Research Article

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In vitro antimicrobial, antioxidant, cytotoxic activities, and wound healing potential of *Thymbra capitata* ethanolic extract

[*Thymbra capitata* etanolik ekstraktının *in vitro* antimikrobiyal, antioksidan, sitotoksik aktiviteleri ve yara iyileşme potansiyeli]

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Abstract

Objectives: In this study, we aimed to detect the chemical compounds of *Thymbra capitata* ethanolic extract (TC-EtOH) as well as to evaluated its antimicrobial, antioxidant, cytotoxic activities, and *in vivo* wound healing effects.

Methods: The chemical composition of TC-EtOH was analyzed by Gas chromatography-mass spectrometry (GC-MS). Antioxidant and antimicrobial properties were determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH), disc diffusion test and broth micro-dilution (minimal inhibitory concentration [MIC]) methods, respectively. Cytotoxic activity was tested on MG63 (human osteosarcoma) and MCF-7 (human breast carcinoma) cells by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Tumor necrosis factor alpha (TNF-α) protein levels were determined by ELISA.

Results: The major components of TC-EtOH were tetratria contane (14.92%), camphor (12.50%), and terpineol (10.77%). TC-EtOH showed powerful antimicrobial activity in *C. Tropicalis* (0.03 mg/mL). The IC_{50} values of the TC-EtOH of the DPPH were determined 21.5 µg/mL. The IC_{50} values were calculated 37.28 and 44.40 µg/mL on the MG63 and MCF-7 cell lines, respectively. It was observed that the wounds treated with TC-EtOH showed a faster healing.

Conclusions: According to results, *T. capitata* species are thought to be natural antioxidants and a novel pharmaceutical compound for the pharmaceutical industry.

Keywords: antimicrobial; antioxidant; cytotoxic activity; *Thymbra capitata*; wound healing.

ÖZ

Amaç: Bu çalışmada *Thymbra capitata* etanolik ekstraktının (TC-EtOH) kimyasal bileşimi, antimikrobiyal, antioksidan, sitotoksik aktiviteleri ve *in-vivo* yara iyileştirici etkileri araştırıldı.

Yöntem: TC-EtOH'ın kimyasal bileşimi gaz kromatografisi-kütle spektrometrisi (GC-MS) ile analiz edildi. Antioksidan ve antimikrobiyal özellikleri sırasıyla 2,2-difenil-1-pikrilhidrazil (DPPH), disk difüzyonu testi ve minimum inhibisyon konsantrasyon (MIC) yöntemleriyle belirlendi. Sitotoksik aktivite MG63 (insan osteosarkomu) ve MCF-7 (insan meme karsinomu) hücre hatlarında 2,3-bis- (2-metoksi-4-nitro-5 sülfophenil) -2H-tetrazolium-5-karboksanilid (XTT) yöntemiyle belirlendi. Tümör nekroz faktörü alfa (TNF-a) seviyesi ELISA metoduyla belirlendi.

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Bulgular ve Tartışma: TC-EtOH'de bulunan ana bileşenler tetratriakontan (%14,92), kafur (%12,50), terpineol (% 10,77)' dür. TC-EtOH, *Candidatropicalis*'e (0,03 mg/mL) karşı güçlü bir antimikrobiyal aktivite gözlendi. TC-EtOH'ın DPPH için IC_{50} değeri 21,5 µg/mL olarak belirlendi. TC-EtOH'ın MG63 ve MCF-7 hücre serileri için IC_{50} değerleri sırasıyla 37,28 µg/mL ve 44,40 µg/mL olarak belirlendi. *In vivo* çalışmalardan elde edilen bulgulara göre, TC-EtOH ile tedavi edilen yaraların kontrol grubuna göre daha hızlı iyileşme gösterdiğini gözlendi.
Sonuç: *T. capitata* türleri doğal antioksidan ve ilaç endüstrisi için yeni bir farmasötik bileşik olabilirler.

Anahtar kelimeler: Antimikrobiyal; Antioksidan; Sitotoksik aktivite; *Thymbra capitata*; Yara iyileşmesi.

