Original article

Phytochemical constituents and anticancer activities of *Tarchonanthus camphoratus* essential oils grown in Saudi Arabia

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**Abstract**

*Tarchonanthus Camphoratus* L. is traditionally known for its various medicinal purposes. In this study, the *T. camphoratus* essential oil (TCEO) was isolated via steam distillation, and its chemical constituents were determined using GC–MS. The *in vitro* antiproliferative effects of TCEO on A549, HepG2, MCF-7 cancer cells, and HUVEC non-tumor cells was investigated using an MTT assay. Flow cytometry analysis was conducted to evaluate cell cycle distribution using propidium iodide staining, and cell death mode using Annexin V-FITC/PI assays. The expression of some apoptosis related genes was investigated using qRT-PCR. Major constituents of TCEO included fenchol, borneol, 3-cyclohexene-1-methanol and 3-ethyl-3-methyl. Cell viability test showed that TCEO is highly effective against MCF-7 cells with IC₅₀ 12.5 µg/mL. Cell cycle arrest at the G1/S phase, and apoptosis mediation were evident in the presence of TCEO. Gene expression analysis of several pro-apoptotic and anti-apoptotic genes revealed the initiation of apoptosis in TCEO-MCF-7 cells. In conclusion, our study confirms the antiproliferative activity of the *T. camphoratus* essential oil.

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1. Introduction

Cancer remains a prevalent, life threatening disease with a high mortality rate worldwide (Siegel et al., 2020). Despite great advances in pharmacology and synthetic drugs, conventional cancer treatments often require highly toxic drugs that have many adverse side effects. Natural products, particularly plants, may be an ideal choice for some patients due to their natural properties (Desai et al., 2008). Essential oils (EO) have been used in the prevention or therapy of several diseases, due to their preservative and medicinal properties. The bioactivity of EO as an antidiabetic, antioxidant, antibacterial, and antiviral have been reported (Edris, 2007). In cancer research, plant EO represent an attractive source of new anticancer compounds that have potential therapeutic applications in chemoprevention. Considered to potentially be more effective than traditional cancer treatments, EO selectively interact with various pathways and cellular mechanisms in a manner that may create synergistic results (Edris, 2007; Bhalla et al., 2013; Blowman et al., 2018).

*Tarchonanthus camphoratus* L. (family Asteraceae) is found in a wide range of environments (e.g., Africa and Arabia), has a grayish appearance, and can grow to be about six meters in height (Van Wyk et al., 1997). Its leaves have been traditionally used to relieve bronchitis, asthma, headaches, inflammations, and abdominal pains (Amabeoku et al., 2000). The essential oil found in *T. camphoratus* displays a wide variety of biological properties (e.g., antibacterial, antimicrobial, antifungal, antioxidant, insecticidal) (Van Vuuren and Viljoen, 2009; Ileri et al., 2010; Aiyeogoro and Van Dyk, 2011). Oxygenated sesquiterpene and monoterpenes have been
documented to be the major constituents of the *T. camphoratus* essential oil (TCEO) (Ali et al., 2013). Other phytochemical investigations on the *T. camphoratus* have detected the presence of reducing sugars, saponins and tannins (Van Wyk et al., 1997). The cytotoxic activity of *T. camphoratus* that has been grown in different regions, using different cell lines has also been reported (Mothana et al., 2009; Ajiegor and Van Dyk, 2011; Ali et al., 2013). According to our literature survey, only some alkaloid and flavones were isolated and characterized from *T. camphoratus* grown in Saudi Arabia by Bishay et al. (2002). However, no report was found regarding the chemical constituents, cytotoxic and apoptosis effects of the essential oils of *T. camphoratus* grown in Saudi Arabia have been published. Therefore, the goal of this study was to determine the chemical profile of the TCEO using gas chromatography and mass spectrometry, while exploring its anticancer activities.

