Antimycobacterial Potential of Essential Oils Used Traditionally for Cough Remedy

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Tuberculosis (TB) is currently a major health hazard due to multidrug-resistant forms of bacilli. Global efforts are underway to eradicate TB using new drugs with new modes of action, higher activity, and fewer side effects. Essential oils from some dietary herbal species have been used as sources of medicine and food preservatives for over 4000 years.

The objective of this research is to investigate the antimycobacterial activity of the essential oils of *Boswellia papyrifera* (Del) Hochst olibanum, *Nigella sativa* seeds and *Eucalyptus camaldulensis* Dehnh leaves, which are used traditionally in treatment of cough and cold.

The essential oils from herbal materials were prepared using hydrodistillation method. The essential oils were analyzed for identification of their chemical composition using GC-MS technique.

The essential oils were tested *in vitro* for their activity against nine clinical isolates and a reference susceptible strain (H37Rv) *M. tuberculosis* in Lowenstein-Jensen (LJ) medium, at concentrations of 75, 50, 30 and 15 µl/mL.

The main constituents identified in the investigated essential oils were p-cymene (45.26%) and thymoquinone (35.35 %) in *N. sativa* oil; octyl acetate (37.3%) and octyl formate (13.3%) in the *B. papyrifera* oil; 1,8-cineole (75%) and p-cymene (16.7%) in *E. camaldulensis* oil.

The investigated essential oils exhibited anti-tubercular activity, in LJ medium, up to concentration of 15 µl/ml for each oil, towards all the tested strains, three of which were multi-drug resistant strains (MDR-TB), as they resist rifampicin, a marker drug for MDR-TB. This research findings may be useful in developing future powerful drugs for MDR-TB from essential oils, the bioactive phytochemical natural products.

Keywords: Tuberculosis, Essential Oils, *Boswellia papyrifera*, *Nigella sativa, Eucalyptus camaldulensis*, Thymoquinone, 1,8-cineole, Octyl acetate

Introduction:

Tuberculosis (TB) is currently a major health hazard due to multidrug-resistant forms of

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bacilli. Global efforts are underway to eradicate TB using new drugs with new modes of action, higher activity and fewer side effects. For this reason, unexplored new sources and previously explored sources were examined and many antimycobacterial compounds have been previously reported (Santhosh and Suriyanarayanan, 2014).

Screening of natural products from higher plants for antitubercular activity were carried out by many researchers (Camacho-Corona et al., 2009; Okemo et al., 2010; Ladda et al., 2012; Nguta et al., 2016).

Essential oils from some dietary herbal species have been used as sources of medicine and food preservatives for over 4000 years (Bakkali et al., 2008; Rota et al., 2008). There are many reports in literature regarding the antimicrobial activity of essential oils (Kofidis et al., 2004; Pandey et al., 2003; Pauli and Schilcher, 2010; Sharifi-Rad et al., 2017). On the other hand, many researchers have recently focused on the antimycobacterial activity of the essential oils (Bueno-Sánchez et al., 2009; Elhassan et al., 2016; Chraibi et al., 2016).

Nigella sativa (black cumin), belongs to the family Ranunculaceae, is an annual flowering plant grows at 20-90 cm tall; it is widely distributed and cultivated in Mediterranean countries, Middle Europe, western Asia and Middle East. The seeds are primarily used as spice and added as food preservatives. They contain about (0.4 to 0.45%) volatile oil (Datta et al., 2012; Sultana et al., 2015). The major components of the essential oil identified were thymoquinone (24.5-57%), ρ-cymene (10.7-40.3%), α-thujene (1.9-8.2%), carvacrol (2,2-4.5%), 4-terpineol (1.9-4.5%) (Burits, 2000; El-Ghorab, 2003).

In the traditional system of medicine, black cumin seeds are effective against cough, bronchitis, asthma, chronic headaches and gastrointestinal problems. It has also been used as a stimulant, diuretic, lactagogue and carminative (Datta et al., 2012; Venkatachallam et al., 2010; Sultana et al., 2015, Hussain et al., 2016).

Previous studies, based on *in-vitro* tests, showed that *N. sativa* seed essential oil and its active constituent, thymoquinone, exhibited significant antibacterial, antifungal as well as anti-yeast activities (Aljabre et al., 2005; Al-Qurashi et al., 2007; Halamova et al., 2010; Halawani, 2009; Shokri, 2014). On the other hand, *N. sativa* essential oil exhibited antibacterial activity against various clinical isolates of bacteria resistant to several antibiotics (Ferdous et al., 1992; Salman et al., 2008).

