


Chemical composition, cytotoxicity and anti-mycobacterium activities of *Clausena anisata* essential oils

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Background: *Clausena anisata* (Willd) Hook has demonstrated several medicinal properties against certain bacterial infections, including tuberculosis. However, scientific validation is imperative to verify its folkloric usage against tuberculosis.

Aim: This study aimed at determining the chemical composition, toxicity and anti-tubercular potentials of the oils extracted from this plant.

Setting: Plant material collection, essential oils extraction and antimycobacterial activity were performed at the University of Fort Hare, Alice, South Africa. This article and selected technical aspect was constructed at the Biomedical Research Centre, Forestry Research Institute of Nigeria.

Methods: Comparative chemical composition of hydrodistilled (HD) and solvent-free microwaved essential (SFME) oils from *C. anisata* was analysed by gas chromatography coupled with mass spectroscopy (GC-MS). *In vitro* minimum inhibitory concentration (MIC) assay was also carried out on the oils against a strain of *Mycobacterium tuberculosis*. The cytotoxicity assay of the oils was also assessed by using human dermal fibroblast (MRHF) cell lines.

Results: Gas chromatography-mass spectroscopy analysis of the essential oils revealed 51 distinct compounds with methyl eugenol and estragole in abundance. The IC₅₀ of volatile oils from SFME and HD methods against the MRHF cell line were 1 216 µg/mL and 383 µg/mL, respectively. On the other hand, both oils and ethambutol (standard drug) showed no activity against the strain of *M. tuberculosis*, but rifampicin suppressed the growth of the organism at 0.25 µg/mL.

Conclusion: This study showed that *C. anisata* is safe for consumption, and further studies of combinative potent but non-toxic oils engaging hydrodistillation method are strongly recommended for enhanced effect against *M. tuberculosis*.

Keywords: solvent-free microwave extraction; tuberculosis; hydrodistillation; medicinal plant; clausena specie; volatile oil.

Introduction

Mycobacterium tuberculosis is the causative pathogen of tuberculosis (TB) that most often affects the lungs (WHO 2018). Tuberculosis remains a significant public health issue in low- and middle-income countries, and as reported by World Health Organization (WHO 2017) it is the leading cause of deaths as a single infectious disease, ranking above human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS). Tuberculosis is responsible for approximately 2 million deaths annually (Ibekwe & Ameh 2015). The WHO (2017) reported 6.3 m new cases of TB amongst HIV-negative people in 2016. According to WHO (2018), about one-third of the world's population has latent TB, which means people have been infected by *M. tuberculosis* but are not yet ill with the disease and cannot transmit it to another person. The WHO African region accounted for 25% of the total number of incident cases globally. In the past two decades, the WHO has listed Nigeria as one of the countries with a high burden of TB (WHO 2015). It has also been estimated that about 80% of South African population is infected with TB, with 88% highest prevalence of latent TB amongst the age group of 30–39 years living in the rural settlements (TB Statistics 2015).

The primary treatment for *M. tuberculosis* disease is chemotherapy (CDC 2016). The recent increase in the widespread existence of extensively drug-resistant tuberculosis (XDR-TB) especially in the developing nations emphasises the need for the development of new drugs to treat this infection. Such new anti-tubercular agents should have novel modes of action

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and full activity against *M. tuberculosis* (WHO 2012). And now, during the time of coronavirus disease 2019 (COVID-19) outbreak, WHO is imploring governments to ensure TB services are maintained. Tuberculosis, being a bacterial disease, shares similar patterns of respiratory symptoms with COVID-19 (a viral disease), and a bit of imitation from the way it incubates in asymptomatic infected persons to the way it sickens symptomatic persons. However, a COVID-19-infected person who is yet asymptomatic may be able to transmit the infection (CDC 2020) as different from the case of TB.

For the fact that chemical agents are engaged in the primary treatment of TB, rifampicin, which is a nucleic acid synthesis inhibitor and believed to inhibit bacterial deoxyribonucleic acid-dependent ribonucleic acid polymerase, has been used as a first-line drug in combination with other therapies for the treatment of TB infections (Shehzad et al. 2013). Ethambutol (EMB), a cell wall inhibitor, is another first-line drug used in combination with isoniazid, rifampicin and pyrazinamide for preventing the emergence of drug-resistant mycobacterium. This drug interferes with the cell wall of *M. tuberculosis* through a synthetic mechanism, thereby inhibiting arabinosyl-transferase (embB), an enzyme involved in cell wall biosynthesis (Teleuti et al. 1997). However, new chemical compounds that might play an important role in the chemotherapy of TB are sourced from natural products and/or their semi-synthetic derivatives (Payan et al. 2009). Therefore, it is important to search for new anti-tubercular agents, preferably those that can be readily produced from medicinal plants.

