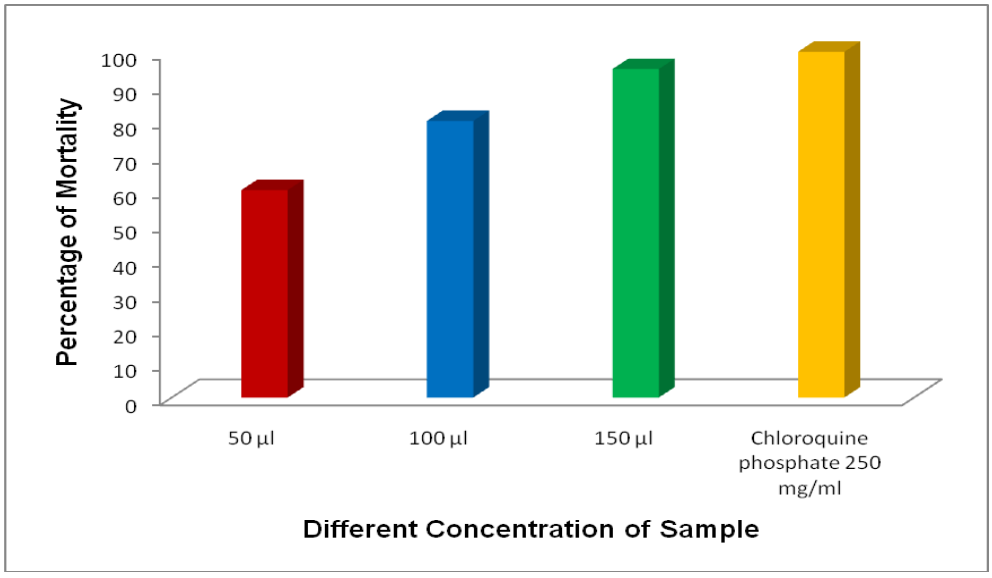


Figure 4. Leaf essential oil of *E. rottleriana* Mortality percentage of fourth instar larvae of *An. stephensi* at different concentration



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