Introduction

Aromatic plants have been used in traditional medicine since the since ancient times [1]. These plants contain essential oils, terpenes, and other non-terpene components [2]. Natural products have been used to prevent and treat the progression of many diseases [3]. Lamiaceae family is the most be aromatic species of great interest [4]. Among these species, thymus, origanum, satureja, thymbra, and coridothymus types are especially important in terms of their wide distribution and economic benefits [5]. In the worldwide, Thymbra herb is categorized into four species; (*Thymbra calostachya (Rech.f.) Rech.f., Thymbra capitata (L.) Cav., Thymbra sintenisii Bornm. and Azn., Thymbra spicata L.*). These species show the greatest variety in the Eastern Mediterranean region. It is widely distributed from Portugal coast (the beginning of the Mediterranean) to Iraq, from Egypt and Algeria to the Black Sea [6]. In Turkey, Thymbra species are represented into five taxon, which are *T. capitata* (L.) Cav., *T. sintenisii* Bornm and Azn. subsp. *sintenisii*, *T. sintenisii* Bornm and Azn. subsp. *isaurica* P. H. Davis, *T. spicata* L. subsp. *intricate* [5], R. Morales, *T. spicata* L. subsp. *Spicata* [7]. Thymbra species have antimicrobial, antioxidant, antiinflammatory, antiparasitic, and antiproliferative activities, among other beneficial biological effects [8–10]. In the literature, there are many studies examining the beneficial biological effects of Thymbra species [3, 11–13]. However, to the best of our knowledge, there are few reports published about the *T. capitata strain.*

The aim of this study was to determine the phytochemical composition, antioxidant, antimicrobial, and cytotoxic activities of *T. capitata* ethanolic extract (TC-EtOH) and to investigate its probable preventive effects.

Materials and methods

Plant material

T. capitata was gathered from Denizli, province in the Southwestern Turkey, in July 2014. M. Çiçek has confirmed the taxonomic recognition of plant. A voucher specimen (*T. capitata*; Herbarium No: 2014-33) was deposited at the Çiçek Herbarium in Department of Biology, Faculty of Arts and Science, Pamukkale University, Denizli, Turkey (PAU-M. Çiçek Herb.). Collection information of *T. capitata;* C2 Denizli: 31 km from Kale to Muğla, 945 m, *Pinusbrutia* clearings, 13.07.2014, *M. Çiçek 2014-33 (Herb. M. Çiçek).*

Preparation of the extract

Aerial parts of plants have parted after drying in the dark under sterile conditions. Plant samples were grinded before extraction process. Twenty grams of the sample were added in 40 mL ethanol for extraction. Ethanol was evaporated using a rotary evaporator (Stuart RE300) under reduced pressure at 30 °C [14].

GC-MS analysis

Chemical composition of TC-EtOH was determined with the method modified by Abay [15]. Agilent Technologies GC 7890A, equipped with 5975 Triple Axis Detector mass spectrometer was employed to perform gas chromatography-mass spectrometry (GC-MS) analysis. DB-WAXetr column (60 m × 0.20 mm × 0.25 µm), electron ionization system and ionization energy of 70 eV were used for GC-MS detection. Helium was the carrier gas at a flow rate of 1 mL/min. The GC oven temperature was kept at 40 °C for 5 min and programmed to 250 °C at a rate of 5 °C/min and kept constant at 250 °C for 20 min. The library search was carried out using NIST.

Antioxidant activity

Radical scavenging activity of extracts was performed with the method modified by Ou et al. [16]. Different concentrations of the extracts were prepared, and then equal volumes (1,000 µL) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and sample solutions were mixed and incubated for 30 min. After incubation absorbance was measured by spectrophotometer at 517 nm. The ascorbic acid as an antioxidant was used to compare the results.

Microbiological activity

Klebsiella pneumoniae (ATCC 10031), Shigellaboydii (ATCC 9905), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 7829), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10987), and *Candida tropicalis* (ATCC 750) were used to determine antimicrobial and antifungal properties of TC-EtOH. All bacteria and fungi were obtained from American Type Culture Collection (ATCC).