Clausena anisata (Willd) Hook. f. ex Benth is a small tree about 10 m in height belonging to the family Rutaceae, and known by local name 'olmaa'ii' in Ethiopia (Afanoromo language), in South Africa as 'Isifudu' (Zulu) and 'umNukelambiba' (Swazi). It is also known as 'Perdepis' (Afrikaans) because the crushed leaves have a characteristic unpleasant horse's urine smell. *Clausena anisata* is abundant in forests and forest margins, riverine thickets and bushveld, from Western Cape up the eastern coast of KwaZulu-Natal, Mpumalanga, eastern Zimbabwe, Mozambique, northwards to Ethiopia and Sudan and westwards to Sierra Leone where it plays a major role in the ecosystem as regulating and supporting services (Van Wyk & Gericke 2000; Van Wyk, Van Oudtshoorn & Gericke 2002). The main stem is smooth, grey to light brown, and the leaves alternate with each leaf having 4–10 pairs of leaflets. The flowers have white petals with numerous yellow stamens. The fruits are berries, initially green, then reddish-black when fully ripe, providing a source of nourishment to many bird and insect species. The wood is yellow, hard and strong and is used for hut-building and the making of knobkerries (Van Wyk et al. 2002).

South African traditional healers have been engaging in the use of *C. anisata* leaves, bark and root to treat a variety of ailments including cough and TB (Van Wyk & Gericke 2000; Van Wyk et al. 2002). The various morphological parts of the plant have been identified to be effective remedies against

worm infections, respiratory ailments, heart disorders, hypertension, malaria fever, rheumatism, insanity, convulsion and other inflammatory conditions (Hamza, Van den Bout-Van den Beukel & Matee 2006; Hutchings et al. 1996). In many African countries, especially South Africa, many people still use phytomedicine as an alternative or to supplement western drugs (Grierson & Afolayan 1999; Lawal, Grierson & Afolayan 2014). The inhibitory properties of medicinal plants against *M. tuberculosis* have been reported by many researchers both in South Africa and in other countries (Payan et al. 2009), but there is a dearth of information on the inhibitory properties of *C. anisata*, which is popular ethnomedicinally for the treatment of TB, especially in South Africa.

The aim of this study, therefore, was to evaluate *C. anisata* essential oils for their antimycobacterium and cytotoxicity activities.

Materials and methods

Plant collection and identification

Identification of the collected plant specimens was done by Prof. D.S. Grierson of the Department of Botany at the University of Fort Hare, South Africa. Voucher specimens with the voucher number (Lawmed 01) were deposited in the Giffen Herbarium of the University of Fort Hare.

Solvent-free microwave extraction

The solvent-free microwave extraction was achieved by using the Milestone Dry Distillation (DIST) (2004) apparatus. The multimode reactor has a twin magnetron (2800 watts [W], 2450 megahertz [MHz]) with a maximum delivered power of 500 W in 5 W increments. Rotating microwave diffusers ensure homogeneous microwave distribution throughout the plasma-coated polytetrafluoroethylene (PTFE) cavity. The attached infrared radiation (IR) sensor was used to regulate the temperature. Constant conditions of temperature and water were guaranteed by the reflux of condensed water, which was achieved by a circulating cooling system at 5 °C. An amount of 250 g of *C. anisata* fresh leaves was placed into the reactor without addition of water or any solvent. The exhaustive extraction of the essential oil was obtained in 30 min.

Hydrodistillation

In brief, 250 g of the fresh leaves were hydrodistilled (HD) for 3 h in an all-glass Clevenger apparatus in accordance with the description of the British Pharmacopoeia (1980). The heat was supplied to the heating mantle (100 °C), and the essential oil was extracted with 5 L of distilled water for 3 h. The essential oil was collected and analysed without delay.