2. Materials and methods

2.1. Plant material

The aerial part of *T. camphoratus* L. was collected from Al Shafa village, 25 km southwest of Al-Taif city, Southwestern region of Saudi Arabia during spring season in March (2019). After collection, the *T. camphoratus* samples identity was confirmed by Prof. Ramzi Mothan, Department of Pharmacognosy, College of Pharmacy, King Saud University. A voucher specimen (No. 15848) was deposited in the herbarium of the Department.

2.2. Volatile oil preparation

The aerial parts were ground (150 g plant material in 0.5 L water), then subjected to hydrodistillation for 3 h in a Clevenger-type apparatus at temperatures ranged from 80 °C to 100 °C. The volatile oil produced was filtered and kept at +4 °C until further investigation.

2.3. GC–MS analysis and compound identification

The chemical constituents of TCEO were determined using gas chromatography and a mass spectrometer (Turbomass, PerkinElmer, Inc., Waltham, MA, USA). The temperature program was set to 40 °C, followed by a 2 min hold, then raised to 200 °C at a rate of 5 °C min⁻¹, which was also then put on hold for 2 min. From 200 °C, the temperature was raised by 5 °C min⁻¹ to 300 °C and held for another 2 min. The TCEO chemical composition were determined by comparing the mass spectra obtained with the mass spectra from the National Institute of Standard and Technology and WILEY Spectral libraries. The mass spectra TCEO compounds were also compared with those of similar compounds in the Adams Library (Adams, 2007) and the Wiley GC/MS Library (McLafferty and Stauffer, 1989).

2.4. Cytotoxic effects of TCEO

The proliferation of breast (MCF-7), liver (HepG2), lung (A549) cancer cell lines, and non-tumorigenic (HUVEC) cells were assessed using MTT assay, according to Kumar et al., (2018), with slight modification. In 96-well plates, cells were plated at 1 x 10⁴ cells/ml then incubated for 24 h. TCEO at 12.5, 25, 50, 100 and 200 μg/mL was added after 24 h of incubation, then allowed to incubate for another 24 h. After 48 h of incubation, MTT solution (5 mg/ml) was added (10 μL/well) and incubated for 2–4 h at 37 °C. After the incubation, formazan crystals end product was dissolved with 0.1 mL acidified isopropanol, and the plate was put on a shaker for 10–15 min. The plate was read using a plate reader (BioTek, USA) at 570 nm. The inhibitory concentration (IC₅₀) was calculated from the dose-dependent curve using OriginPro 8.5 software. The cell viability percentage was calculated using the following formula: Cell Viability % = (O.D. of TCEO wells/O.D. of untreated wells) x 100.

2.5. Cell cycle arrest assessment induced by TCEO

The cell cycle distribution was assessed using MCF-7 according to Alqahtani et al., (2020). In brief, after a 24 h incubation period for TCEO (12.5 and 25 μg/mL), both control and treated – 7 cells were collected, washed twice with cold PBS, fixed in 70% ethanol (stored at –20 °C), and then stored at 4 °C from 0.5 h to 4 h. Fixed cells were incubated with RNase A (100 μg/mL) for RNA degradation, and propidium iodide (PI) (100 μg/mL) (Sigma) for DNA staining. After a 30 min incubation, the DNA content of the samples were evaluated using a flow cytometer (Cytomics FC 500; Beckman Coulter, CA, USA). Data collection and analysis were performed using CXP software V. 3.0.

2.6. Flow cytometry assessment of apoptotic vs necrotic cells

An Apoptosis Detection Kit FITC Annexin V with PI (ThermoFisher Scientific, CA, USA) was used per the manufacturer’s instructions to validate evidence of early vs late apoptosis and necrosis in the cancer cells. Briefly, MCF-7 cells were seeded in 6-well culture plates (2 x 10⁵ cells/well) for 24 h, then treated with TCEO (12.5 and 25 μg/mL) for another 24 h. After incubation, cells were collected and washed twice with PBS, then resuspended in Annexin V-binding buffer (100 μL). Thereafter, 5 μL of each Annexin V-FITC and PI dyes were added and incubated for 15 min in the dark. Apoptotic vs necrotic cell populations were analyzed via flow cytometry (Cytomics FC 500; Beckman Coulter, CA, USA). Data collection and analysis were performed using CXP software V. 3.0.