Disc diffusion assay

The antimicrobial activity of TC-EtOH was evaluated by using disc diffusion method [17], in which 100 µL of suspension containing 10^8 CFU/mL of bacteria and 10^6 CFU/mL of fungi spread on Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) were used, respectively. After the impregnation of the disc (6 mm diameter) with 20 µL at 50 mg/mL concentration, it was added on the inoculated agar. Ethanol was used as negative control. Commercially available cefoperazone/sulbactam (105 µg) and fluconazole (25 µg) discs were employed for positive control of the bacteria and fungi, respectively. Regarding the zones of growth inhibition around the disks; bacteria's were measured after 24 h incubation at 37 °C, whereas fungi's were measured after 28 h incubation at 37 °C. All the assays were done in triplicate [18].

Micro-well dilution assay

Antimicrobial activities of TC-EtOH were determined by broth micro dilution technique, which was performed according to CLSI protocol [19]. Minimal Inhibitory Concentration (MIC) values of TC-EtOH were performed by micro-well dilution method. Sample concentration range (0.03–2 mg/mL) was prepared with Müller–Hinton Broth (MHB). The suspensions were adjusted to 0.5 McFarland standard turbidity. Ninety five microliters of MHB and 5 µl of the inoculums were added into each well; whereas 195 µL of Nutrient broth without compound and 5 µL of the inoculum were used as negative control. Piperacillin/tazobactam (8/1) and fluconazol were used as positive control for bacteria and fungi, respectively.

In vivo wound healing activity

This research was carried out in Sivas Cumhuriyet University Animal Laboratory in accordance with the guidelines of Sivas Cumhuriyet University Animal Experiments Local Ethics Committee. The research was conducted in accordance with ethical rules with the decision of Sivas Cumhuriyet University Animal Experiments Local Ethics Committee dated 06.08.2015 and number 65202830–050.04.04/69.

Male Wistar albino rats (n=16) weighed (250–275 g) were used. Standard laboratory diet and requested drinking water were used to feed all animals, so that they can always be internal. Rats were housed such that eight animals per cage. Rats capable of normal activity in cages at 22 ± 2 °C, humidity (50–70%) and on 12 h day/night set will be kept in the room [20].The anesthesia of the rats was performed with ketamine hydrochloride (90 mg/kg). Anaesthetized animals were made 3 cm full thickness incision and a punch biopsy was opened $1 \times 1\ cm^2$. Two groups of eight rats were formed by randomly assigning the animals. While one was the control group, the other group was treated with TC-EtOH. Sterile solution of extracts was applied topically to each wound of the animals at a dose of 50 µL/wound once daily for seven days. The animals of all groups were euthanized in accordance with the procedures on seventh day, their cardiac blood and tissue samples were taken. Blood samples were centrifuged at 1500 × g for 10 min. TNF-α values were determined by ELISA according to the manufacturer's instructions (BOSTER,Rat TNFα ELISA Kit, Cat No:EK0526) [21]. Tissue samples were examined histopathologically. The changes in the injured area were regularly measured and the data were calculated according to the following Eqn.: wound contraction=(healed area/total wound area) × 100. The wound healing was compared to the control group (healed area = wound area – present wound area) [22].

Cell culture

Human osteosarcoma (MG63, ATCC-CRL-1427), human breast adenocarcinoma (MCF-7, ATCC-HTB-22), and mouse fibroblast (ATCC-CRL-6364) cell lines were purchased from ATCC. Cells were grown at 37 °C in a humidified incubator in 5% CO_2. All media were supplemented with 1% penicillin (100 U/mL) and streptomycin (100 µg/mL), and 10% fetal bovine serum (FBS). Live cell as control was considered as 100%.

Cytotoxicity assay

2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method was used to evaluate cytotoxic activity. All cells were seeded in a 96-well plate in growth medium then treated with different concentrations of test compounds and incubated in a humidified CO_2 atmosphere at 37 °C for 24 h. Following the incubation, 100 µL XTT solution was added to each well for another 2 h incubation. The optical density values were measured at 475 nm with a microplate reader [23].

Statistical analysis

Data were expressed as mean ± standard deviation [±SD]. Statistical analysis were performed using the Sigma Plot 12.0. All determinations were computed independent triplicated [n=3].

Results and discussion

Since ancient times, aromatic plant species, which are defined as "thyme" have been used to treat a range of symptoms and complaints in different diseases [24–26]. Lamiaceae species possess antimicrobial, antioxidant, and antitumoral activities [27].

