Research Paper

# Antimicrobial, Antioxidant, Cytotoxic and Wound Healing Effects of *Thymbra sintenisii* Extract

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**Dried leaves of the *Thymbra sintenisii* have long been used traditionally in the form of herbal tea and as an antiseptic against flu in Anatolia. In the present study, *Thymbra sintenisii* extract's antimicrobial, antioxidant, cytotoxic and wound healing effects were investigated. Aerial parts of *Thymbra sintenisii* were extracted with ethanol and the chemical composition was analysed using gas chromatography-mass spectrometry. Antimicrobial activity of the ethanol extract of *Thymbra sintenisii* was evaluated using micro-well dilution method and radical scavenging activity was measured using a spectrophotometric method. Cytotoxic activity of ethanol extract of *Thymbra sintenisii* in MCF-7 and MG63 cell lines were evaluated using the XTT assay. Results revealed that thymol was the major component by 64.97 %. Ethanol extract of *Thymbra sintenisii* displayed strong antimicrobial activity against *Candida tropicalis* (0.06 mg/ml) and moderate activity against *Shigella boydii* and *Pseudomonas aeruginosa* (0.5 mg/ml) strains. $IC_{50}$ values of the ethanol extract of *Thymbra sintenisii* were against MG63, 37.28 µg/ml; MCF-7, 44.40 µg/ml and L929, 44.84 µg/ml at 24 h. Significantly faster healing was observed in wound area of the treatment group when compared to the control group. Wound area measurements showed that ethanol extract of *Thymbra sintenisii* reduced the total wound area by 30 % while that of the untreated reduced by 7 % on end of the seventh day. Antimicrobial, antioxidant, cytotoxic and wound healing activities of *Thymbra sintenisii* appeared to be quite remarkable. The results indicated that ethanol extract of *Thymbra sintenisii* could be of therapeutic potentials.**

**Key words: Antimicrobial, antioxidant, cytotoxic activity, *Thymbra sintenisii*, wound healing**

Plants and bioactive substances from plants were used as traditional medicine to treat diseases throughout history. Nowadays, treatment with plants still continues significantly in various cultures. According to the World Health Organization, approximately 65 % of the world population uses plants for treatment purposes[1]. There have been many studies related to the biological effects of plants. Therefore, extracts and essential oils obtained from plants have been used as anticancer agents in addition to antimicrobial, antiviral and antiparasitic effects. *Thymbra sintenisii* is thought as an important branch of Lamiaceae family[2-4].

*Thymbra* species are represented in the flora of Turkey by two types and 4 taxon, which are *T. spicata* L. (var. *spicata*, var. *intricata* P.H. Davis) and *T. sintenisii* Bornm. and Aznav. (subsp. *sintenisii*, subsp. *isaurica* P.H. Davis). The dried leaves of the plant, known as "white *zahter*" colloquially, are used in the form of a herbal tea in Anatolia and as an antiseptic against flu[5,6]. The biological and antimicrobial activities of *Thymbra* species have been reported in several studies[7-9]. However, only a few reports were published on *T. sintenisii*. Therefore, the aim of the study was to evaluate antimicrobial, antioxidant, cytotoxic and wound healing activities, as well as the phytochemical composition of ethanol extract of *T. sintenisii* (TSEE).

## MATERIALS AND METHODS

Ethanol, trypan blue solution, dimethyl sulfoxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and other chemicals were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained

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from Biochrom (Berlin, Germany). Penicillin and streptomycin were purchased from Gibco (Paisley, UK) and trypsin ethylenediaminetetraacetic acid solution from Biological Industries (Kibbutz Beit Haemek, Israel). Dulbecco's modified eagle medium was obtained from Lonza (Verviers, Belgium). TNF-α ELISA kit was purchased from Yehau (China).