Gas chromatography–mass spectroscopy analysis

The chemical composition profiling of essential oils extracted through hydrodistillation (HD) and solvent-free microwaved essential (SFME) was achieved through gas chromatography–

mass spectroscopy (GC–MS) analysis. This was carried out by using an Agilent (6890N) GC with chlortetracycline (CTC) Combi Pal Autosampler and Agilent (5975B) MS. Identification of the chemical components of the essential oils was accomplished by corresponding their mass spectra and retention indices with those of the Wiley 275 library (Okoh, Sadimenko & Afolayan 2010; Omoruyi, Anthony & Bradley 2014). The column type was Zebron (ZB) 274305, Semi volatiles (30 m, 0.25 mm identification (ID), 0.25 μ m film thicknesses). The spectrograms of each identified compound were determined by the integration of the peak areas. The initial temperature was 280 °C; pressure, 96.9 kN/m²; split ratio, 20:1; split flow, 19/9 mL/min; total flow, 23.7 mL/min; column flow, 1 mL/min; nominal initial pressure, 52.4 kN/m²; average velocity, 36 cm/s; gas type, Helium; MS mode, EI+; scanning mass range, 35 m/z – 600 mass per charge number (m/z); solvent delay, 4.65 min.

Cytotoxicity assays

Cell culture conditions

The adherent human dermal fibroblast (MRHF) cell line was routinely maintained in 10-cm culture dishes without antibiotics in Dulbecco's Modified Eagle Medium (DMEM) cell culture medium containing 4500 mg/L glucose, 4 mM glutamine and sodium pyruvate (HyClone Laboratories, Inc., South Logan, Utah, United States), and 10% foetal bovine serum (Gibco, Grand Island, New York, United States) in a humidified incubator and 5% CO₂ at 37 °C.

Sample preparation

Volatile oils extracted by using SFME and hydrodistillation methods were reconstituted in absolute ethanol as stock concentrations of 100 mg/mL. Stock concentrations were stored at –80 °C to prevent evaporation.

Screening protocol

Working concentrations (500 μ g/mL) of the volatile oils were prepared from the stock concentrations in complete medium, which were serially diluted to concentrations between 1.95 μ g/mL and 250 μ g/mL. Absolute ethanol (0.5%, volume per volume [v/v]) and melphalan (50 μ M and 100 μ M) were used as negative (vehicle) and positive controls, respectively.

Cells were seeded into a 96-well microtitre plate at a cell density of 30 000 cells/mL, by using 200 μ L of the cell suspension per well (6000 cells/well). The microtitre plate was incubated for 24 h in a humidified incubator and 5% CO₂ at 37 °C, prior to the addition of the volatile oils. Spent medium was aspirated from the wells and replaced with the appropriate treatment. The plate was returned to the incubator, and the cells were incubated for 24 h.

Methylthiazol tetrazolium assay

After 24 h, treatments were removed via aspiration, 200 μ L MTT 0.5 mg/mL (Holst-Hansen & Brünner 1998) dissolved in complete medium, added to each well and

incubated for 3 h at 37 °C. Methylthiazol tetrazolium (MTT) was removed via aspiration, 100 μ L DMSO was added to each well to dissolve the formazan crystals and the absorbance was read at 540 nanometer (nm) by using a BioTek® PowerWave XS spectrophotometer.

Data analysis

Cell viability was determined by using four replicate wells for each concentration. Vehicle control wells were considered to have 100% cell viability (i.e. the mean OD of the untreated wells = 100% viability). Cell viability in test wells was calculated relative to the vehicle control and expressed as a percentage. IC₅₀ was calculated by using ED₅₀ V10 add-in on Excel.

Antimycobacterium assays

Microorganism, growth conditions and media

The *M. tuberculosis* strain H37 was obtained from the National Health Laboratory Services (NHLS), Port Elizabeth, South Africa. *Mycobacterium tuberculosis* was grown in a culture broth containing Difco™ Middlebrook 7H9 broth (BD), glycerol, Tween®80 and Middlebrook albumin-dextrose-catalase (ADC) growth supplement (Sigma) at 37 °C.

Control antibiotics

Rifampicin and EMB dihydrochloride (Sigma) were used as antibiotic positive controls against *M. tuberculosis*. Rifampicin and EMB were dissolved in sterile water at stock concentrations of 1 mg/mL and 2 mg/mL, respectively. From pre-screening, working concentrations of 16 μ g/mL and 128 μ g/mL were prepared for rifampicin and EMB, respectively, in culture broth (without Tween®80) and filter sterilised (0.2 μ M filter).