2.7. Quantitative Real-time PCR (qRT-PCR)

For qRT-PCR analysis, MCF-7 cells (2 x 10⁵ cells/ml) in a 6-well plate were exposed to 12.5 μg/ml or 25 μg/ml concentrations for 24 h. RNA from the exposed and non-exposed MCF-7 cells was extracted using a TRIzol reagent. One microgram of purified RNA was then converted to cDNA using a specific kit (promega transcriptor). Data from each sample was then run in triplicate with specific primers of *Bcl-2*, *P53* and caspases 9,8,3 using Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, CA, USA) according to Semlali et al., (2016).

2.8. Statistical analysis

Data was analyzed for statistical significance using the student’s t-test. Values of each triplicate experiment are presented as mean ± SD. Statistical analysis and the IC₅₀ values were generated using OriginPro 8.5 software.

3. Results

3.1. TCEO chemical composition

The chemical content, retention times and area percentages of TCEO are presented in Table 1. The identified compounds are represented in order of their elution on the HP Innowax column. A total of 29 compounds, representing 98.99% of the total oil, could be identified. Fenchol (37.3%) was the primary constituent, 3-
Cyclohexene-1-methanol (25.7%) was a moderate constituent, and borneol and 3-ethyl-3-methylcyclohexene (9.7% and 6.8%, respectively) were low constituents (Table 1 and Fig. 1). Other compounds present in fairly good amounts were 8-Hydroxycarvotanacetone (2.4%), 2-naphthalenemethanol (2.3%), 1, 2, 4-Trihydroxymenthane (1.8%) and caryophyllene oxide (1.7%).
3.2. TCEO decreases cell proliferation in a dose-dependent manner

The cytotoxicity of cell lines treated with TCEO for 48 h are presented in Fig. 2. The TCEO exhibited a concentration-dependent inhibitory effect on cell proliferation in all cell lines after 48 h of incubation. The calculated IC_{50} values for all tested cells are displayed in Table 2. It was shown that inhibitory effect of TCEO was particularly observed in MCF-7 breast carcinoma cells with (IC_{50} = 12.5 \mu g/mL), indicating that TCEO was more cytotoxic in MCF-7 cells compared to the other cell lines. We therefore chose to focus on breast carcinoma MCF-7 cells as cell line models in this study.

3.3. TCEO causes G1/S cell cycle arrest in MCF-7 cells

To explore whether TCEO proliferation inhibition on MCF-7 cells causes cell cycle arrest, flow cytometric exploration with PI staining was performed. As shown in Fig. 3, dose-dependent increases in the G1 phase of treated MCF-7 cells was observed. The G1 phase cells proportion increased approximately by 5% and 16% in the presence of 12.5 and 25 \mu g/mL of TCEO, respectively, compared with the control. This increase was conjugated with significantly fewer cells in the other phases (Fig. 3).

3.4. Annexin V-FITC apoptosis detection

To further examine whether apoptosis had been induced in MCF-7 cells, an annexin V-FITC staining assay was performed. As shown in Fig. 4, treatment of MCF-7 cells with 12.5 and 25 \mu g/mL of TCEO showed increases in the amount of early apoptosis (from 1.2 ± 0.14% in the control to 2.2 ± 0.21% and 3.6 ± 0.28%, respectively). A remarkable increase in late apoptotic cells was also observed (from 1.8 ± 0.21% in the control to 13.1 ± 0.7% with p < 0.05, and 36.2 ± 2.8% with p < 0.001, respectively). Thus, the antiproliferative impact of TCEO on MCF-7 cells could be correlated with induction of apoptosis.