**Plant material and extract preparation:**

Aerial parts of *T. sintenisii* were collected during August 2016 from Siirt city located in the Southeastern Anatolia region of Turkey. The plant material was identified at the Pamukkale University and verified using Flora of Turkey. Dried samples of specimen were deposited at the Pamukkale University herbarium, Turkey (M. Çiçek 2014-36 Herb) for future reference. Plant samples were dried at room conditions. Aerial parts of *T. sintenisii* were ground in a Waring blender and extracted with ethanol using a Soxhlet apparatus. Solvent was evaporated using a rotary evaporator (Stuart RE300) under reduced pressure at 30°[10].

**Analysis and identification of *T. sintenisii* extract:**

Chemical composition of TSEE was evaluated according to the method by Abay *et al*[11]. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent Technologies GC 7890A equipped with 5975 Triple Axis Detector mass spectrometer. For GC-MS detection, DBWAXETR column (60×320×0.25 m), electron ionization system and ionization energy of 70 eV were used. Helium was the carrier gas at a flow rate of 1 ml/min. The column temperature was operated under the same conditions as described above.

**Antimicrobial assay:**

Bacterial and fungal strains used were as follows, *Klebsiella pneumoniae* (ATCC 10031), *Shigella boydii* (ATCC 9905), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 7829), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10987), *Candida tropicalis* (ATCC 750) were obtained from American Type Culture Collection (ATCC) and clinical isolates of *P. aeruginosa, Acinetobacter baumannii*, *S. aureus* were obtained from clinical microbiology laboratories of Cumhuriyet University Hospital. Microorganisms were identified using the BD Phoenix 100 (Becton Dickinson, Sparks, MD, USA) automatized microbiology system.

The antimicrobial activity of TSEE was evaluated on test bacteria using Kirby-Bauer disc diffusion method[12]. In this method 100 µl of suspension containing $10^8$ CFU/ml of bacteria on Mueller-Hinton broth (MHB) was used. After the impregnation of the disc (6 mm in diameter) with 20 µl at 50 mg/ml extract, was placed on the inoculated agar. The solvent in which the extract was dissolved was used as the negative control. Commercially available cefoperazone/sulbactam (105 µg) and fluconazole (25 µg) discs were used as positive control for bacteria and fungi, respectively. The zones of inhibition around the disks were measured after 24 h of incubation at 37° for bacteria and 48 h for fungi at 28°. All the assays were done in triplicate. The samples exhibiting antibacterial activity ≥7 mm in preliminary screening were used to determine minimum inhibitory concentration (MIC).

The broth microdilution method was employed for the determination of antimicrobial activities of TSEE according to the protocol of Clinical and Laboratory Standards Institute[13]. MIC values of the TSEE were determined using the micro-well dilution method for the bacterial and fungal strains, which were found to be sensitive to TSEE in the disc diffusion assay. MIC values were determined by the serial dilution technique using 96-well microtiter plates. Serial two-fold dilutions were prepared in 96-well plates with MHB at a concentration varying between 0.03 to 2 mg/ml. The inocula of the strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The 96-well plates were prepared by adding 95 µl of MHB and 5 µl of the inocula into each well. One hundred microlitres of TSEE dilutions were added to the wells and incubated at 37°, for 24 h. The last well containing 195 µl of nutrient broth without the compound and 5 µl of the inocula on each strip was served as the negative control. Piperacillin/tazobactam (8/1) and fluconazole were used as positive control for bacteria and fungi, respectively. A microplate reader (Thermo Scientific microplate photometer, Multiskan FC, USA) was used to measure the absorbance of the plates at 570 nm after incubation for 24 h at 37°. The optical density at the 24th h of the inoculum remained the same or decreased at the MIC, which was detected as the lowest concentration of the compounds, when compared to the reading at the beginning.

**Radical scavenging activity:**

The modified method of DPPH of Yu *et al.*[14] was used for radical scavenging activity of TSEE. Varying concentrations of TSEE were prepared. Equal volumes (1000 µl) of DPPH and sample solutions were incubated for 30 min with stirring. After incubation,

DPPH was measured using spectrophotometer at 517 nm wavelength. DPPH solution and solvent were used as negative controls while the ascorbic acid used as the positive control. Samples and radical scavenging activity of the standard were calculated accordingly.