Sample preparation

Working concentrations of 500 μ g/mL were prepared in culture broth (without Tween®80) and filter sterilised (0.2 μ M filter) from the reconstituted stock concentration of both volatile oils obtained from solvent-free microwave extraction and hydrodistillation.

Microbroth dilution method

The assay was performed as described by Franzblau et al. (1998) with modifications. The outer perimeter wells of clear 96-well plates were filled with sterile water to minimise evaporation of medium in experimental wells during incubation. In brief, culture broth (100 μ L) was added to all experimental wells (i.e. volatile oils and antibiotics) except to the highest volatile oil and antibiotic concentration wells to which 200 μ L of the working concentrations was added. Serial dilutions (1:2) were prepared for the volatile oils (3.9 μ g/mL – 250 μ g/mL), rifampicin (0.0625 μ g/mL – 8 μ g/mL) and EMB (0.25 μ g/mL – 32 μ g/mL). The *M. tuberculosis* culture was assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08–0.1; equivalent to $\sim 1.5 \times 10^8$ cells/mL) and 100 μ L added to each test well.

The following controls were prepared:

- Antibiotic/medium control (100 μ L medium + 100 μ L of highest antibiotic concentration);
- Volatile oil/medium control (100 μ L medium + 100 μ L of highest volatile oil concentration);
- 0.5% absolute ethanol control (100 μ L medium + 100 μ L 1% absolute ethanol); and
- Micro-organism growth control (100 μ L medium + 100 μ L *M. tuberculosis* inoculum).

Plates were sealed with microplate sealing tape and incubated at 37 °C for 7 days.

CellTiter-Blue® cell viability assay

After treatment, 40 μ L of CellTiter-Blue® Reagent (Promega) and 12.5 μ L 20% Tween® 80 were added to each well. Wells were observed for colour change, where a blue colour represents no growth (non-viable cells) and a pink colour represents growth (viable cells). After 6 h, *M. tuberculosis* cells were killed by adding 35% formaldehyde to reach a final concentration of 10% in each well. This was done to eliminate the biosafety hazard of *M. tuberculosis* aerosols. The absorbance was read at 570 nm and at a reference wavelength of 600 nm by using a BioTek® PowerWave XS spectrophotometer.

Ethical consideration

Ethical clearance was not needed for the study.

Results

Chemical composition

The essential oils of *C. anisata* obtained by HD and solvent-free extraction (SFME) process are shown in Table 1. The results obtained by GC-MS analyses of the oils from both extraction methods showed a total of 51 distinct molecular compounds; 36 and 22 chemical compounds were identified in the essential oils extracted through HD and SFME respectively whilst oils of both extraction methods shared 8 similar compounds namely, α -pinene; limonene; 1, 8- cineole; bornyl acetate; methyl eugenol; cis-methyl isoeugenol; germacrene-D; and (+) spathulenol. A good number of chemical compounds with substantial amounts were found in the essential oils of *C. anisata*, and higher percentage composition of these compounds above 1% in HD essential oils, which are shown in Figure 1, are methyl eugenol, 36.04%; 4-allyl anisole (estragole), 26.43%; Limonene, 10.99%; β -myrcene, 8.28%; germacrene-D, 2.08%; trans-methyl isoeugenol, 1.97%; and β -cubebene, 1.15%; whilst in SFME are methyl eugenol, 76.60% and methyl charicol (estragole), 14.48%. Methyl eugenol and estragole were the most abundant compounds present in both oils.

Results of cytotoxic activity of *C. anisata* essential oils at the concentrations range of 1.95 μ g/mL – 500 μ g/mL are graphically shown in Figure 2. The cytotoxic activity of *C. anisata* essential oils on MRHF cell line was evaluated by

using the MTT assay. The essential oils from both extraction methods showed significant effect on MRHF cells at 250 μ g/mL and 500 μ g/mL. The highest cytotoxic activity of the oils was found at 500 μ g/mL concentration as Figure 2 depicts the % cell viability of SFME and HD reduced to approximately 81% and 39%, respectively. The cytotoxic effects of volatile oil obtained by solvent-free microwave extraction against the MRHF cell line between 1.95 μ g/mL and 125 μ g/mL showed approximately 100%. It was observed that the percentage of cell viability shown in Figure 2 decreased with increasing concentration of volatile oil from concentrations above 125 μ g/mL.