Determining chemical composition of TC-EtOH

Phytochemical composition of TC-EtOH was analyzed by GC-MS. TheTC-EtOH has approximately 53 different compounds (Table 1), and approximately 90% of compounds were identified (Figure 1). Tetratria contane (14.92%), camphor (12.50%), terpineol (10.77%) were major components of TC-EtOH. Machado et al. investigated chemical composition of *T. capitata*, and they reported that the carvacrol was as the dominant compound in *T. capitata* [28]. Another study, Ali et al. demonstrated that terpinene (5.8%), *p*-cymene (7.7%), and carvacrol (66%) were the

major components in these species. Miguel et al. reported that carvacrol, thymol, *p*-cymene, thymol, and camphor were as major compounds of the Thymbra and Tymus species [8]. Barberan et al. showed that *T. capitata* species have high amount of flavonoid [29].Our results were different from the previous reports. This situation may arise from geographical origin, extraction methods, as well as the harvest time [27].

Determination of the antioxidant activity of TC-EtOH

DPPH method is widely used to determine for scavenging free radicals [30]. Radical scavenging activity of TC-EtOH (IC_{50}) was foundbe 21.5 µg/mL, while IC_{50} value of the ascorbic acid was found to be 5.02 µg/mL. Galego et al. have examined on of *T. capitata's* essential oils gathered from Portugal region [24]. The comparison of DPPH activity according to butylated hydroxyanisole (BHA) showed that it has high antioxidant scavenging activity. Saidi et al. have reported that the DPPH value of essential oil from *T. capitata* as 1.28 µmol/mL [31]. Faleiro et al. have studied antioxidant and antimicrobial properties of *T. capitata (L.) Cav*. IC_{50} value was found to be 27.84 µg/mL [25]. It is clear that results of our radical scavenging activity were similar with previous reports.

Determination of the antimicrobial activity ofTC-EtOH

The activity was assessed by the presence or absence of inhibition zones and MIC values. The MIC values detected at the lowest concentrations without visible growth were defined as concentrations completely inhibiting microbial growth (Table 2). Results showed that TC-EtOH exhibited antibacterial activity against *C. tropicalis* (0.03 mg/mL), and also against bacterial strains (0.25–1 mg/mL). Previous studies have reported that various extracts obtained from *T. capitata* herb were effective on microorganisms [32,33].

Determining the wound healing activity of TC-EtOH

In comparison to the control group, the treated group showed a more accelerated wound healing process in the rats [Healed area = wound area - present wound area, (Supplementary 1).In the process of wound healing, reformation of vascular structures is very important for

Table 1: GC-MS identified phytochemicals present in *Thymbra capitata* ethanolic extract.

Number	RT, min	Area%	Name
1	10.96	12.52	Camphor
2	11.69	3.24	Borneol
3	12.15	2.40	Verbenyl, ethyl ether
4	12.53	10.77	α-Terpineol
5	13.18	2.80	Verbenone
6	13.47	1.96	2-Hydroxycineole acetate
7	14.15	0.64	Carvone
8	15.13	0.82	Bornyl acetate
9	15.47	0.55	Thymol
10	17.09	0.73	2-Caren-4-ol
11	17.26	0.96	α-Bourbonene
12	17.48	0.36	Methyleugenol
13	17.94	1.42	8-Hydroxycarvotanacetone
14	18.59	0.47	Aromandendrene
15	19.41	0.90	α-Bourbonene
16	19.69	0.25	Isoshyobunone
17	19.97	0.69	Caryophyllene oxide
18	20.08	0.34	α-Bourbonene
19	20.18	0.27	3-Carene, 4-isopropenyl-
20	20.31	0.56	Limonen-6-ol, pivalate
21	20.42	9.00	Caryophyllene oxide
22	20.54	0.56	α-Gurjunene
23	20.70	1.02	Viridiflorol
24	20.99	0.15	Santalol
25	21.16	0.96	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl-
26	21.44	1.17	Longipinocarveol
27	21.63	0.81	Caryophyllene oxide
28	21.72	0.14	Dodecane, 5,8-diethyl-
29	21.89	0.84	5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one
30	22.05	1.46	Isoaromadendrene epoxide
31	22.74	1.91	Acetic acid, 2,6,6-trimethyl-3-methylene-7-(3-oxobutylidene)oxepan-2-yl ester
32	23.39	0.26	Heptadecane
33	24.09	0.18	α-Muurolene
34	24.35	0.71	Hexahydrofarnesyl acetone
35	25.51	0.11	Octadecane
36	28.21	0.24	Hexadecanoic acid, ethyl ester
37	31.39	0.45	Heneicosane
38	32.48	0.08	Ethyl Oleate
39	32.77	0.08	Stearic acid, ethyl ester
40	32.83	0.30	Heptadecane, 9-hexyl-
41	33.13	0.06	13-Heptadecyn-1-ol
42	33.79	0.24	1-Eicosanol
43	33.90	0.73	Pentacosane
44	34.55	0.26	4,8,12,16-Tetramethylheptadecan-4-olide
45	36.05	1.05	Hexacosane
46	37.28	0.40	Heptacosane
47	38.74	2.76	Octacosane
48	40.00	0.63	Nonacosane
49	40.48	0.60	Hentriacontane
50	41.78	0.17	Hentriacontane
51	42.68	14.92	Tetratriacontane
52	51.69	0.31	Tetracontane
53	58.19	3.09	Tetratetracontane