3.5. Effect of TCEO on the expression of apoptosis related genes in MCF-7 cells

To examine the mechanism by which TCEO-induced apoptosis, the expression levels of various pro-apoptotic and anti-apoptotic genes was quantified using qRT-PCR analysis. Treatment of MCF-7 cells with TCEO 12.5 and 25 \mu g/mL for 24 h caused a dose-dependent decrease in anti-apoptotic Bcl-2 gene expression (p < 0.05), however, treated MCF-7 cells showed PS3, Bax and caspases 8, 9, 3 upregulation by TCEO relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control (Fig. 5).

4. Discussion

Plants have been investigated across the world to see if they can provide novel, anticancer agents with few side effects (Biersack and Schobert, 2012; Dall’Acqua, 2014; Khazir et al., 2014; Negi et al., 2015). Several studies of various plant species have demonstrated that essential oil compounds show promising antitumor activity, and could be used as a substitution to or in addition to conventional anticancer therapies (Andrade et al., 2018). It is well-known that harsh conditions are considered as positive factors for plants to produce numerous secondary metabolites that help them to adapt to these conditions. Most plants in Saudi Arabia grows under extreme temperatures and water scarcity which perhaps make them medicinally promising (Harlev et al., 2012). Therefore, more studies investigating other EO for their anticancer properties could expand the present knowledge of these mechanisms.

Our GC/MS analysis showed that fenchol (37.3%) was the major constituent in TCEO, followed by 3-cyclohexene-1-methanol (25.7%) and bornel (9.6%) (Table 1). Our results are partly in agreement with a study from Ali et al. (2013), who reported that endo-fenchol (21.2%), trans-pinene hydrate (8.8%), and carvophyllene oxide (7.5%) were the main constituents in the T. camphoratus essential oil grown in Yemen (Ali et al., 2013). Fenchol was also the main constituent of T. camphoratus grown in Kenya, but the percent (15.9%) was different from what we found in this study (37.3%) (Matasyoh et al., 2007). In addition, similar chemical constituents between our GC–MS data of TCEO and the results obtained by Costa et al. (2008), were observed. T. camphoratus grown in South Africa were found to have a different essential oil chemical composition (Nanyonga et al., 2013). These variations in chemical composition could be attributed to several factors, including geographical location, ecological conditions, harvesting conditions, postharvest waiting times until distillation, distillation techniques, or storage (Castelo et al., 2012; Shams et al., 2016).

This study also demonstrated that TCEO had significant dose-dependent, antiproliferative effects on all tested cell lines, with MCF-7 as the most responsive. These results correspond with results from other studies that have reported strong anticancer activities against various cancer cell lines in the EO from several plant species (Gautam et al., 2014).

Particularly, in the work of Nanyonga et al. (2013), EO from T. camphoratus grown in South Africa displayed cytotoxic activity with IC_{50} values above 100 \mu g/ml against human embryonic kidney (HEK 293) and hepatocellular carcinoma cells (HepG2). The cytotoxic effects of EO taken from T. camphoratus grown in Yemen have been reported against colon cancer cells (HT29) with an IC_{50} = 84.7 \mu g/mL (Ali et al., 2013). Our results showed that TCEO had stronger cytotoxicity in terms of the IC_{50} value against different cancer cells. These differences between IC_{50} values could be attributed to a quantitative variation of chemical constituents among these oils, as well as cancer cells used, and the duration of treatment.

Cell cycle arrest analysis was conducted to evaluate the role of TCEO in cell death. It is well known that cell cycle control represents an important regulatory mechanism for cell growth, and that disorganization in cell cycles lead to tumorigenesis (Golias et al., 2004). Indeed, it has been documented that several EO from differ-
ent plants have exerted cell death through cell cycles disruption (Singh et al., 2002; Saleh et al., 2017). Here, we mentioned that TCEO exerted G1 cell cycle arrest in MCF-7 cells. This sort of cell cycle arrest at G1 phase has been described for essential oils (Cai et al., 2013; Chung et al., 2019).