There is a significant difference only between HD and SFME at 7.81 μ g/mL, 500 μ g/mL, and positive control at 100 μ M Melphalan.

Antimycobacterial activity

The results from the antimycobacterial assay are presented in Plates A.1 and A.2 for both oils and standard drugs, respectively (Appendix 1). Wells A1–12 and H1–12 contain sterile water. The 96-well microtitre plate showed all the concentrations (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250 μ g/mL in wells labelled 5 to 11) for both solvent-free microwave extraction volatile oil (represented in wells B, C, D), and HD volatile oil (represented in wells E, F, G). The mycobacterial growth in these wells formed as pink colour indicating growth or viable cells, except in well 2 (control 1), which contained 100 μ L medium and 100 μ L of 500 μ g/mL volatile oil with no *M. tuberculosis*, are observed in the Plate A.1. As none of the wells treated with the essential oils is noted to inhibit *M. tuberculosis* growth, this study observes a minimum inhibitory concentration (MIC) > 250 μ g/mL, which is similar to the case of one of the standard drugs, EMB (represented in wells E, F, G), observation of concentration between 0.063 μ g/mL and 8 μ g/mL. In contrast, the concentrations (0.063 μ g/mL, 0.125 μ g/mL, 0.25 μ g/mL, 0.5 μ g/mL, 1 μ g/mL, 2 μ g/mL, 4 μ g/mL, 8 μ g/mL contained in wells 4–11 of Plate A.2) available for the other standard drug, Rifampicin, showed observable and significant colour change from pink to blue between 0.25 μ g/mL and 8 μ g/mL, as blue colour indicates no growth or non-viable cells of *M. tuberculosis*. This study records antimycobacterial activity at MIC = 0.25 μ g/mL for rifampicin.

Discussion

Chemical analysis of *C. anisata* oil showed that methyl eugenol and estragole are the most abundant compounds in this study. The oil extracted by HD method yielded clearer and more chemical compounds than oil extracted by solvent-free microwave extraction method. This agrees with Cook and Lanaras (2016) who noted the high yields and good recovery constituents of essential oils extracted by HD method. Several compounds of Clausena (*C. dentata*, *C. dimidata*, *C. engleri* and *C. indica*), similar to this study, have been reported in which the major compounds are estragole (Ekundayo, Oguntimein & Hammerschmidt 1986)

TABLE 1: Chemical composition of essential oil of *C. anisata* obtained by solvent-free extraction and hydrodistillation.