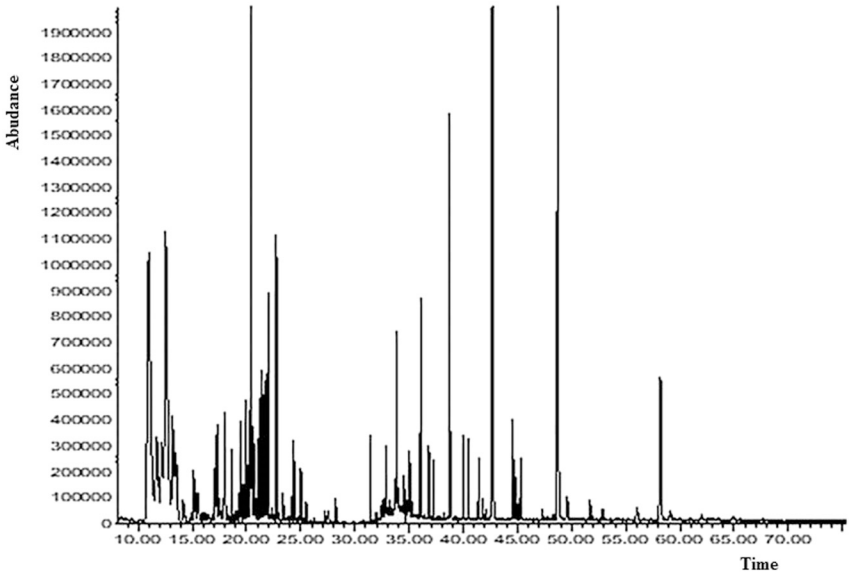

Figure 1: The major components identified by gas chromatography-mass spectrometry from samples of TC-EtOH. Chromatographic profiles of samples from *Thymbra capitata*.

Table 2: Antimicrobial screening of TC-EtOH. Antimicrobial activity of TC-EtOH performed by disc diffusion and micro-well dilution assay. Cefoperazone/sulbactam and fluconazole were used for positive control of the bacteria and fungi, respectively.

Bacteria	Zone of Inhibition, mm		MIC, mg/mL	
	Extract	Control[a]	Extract	Control[b]
Klebsiella pneumoniae	22.3 ± 0.5	26.6 ± 2.0	0.5	<0.03
Shigella boydii	17.3 ± 0.5	25.0 ± 1.7	0.5	<0.03
Pseudomonas aeruginosa	11.3 ± 1.5	24.6 ± 1.1	1.0	<0.03
Proteus vulgaris	14.0 ± 1.0	28.6 ± 1.1	1.0	<0.03
Staphylococcus aureus	29.6 ± 2.0	19.6 ± 1.5	0.25	<0.03
Bacillus cereus	16.0 ± 1.7	32.3 ± 0.5	0.5	<0.03
Fungus	Extract	Control[c]	Extract	Control[d]
Candida tropicalis	12.8 ± 0.4	13.1 ± 0.8	0.03	<0.03

[a]Cefoperazone/ Sulbactam 2:1, [b] Piperasilin/Tazobactam(8:1), [c]Flucanazol disk, [d]FlucanazolMIC, minimal inhibitory concentration.