Eucalyptus camaldulensis Dehnh (river red gum), belongs to the family Myrtaceae, is one of the most widely distributed *Eucalyptus* species that grows to a height of 20 m tall, with a trunk diameter of 1-2 m (FAO, 2001; Brocker, 2002; Gebretsadik, 2013).

The major components in the eucalyptus leaves essential oil reported were 1,8-cineole (27.22-40.05%), p-cymene (17.50-19.16), α-pinene (5.25-14.68%),γ-terpinene (9.42-33.03%) and other constituents (Anwar et al., 2010; Siramon et al., 2013).

Several studies reported that *E. camaldulensis* leaves essential oil displayed antibacterial activity (Ayepola and Adeniyi, 2008, Mohammed et al., 2012), antifungal activity (Falahati et al., 2005; Katool, 2011; Siramon et al., 2013) as well as other biological activities (Silva et al., 2003; Siramon et al., 2009; Fathi and Shakarami, 2014).

In addition to its powerful antiseptic property, *Eucalyptus* oil and fresh leaves are used widely, in steam inhalation, for treatment of cough and cold, sore throat and other infections of the respiratory tracts (Cimanga et al., 2002; Anwar et al., 2010).

Boswellia papyrifera (Del) Hochst (Burseraceae), which is distributed in Central and Eastern Africa, is a deciduous, gum-producing, perennial tree, which is tapped on the stem for a kind of oleo-gum-resin called olibanum or frankincense (Vollesen, 1989; Ammon et al., 1993; Melese, 2007).

The composition of the frankincense essential oil differs according to the climate, harvest conditions, and geographical source (Yates and Wenninger, 1970). Generally, the major constituents in *B. papyrifera* essential oil were *n*-Octyl acetate (more than 60%), followed by *n*-octanol (5.2 -17.8%), incensol acetate (1.7-10.8%) and others (Hamm et al., 2005; Camarda et al., 2007; Shimelis et al., 2012) .

According to several published reports, the frankincense essential oil exhibits in vitro antibacterial, antifungal and other biological activities (Sharma et al., 1988; Ammon et al., 1993; Abdoul-latif et al., 2012; Mikaeil et al., 2003).

Traditionally olibanum is used in treatment of diarrhea, asthma, and bronchitis. It is also used in medicinal preparations for the treatment of amenorrhoea (Dekebo et al., 2012).

The essential oils of *B. papyrifera* (Del) Hochst olibanum, *N. sativa* seeds and *E.camaldulensis* Dehnh leaves are used traditionally in the treatment of some respiratory conditions, including cough.

The objective of this research is to investigate the antimycobacterial activity of the essential oils of *Boswellia papyrifera* (Del) Hochst olibanum, *Nigella sativa* seeds and *Eucalyptus camaldulensis* Dehnh leaves.

Materials and methods:

Plant materials

Eucalyptus camaldulensis leaves were collected from Kenana Eucalyptus forest, Central Sudan; *Nigella sativa* seeds were collected from Northern Sudan whereas the Frankincense of *Boswellia papyrifera* was purchased from a local market in Khartoum and authenticated at Medicinal and Aromatic Plants Institute, National Research Centre, Khartoum, Sudan (Voucher no. K/96/12).

Test micro-organisms

The test micro-organisms, comprised nine clinical isolates and a reference susceptible strain (H37Rv) *M. tuberculosis*, were provided by -Tuberculosis Reference Laboratory – Sudan National Public Health Laboratory.

Essential oil isolation

One hundred grams of each provided herbal sample were subjected to hydro-distillation for 4 h using Clevenger's apparatus. The yield of the oil (v/w %) was calculated based on the plant dry matter. The distilled essential oil was dried over anhydrous sodium sulphate, filtered and stored in a sealed vial at 4° C. The yield for the oils was calculated in triplicates. The results were expressed as mean ± standard deviation.

Physical properties determination

The physical parameters (Refractive index, specific gravity and Solubility) of the oils under investigation were determined as described in the British Standards (BS 2073).