No.	Chemical compounds	RT/min		SFME		HD	
		SFME	HD	Area %	KI Values	Area %	KI Values
1	Alpha-pinene†	5.70	5.70	0.34	1026	0.56	1026
2	Limonene†	7.74	7.90	0.71	1128	10.99	1135
3	1, 8-Cineole†	7.82	8.12	0.23	1132	0.68	1147
4	(-)-Bornyl acetate†	13.70	13.75	0.69	1368	0.46	1346
5	Methyl eugenol†	16.75	13.75	76.60	1507	36.04	1507
6	Germacrene-D†	18.10	18.38	0.34	1551	2.08	1563
7	Cis-methyl isoeugenol†	18.28	18.28	0.77	1572	0.70	1552
8	(+) Spathulenol†	20.00	20.00	0.18	1632	0.84	1639
9	Methyl chavicol (Estragole)	11.83	12.04	14.48	1332	-	-
10	Beta-pinene	6.62	-	0.11	1072	-	-
11	Myrcene	6.80	-	0.45	1081	-	-
12	Linalyl acetate	9.34	-	0.53	1192	-	-
13	P-Mentha-2, 8-dien-1-ol, trans-(+,-)-	9.90	-	0.13	1235	-	-
14	2,3-epoxygeranial	10.25	-	0.17	1253	-	-
15	Camphor	10.53	-	0.62	1267	-	-
16	2,6-Dimethyl-1,5,7-octatrien-3-ol	10.68	-	0.40	1274	-	-
17	Borneol L	11.12	-	0.23	1296	-	-
18	Verbenon	12.01	-	0.81	1340	-	-
19	Cis-Citral	12.61	-	0.27	1370	-	-
20	Geranial	13.29	-	0.51	1404	-	-
21	2-Undecanone	13.84	-	0.44	1383	-	-
22	Triphenylphosphine oxide	32.58	-	1.00	2246	-	-
23	4-Allyl anisole (Estragole)	-	12.04	-	-	26.43	1342
24	Beta-myrcene	-	6.92	-	-	8.28	1087
25	(+)-3-Carene	-	7.31	-	-	0.62	1106
26	Nonanal (n-nonylaldehyde)	-	9.44	-	-	0.64	1287
27	L-camphor	-	10.52	-	-	0.15	1266
28	Citronellal	-	10.56	-	-	0.31	1269
29	Cyclopropane, methylmethylene-	-	10.66	-	-	0.58	-
30	4-Allyl anisole (Estragole)	-	12.04	-	-	26.43	1342
31	P-allylAnisole	-	12.12	-	-	0.46	1346
32	Beta.-sesquiphellandrene	-	17.75	-	-	0.19	1536
33	Beta.-cubebene	-	18.32	-	-	1.15	1560
34	Trans-methyl isoeugenol	-	18.52	-	-	1.97	1582
35	Bicyclogermacrene	-	18.61	-	-	0.45	1573
36	Beta.-bisabolene	-	18.75	-	-	0.13	1579
37	Delta.-Cadinene	-	18.93	-	-	0.18	1587
38	Beta.-sesquiphellandrene	-	19.05	-	-	0.11	1592
39	1-Formyl-2,2,6-trimethyl-3-cis-(3-methyl-2-butene-1-yl)5-cyclohexene	-	19.33	-	-	0.07	-
40	4-Mercaptotoluene (p-thiocresol)	-	19.69	-	-	0.14	-
41	Germacrene B	-	19.79	-	-	0.73	1624
42	2,3-Dibromo-8-phenyl-p-menthane	-	20.06	-	-	0.19	1634
43	Oplopenone.Beta.-oplopenone	-	20.21	-	-	0.14	1642
44	Caryophyllene oxide	-	20.25	-	-	0.22	1643
45	Zingiberenol	-	20.30	-	-	0.19	-
46	Salvial-4(14)-en-1-one	-	20.44	-	-	0.14	-
47	(+)-.Alpha.-longipinene	-	21.06	-	-	0.34	1678
48	Aromadendrene	-	21.16	-	-	0.25	1683
49	Salvial-4(14)-en-1-one	-	21.38	-	-	0.12	-
50	T-murolol (1-naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-)	-	21.55	-	-	0.42	1700
51	1H-indole-3-carboxylic acid, 4-hydroxy- (4-hydroxyindole-3-carboxylic acid)	-	21.64	-	-	0.20	-

KI, Kovats index; RT, retention time; HD, hydrodistilled; SFME, solvent-free microwaved essential.

†, compound found in oil from both extraction methods.

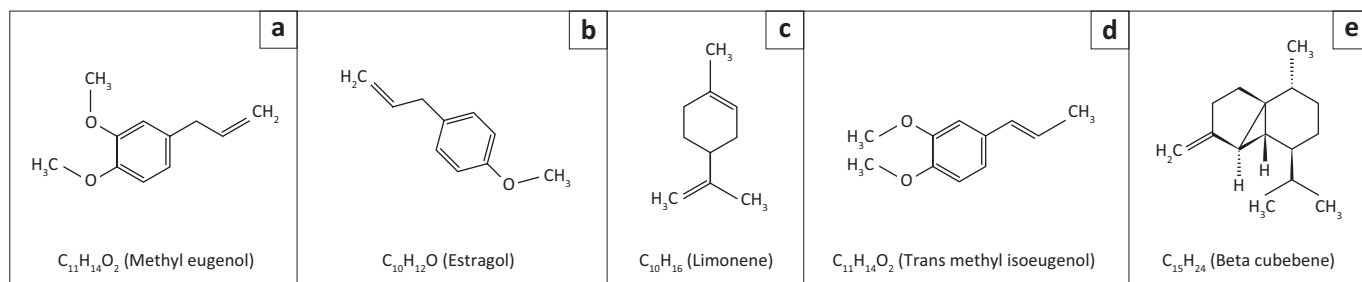
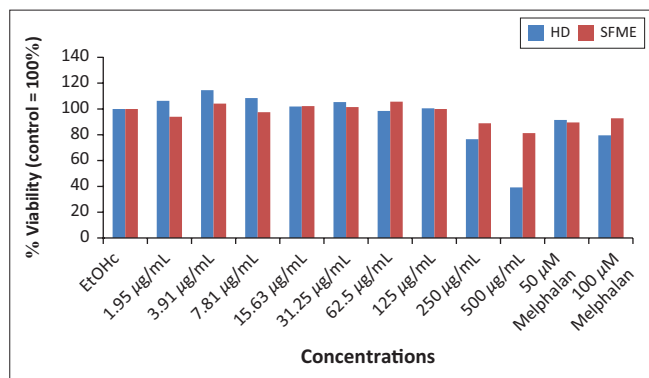