Apoptosis is an essential cell death process and its induction in cancer cells has become a promising strategy and a target pathway for the development of new anticancer drugs (Bremer et al., 2006; Pfeffer and Singh, 2018). In their review, Blowman et al. (2018), reviewed that several EO can induce different pathways of apoptosis. Therefore, the manner of cell death induced by TCEO in MCF-7 cells was also inspected. In this study, apoptosis induction was confirmed by Annexin V staining and supported by RT-PCR data. It is well known that Bcl-2 family genes play a crucial role in the initiation of apoptosis. In addition, the elevated levels of activated caspases ultimately lead to apoptotic cell death as a results of decrease or increase expression of these genes (Ola et al., 2011; Kale et al., 2018). Based on this, our gene expression results showed that the level of Bcl-2 decreases, while that of Bax increases in MCF-7 treated cells. Furthermore, treatment of MCF-7 cells with TCEO triggers a dose-dependent expression of P53, caspases-9,8 and 3, confirming the involvement of apoptosis in TCEO-induced cell death. Our data are in line with different studies that reported the involvement of EO in inducing apoptosis through the alteration of the expression levels of Bcl-2 and Bax genes (Gautam et al., 2014; Russo et al., 2018).

The essential oils anticancer activities of many plant species have been referred to the presence of monoterpenes and its deriva-
tives in their constituents (Sobral et al., 2014). Our GC–MS data on the essential oil compositions of *T. camphoratus* showed that fenchol and borneol monoterpenes were the primary compounds. It was found that some of these compounds detected in TCEO were previously known to exhibit anticancer activities. Among these ingredients, borneol compound which represents 9.66% of all constituents of TCEO. A recent study established that borneol promoted apoptosis, downregulated the expression of Bcl-2 and upregulation of Bax and caspase-3 in glioma cells (Wang et al., 2020). Additionally, cadinene and linalool compounds were also found to exert anticancer activities through apoptosis induction in different cancer cells (Hui et al., 2015; Iwasaki et al., 2016).

According to the obtained results, it could be predicted that the anticancer activity of TCEO is due to these constituents, which can synergistically perform these effects with remaining compounds.

### 5. Conclusion

Our results established the presence of fenchol, borneol and 3-cyclohexene-1-methanol as a main constituent of TCEO. The anticancer activity of the essential oils extracted from *T. camphoratus* is mediated through halting cell cycle in dose-dependent manner that subsequently prompted apoptosis. Our results indicate the potential applications of TCEO in complementary and alternative herbal treatment especially in cancer treatments. The presence of several constituents in TCEO such as fenchol, borneol, cadinene and linalool may serve as an efficient natural antitumor com-

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**Fig. 4.** Apoptotic effect of TCEO treatment on MCF-7 cells. (A) Dot plots of MCF-7 cells treated with 12.5 and 25 μg/mL of TCEO for 24 h. A1: necrotic cells, A2: late apoptotic cells, A3: Viable cells, and A4: early apoptotic cells. (B) The bar graphs show the percentages of viable, early, late and necrotic cells for non-treated and treated MCF-7 cells.

**Fig. 5.** Apoptosis related genes expression in MCF-7 treated cells with TCEO. Cells were treated with a concentration for 24 h. Fold changes in P53, Bax, Bcl-2, Casp3, 9 & 8 genes relative to untreated cells were quantified using qRT-PCR. Statistical significance is expressed as *p < 0.05, **p < 0.01.*
pounds to enhance the human health. Similar investigations using different types of cancer cell lines should be performed to further confirm our findings and explore the detail mechanism for possible pharmaceutical application.

Declaration of Competing Interest

The authors declare that no conflict of interest.

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