GC-MS analysis of the oils

The essential oils were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using HP 6890 (GC) and HP 5973 (MSD). The sample was dissolved in dichloromethane (1%) and injected at 250 °C (Injector temperature) into a capillary column type HP-1(30 m X 0.25 mm i.d, 0.25 µm film thickness, stationary phase (95 % diethyl-5% diphenylsiloxane), using helium as a carrier gas at a flow rate of 1 ml/min. The injected volume was 1 µl and the injection mode used was splitless. The oven temperature was programed from 45–280 °C at the rate of 4 °C/min. Detector temperature was 250 ^{o}C. The MS was operated in the El mode at 70 ev. The mass and scan range was set at *m/z* 30-500.

Identification of essential oils components

The essential oils constituents were identified by their retention time and computer matching of their mass spectra with those found in NIST and Wiley library database.

Assessment of essential oils antimycobacterial activity

The three essential oils were tested *in vitro* for their activity against nine clinical isolates and reference susceptible strain ($H_{37}R_v$) *M. tuberculosis* in Lowenstein-Jensen (LJ) medium.

The media containing different concentration of essential oils (75, 50, 30 and 15 µl/ml) were inoculated with the different strains of *Mycobacterium* suspensions, the concentrations of which was equivalent to McFarland No. 0.5, then were incubated at 37 °C for up to six weeks. All isolates were tested for susceptibility to the antitubercular first-line drug, rifampicin (RIF).

Evaluation of antitubercular activity was determined using the absolute concentration method which is expressed in terms of the minimal inhibitory concentration (Rieder et al. 2007).

Results and discussion:

Hydro-distillation of *N. sativa* seeds yielded (0.2 ± 0.03 %) pale brown liquid oil. The refractive index and specific gravity of the oil were found to be 1.48477 and 0.86009, respectively, at 25 °C. The oil was soluble in 2 volumes of alcohol 80%.

Gas chromatographic profile of hydro-distilled *N. sativa* essential oil showed detection of twelve compounds, their retention times and percentages are shown in Table 1.

The main constituent identified in the present investigated essential oil of *N. sativa* seeds was p-cymene (45.26%). The oil was characterized by the presence of high content of thymoquinone (35.35 %), in contrast to some previously investigated essential oils, from the plant cultivated in other countries, in which thymoquinone was detected in very low amount (up to1.65 %) or as a trace compound (Nickavara et al., 2003; Wajs et al. 2008; Toma et al., 2010).

B. papyrifera olibanum, upon hydro-distillation, yielded (4.20±0.11%) colorless liquid oil. The refractive index and specific gravity of the oil were found to be 1.47520 and 0.885, respectively, at 25 °C. The oil was soluble in 2 volumes of alcohol 80%.

Gas chromatographic profile of hydro-distilled *B. papyrifera* essential oil showed detection of thirty-four compounds. The main identified constituents with their retention times and percentages are shown in Table 2. Octyl acetate (37.3%) was the major constituent in the *B. papyrifera* essential oil, followed by octyl formate (13.3%). Diterpenes represent about (20%) of the oil.

Hydro-distillation of *E. camaldulensis* leaves yielded (1.5±0.05 %) colorless oil. The refractive index and specific gravity of the oil were found to be 1.46840 and 0.899, respectively, at 25 °C. The oil was soluble in 1.5 volumes of alcohol 80%. The gas

chromatogram of the hydrodistillate of *E. camaldulensis* oil revealed the presence of 1,8-cineole (75%), p-cymene (16.7%), β-phellandrene (4.0%) as the main constituents (Table 3).

Evaluation of antitubercular activity

The essential oils were tested *in vitro* for their anti-mycobacterial potential, along with rifampicin as standard drug, against nine clinical isolates and a reference susceptible strain ($H_{37}R_v$) *M. tuberculosis* in LJ medium at concentrations of 75, 50, 30 and 15 µl/mL.

All investigated essential oils exhibited pronounced anti-tubercular activity towards all the tested strains of *M. tuberculosis* isolates, at all concentration up to 15µl/ml, for each oil. Table 4 shows the anti-mycobacterial potential of the three oils, at 15µl/ml, against the tested strains. Interestingly, three of the tested strains were MDR-TB (showed resistance towards rifampicin).

In many essential oils, the antimicrobial activity is due to the presence of terpenoids such as monoterpenes, sesquiterpenes or related alcohols and phenols (Trombetta, et al., 2005; Koroch et al., 2007; Bueno-Sánchez et al., 2009).