FIGURE 1: Chemical structures of some monoterpenoids and sesquiterpenoids identified with high percentages in the essential oil of *C. anisata* obtained by hydrodistillation and solvent-free microwave extraction by using gas chromatography–mass spectroscopy analysis.



HD, hydrodistilled; SFME, solvent-free microwaved essential; EtOHc, ethanol control.

FIGURE 2: Cytotoxicity effects of volatile oil obtained by solvent-free microwave extraction and hydrodistillation against the human dermal fibroblast cell line.

and β -pinene (Senthilkumar & Venkatesalu 2009). Methyl eugenol is a major compound extracted through HD and SFME in this study at 36.04% and 76.60% respectively, but identified in trace amount (0.3%) in the leaf essential oils of *C. engleri* from Vietnam (Trung et al. 2014). Volatile oil of *C. anisata* extracted from HD in this study revealed β -myrcene at 8.28%, whilst Zhou et al. (2008) observed that a steam-distilled oil of *C. dentata* from China exhibited β -myrcene (51.5%) as its main compound, and constituents of the water-distilled oil of *C. indica* of Chinese origin comprised β -myrcene at 5.03%. Trung et al. (2014) identified the same compound in the leaf essential oils of *C. dentata*, *C. dimidiata* and *C. indica* at 14.3%, 1.4% and 3.3%, respectively. Another report (Zhou et al. 2008) identified β -myrcene (23.46% and 0.1%) as the compounds in the fruit and leaf oils of *C. indica*, respectively. Furthermore, in this study, α -pinene composition is observed in trace amount to be 0.56% and 0.34% in HD and SFME respectively but in substantial amount (21.7%) in *C. dentata* (Trung et al. 2014). The observation in this study is in agreement with the previous report on *C. anisata* from different geographical regions in the south of Croatia (Jekovic & Mastelic 2001).

Cell viability and/or proliferation rates of cells are good indication of cell health (Aslantürk 2018). In this study, the IC_{50} values of *C. anisata* essential oils extracted by SFME and HD methods were 1 216 μ g/mL and 383 μ g/mL, respectively. Extracts that demonstrate an LC_{50} value greater than 100 μ g/mL were considered as non-toxic, and an LC_{50} value less than 100 μ g/mL as toxic (Meyer et al. 1982).

Therefore, *C. anisata* is considered safe for consumption. However, from the findings of Makirita, Chauka and Chacha (2016), *C. anisata* leaf chloroform and *C. anisata* twigs ethyl acetate extracts, which demonstrated an LC_{50} value of 3.5761 μ g/mL and 6.1276 μ g/mL respectively, were much more cytotoxic and described as having anticancer prospects according to Meyer et al. (1982) who pointed out that extracts with an LC_{50} value less than 20 μ g/mL are reckoned as potential anticancer agents.

The *in vitro* antitubercular activity evaluated against *M. tuberculosis* showed that *C. anisata* volatile oils and EMB have no antimycobacterial effect against *M. tuberculosis* strain. This observation supported the report of York, Van Vuuren and De Wet (2012) that aqueous and organic extract of *C. anisata* leaf is inactive against *M. smegmatis* but in contrast to Gizachew, Mirutse and Tilahun (2013) who reported that MeOH and aqueous extracts of *C. anisata* leaf growing in Ethiopia showed promising growth inhibition on *Mycobacterium bovis* (SB 1176). The result of this study support the report that geographical location of plants influences its chemical composition and its bioactivity (Jekovic & Mastelic 2001). The medicinal plant bioactivity depends on the chemical constituents. This may be responsible for the resistance of *M. tuberculosis* strain to the oils. This specific strain of *M. tuberculosis* might also be resistant to EMB. The low activity recorded by the oil and EMB could be because of strong resistance developed by *M. tuberculosis*.