the nutrition of the damaged tissue. Angiogenesis is to be closely associated with inflammation. Monocyte/macrophages are essential for the wound healing and the accumulation of monocyte/macrophage in the region of the wound is very important for capillary organization and collateral formation. These inflammatory cells mediate the formation of a series of angiogenic cytokine and growth factors. Therefore, determination of TNF-α level would be an important determinant for the wound healing [34]. In this study, using an animal model of excisional wound healing, we found that TC-EtOH application caused significant healing in all wound models compared to the control group (Supplementary 2). The capability TC-EtOH is crucially dependent on the modulation of the inflammatory factor TNF-α. The changes in the levels of TNF-α was examined and we found that the level of TNF-α levels decreased significantly.

Mohamad et al. showed that the ethanolic extract of *T. spicata* has wound healing activity [35].Tutar et al. demonstrated that the ethanol extract of *T. sintenisii* was reduced the total wound area [36].

Determination of the cytotoxic activityofTC-EtOH

Cancer is a pathological condition that occurs with a genetic and developmental process [37]. Anticancer drugs have toxicity and serious side effects for normal cells due to the chronic use [38]. Developing a new generation of anticancer agents became a quite popular research area. In the study, the cytotoxic activity of TC-EtOH was tested on MCF-7, MG63, and L929 cells. The cell proliferation

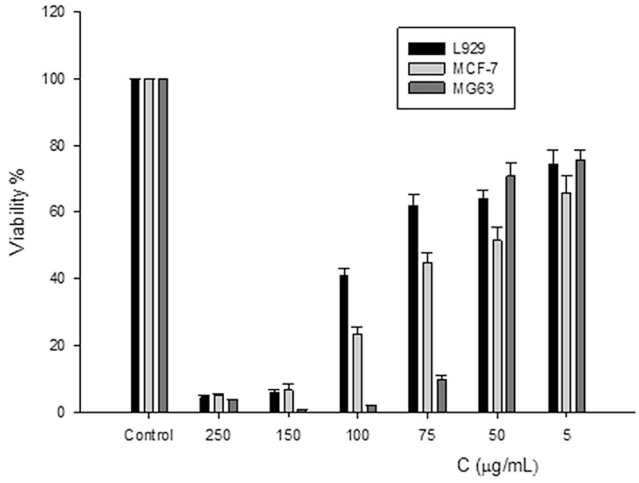

Figure 2: Cell cytotoxicity assessment by XTT assay. The data represents the viability of MCF-7, MG63, and L929 cells treated for 24 h with TC-EtOH. The experiments were performed at three times for each experimental group. Data are shown as the mean ± SD.

percentage of the control group has been considered as 100%. IC_{50} values were found of TC-EtOH37.28, 44.40 and 44.84 µg/mL on MG63, MCF-7, and L929 cells, respectively (Figure 2). TC-EtOH was reduced cell viability on MG63 and MCF-7 cells a dose-dependent manner. It has been observed that the TC-EtOH was more effective on MG63 than MCF-7 cells. Miguel et al. showed that *T. capitata* has antiproliferative activity on THP-1 cells for 24 h. The essential oils from *T. capitata* were decreased viability of THP-1 cells in a dose-dependent manner [8]. Delgado-Adamez et al. investigated the cytotoxic activities of essential oil of *T. capitata* and Thymus species on HeLa (adherent cells) and U937 (free-floating cells). They reported that these essential oils were decreased cell viability of both tumor cells in a dose-dependent [33]. Alexa et al. reported that Thyme and sage essential oils exhibited antiproliferative activity on A375 human melanoma and B164A5 mouse melanoma cells [27]. The bioactive compounds of thymus endemic species show antiproliferative activities against on two human lung cancer cell lines (A549 and NCI-H226) [39], human colon cancer (HCT116) cells [40], A375 human melanoma cells [41], MCF-7-breast and LNCaP-prostate cancer cell lines [42].

Conclusion

The results of this study revealed that the TC-EtOH established interesting biological effects. TC-EtOH displayed strong antioxidant and antimicrobial activities. Moreover, results clearly showed that the TC-EtOH has *in vitro* cytotoxic activity and *in vivo* wound healing activity. However, more studies are needed to elucidate their mechanism in this area.

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