The anti-mycobacterial activity of the black cumin essential oil is attributed to the presence of a high percentage of thymoquinone (35.35 %) which in a previous study showed activity against *M. tuberculosis* with MIC of 20 µg/ml (Randhawa, 2011); whereas the activity of *E. camaldulensis* essential oil may be due to the presence of high content of the oxide 1,8-cineole (75%) which was known for their pronounced antimicrobial activity as well as other biological activity (Asanova et al., 2003; Cermelli et al., 2008; Bastos et al., 2010). On the other hand, it was reported that 1,8-cineole exhibited additive and synergistic effects in combination with hydrocarbons sesquiterpene and monoterpene constituents in the oil (Delaquis et al., 2002). This phenomenon may increase its antimicrobial efficacy which explains the growth inhibition of the resistant mycobacterial strains by *E. camaldulensis* essential oil. The finding in the present study is in accordance with the results obtained in a previous study (Lawal et al., 2012), in which different *camaldulensis* leaves organic solvents extracts (hexane, chloroform and methanol extracts) and isolates exhibited antimycobacterial activity at concentrations (4-64 mg/ml).

Anti-mycobacterial activity of *B. papyrifera* may be attributed to the major constituent, octyl acetate (37.26%) or to the presence of remarkable diterpenes constituents (20%) since different diterpenes from various plants exhibited antimycobacterial activity (Santhosh and

Suriyanarayanan, 2014); or may be attributed to the synergistic effects of the essential oil constituents.

The antimycobacterial potential of the present investigated oils indicates that essential oils and their components may provide natural alternatives to tuberculosis drugs and a new source of drugs for Multi-drug resistant *M. tuberculosis* strains.

Conclusion:

The present investigation results may be useful in developing future powerful antitubercular drugs from essential oils, the natural bioactive phytochemicals, and may provides important baseline information for further research.

Table1. *N. sativa* seeds essential oil composition

Peak No.	R. time	Compound	Percentage
1	12.91	1,5-decadiene	9.9
2	13.30	α-pinene	1.5
3	15.93	β-phellandrene	0.46
4	16.17	β- terpinene	1.45
5	18.98	Terpinolene	0.11
6	19.59	p-cymene	45.26
7	19.82	Dihydrocarvone	0.85
8	24.54	4-Thujanol	0.33
9	26.16	p-Menth-1(7)-en-9-ol	3.10
10	35.78	Thymoquinone	35.35
11	41.77	α-Longipinene	0.27
12	45.58	β-neoclovene	1.42

Table 2. The main constituents in *B. papyrifera* olibanum essential oil

No.	R T	Compound	Area,%
1	10.750	α-pinene	2.14
2	13.947	Limonene	5.52
3	15.413	Octyl formate	13.25
4	19.755	Octyl acetate	37.26
5	29.270	Spathulenol	2.75
6	29.415	Globulol	2.09
7	30.994	β-eudesmol	3.39
8	36.797	Cembrerne	0.96
9	37.398	Neocembrene A	2.68
10	38.484	verticilla-4(20),7,11-triene	6.85
11	40.756	Allylcembrol	0.69
12	41.00	Incensole	3.29
13	41.426	Incensyl acetate	5.20

Table 3. Essential oil composition of *E. camaldulensis*

Peak No.	RT	Compound	Area, %
1	12.92	α -Thujene	0.137
2	13.33	α-Pinene	1.611
3	16.17	β -Pinene	0.158
4	18.23	Terpinolene	0.742
5	19.54	p-Cymene	16.710
6	19.93	β-Phellandrene	3.990
7	20.18	1,8-cineole	74.814
8	21.95	ϒ- Terpinene	0.487
9	47.71	(+)-Aromadendrene	0.192
10	48.74	Aromadendrene	0.188
11	55.93	Spathulenol	0.649
12	56.40	Cedr-8-en-13-ol	0.278
13	6062	α -Eudesmol	0.182

Table 4. Antitubercular activity of the oils at 15µl/ml against *M. tuberculosis* strains

No.	Strain code	*Nigella sativa*	*Eucalyptus camaldulensis*	*Boswellia papyrifera*	Rifampicin
1	**1303**	S	S	S	S
2	**150904**	S	S	S	S
3	**1098**	S	S	S	S
4	**1240**	S	S	S	**R**
5	**0115**	S	S	S	S
6	**1214**	S	S	S	**R**
7	**1081**	S	S	S	S
8	**150**	S	S	S	S
9	**153**	S	S	S	R
10	**H_{37} R_v**	S	S	S	S

R= resistant, S= sensitive

Competing interests
The authors declare that they have no competing interests.

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