Conclusion

This study showed that *C. anisata*, having methyl eugenol and estragole in abundance, is safe for consumption considering its various therapeutic uses; however, no remarkable antimycobacterial activity was recorded on the oil of the plant. The HD yielded purer and more chemical compounds than solvent-free microwave extraction. Combinative potent oils by HD method can be considered for synergetic and enhanced effect against *M. tuberculosis*. Further studies in this regard are strongly recommended.

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Competing interests

The author has declared that no competing interests exist.

Authors' contributions

I.O.L. designed the study, coordinated plant material collection, carried out all the experiments and drafted the manuscript.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Disclaimer

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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Appendix starts on next page→

APPENDIX 1

Antimycobacterium activity on 96-well microtitre plate

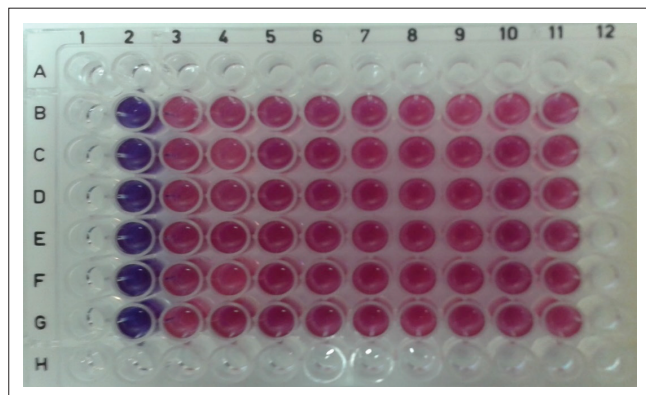


FIGURE 1-A1: Plate A.1 – Inactivity effect of volatile oils from solvent-free microwaved essential and hydrodistilled on *Mycobacterium tuberculosis* on a 96-well microtitre plate showing: Well A1–12; H1–12: Sterile water; well 2 (Control 1): 100 μ L medium and 100 μ L of 500 μ g/mL volatile oil; no *Mycobacterium tuberculosis*; well 3 (Control 2): 100 μ L medium and 100 μ L *Mycobacterium tuberculosis* inoculum; well 4 (Control 3): 100 μ L medium containing 1% absolute ethanol and 100 μ L *Mycobacterium tuberculosis* inoculum; wells 5–11: (250, 125, 67.5, 31.3, 15.6, 7.8, 3.9 μ g/mL); B, C, D: oil from solvent-free microwaved essential and E, F, G: oil from hydrodistilled.

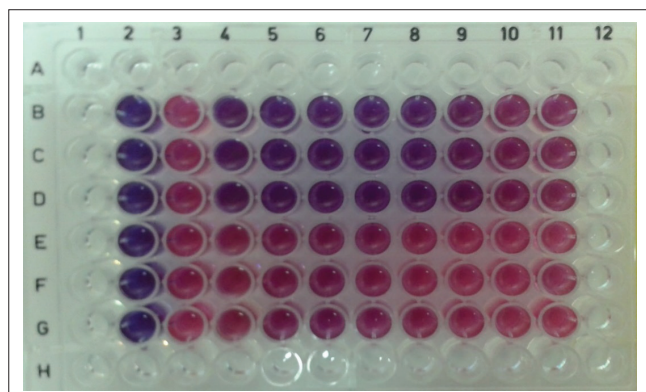


FIGURE 2-A1: Plate A.2 – Susceptibility effect of rifampicin and weak inhibitory effect of Ethambutol on *Mycobacterium tuberculosis* on a 96-well microtitre plate: Well A1–12; H1–12: Sterile water; well 2 (Control 1): 100 μ L medium and 100 μ L of 500 μ g/mL volatile oil; no *Mycobacterium tuberculosis*; well 3 (Control 2): 100 μ L medium and 100 μ L *Mycobacterium tuberculosis* inoculum; well 4 (Control 3): 100 μ L medium containing 1% absolute ethanol and 100 μ L *Mycobacterium tuberculosis* inoculum; wells 5–11: (250, 125, 67.5, 31.3, 15.6, 7.8, 3.9 μ g/mL); B, C, D: oil from solvent-free microwaved essential and E, F, G: oil from hydrodistilled.