***In vivo* wound healing activity:**

Male Wistar rats (n=16) weighing between 250 and 275 g were used. This study was conducted within strict adherence to the principles of laboratory animal rules of Cumhuriyet University and the standards for Animal Experiments and Animal Care. All animals were maintained on standard pellet diet and water throughout the experiments and were housed 8 animals per cage. Rats were capable of normal activity in cages at 22±2°, with humidity (50-70 %) under a 12 h light/dark cycle[15].

Rats were anaesthetized with ketamine hydrochloride (90 mg/kg) and were then incised with 3 cm full thickness incision and a punch biopsy was opened 3×3 $cm^2$. Animals were randomly assigned to 2 groups of 8 rats each. Group 1 served as the control, whereas the other group was TSEE treated. Sterile solution of TSEE was applied topically to each wound of the animals at a dose of 50 µl/wound once daily for 7 d. The animals in all groups were euthanized in accordance with the procedures on day 7 and cardiac blood was taken. Tubes were centrifuged at 3000 rpm for 10 min. Serum was stored at –80° until measurement. TNF-α was determined using ELISA according to a previously described method[16]. Animals of all groups were appropriately terminated on d 7 of the experiment and tissue samples were taken from the wounds and were examined histopathologically. The changes occurred in wound area were regularly measured and calculated according to the following Eqn., wound contraction=(healed area/total wound area)×100 and wound healing was compared with the control group graphically (healed area=wound area–present wound area)[17].

**Cytotoxic activity and cell culture:**

MCF-7 (human breast cancer cell line, ATCC HTB-22), MG63 (human osteosarcoma cell line, ATCC CRL-1427), L929 (mouse fibroblast cell, ATCC CCL-1) cells were purchased from ATCC. Cells were grown at 37° in a humidified incubator (5 % $CO_2$). All media were supplemented with 1 % penicilin (100 U/ml), streptomycin (100 µg/ml), and 10 % FBS. Cytotoxicity was quantitatively evaluated by XTT method. The cells were seeded in 96-well plate in growth medium then treated with different concentrations of test compounds and incubated in a humidified $CO_2$ atmosphere at 37° for 24 h. After the incubation, 100 µl XTT was added to each well for another 4 h incubation. The optical density values were measured at 475 nm using a microplate reader[18].

**Statistical analysis:**

Data were expressed as the arithmetic mean±standard deviation (SD). One way ANOVA and post-hoc Tukey analyses were used to reveal the relationships between groups. The differences were accepted as significant for $p<0.05$. Statistical analysis was performed with SPSS for windows 22.0 package. All determinations were computed three times.

## RESULTS AND DISCUSSION

In Turkey there are many aromatic plant species belonging to Lamiaceae family defined as “thyme”. However, the species containing thymol/carvacrol type of essential oil are regarded as "thyme". Among these species, *Thymus*, *Origanum*, *Satureja*, *Thymbra*, and *Coridothymus* types are especially important in terms of their wide distribution and economic benefits[19]. According to the statement of World Health Organization, the increase of the resistance to antibiotics arising in recent years is accepted as one of the most significant problem for human health[20]. In this regard, potential effects of plant extracts on microorganisms have been studied by many researchers[21]. Some studies reported the presence of antibacterial and antifungal activities of several types belonging to *Thymbra* species. *Thymbra* species can be considered as a natural antimicrobial agent source[22-24]. The phytochemical composition of TSEE was analysed by gas chromatography. The major components of TSEE were thymol (64.97 %), p-tert-butylcatechol (11.99 %), borneol (2.25 %; Table 1). Khoury *et al.* reported thymol and borneol were the major components of the essential oil of some species from Lamiaceae collected from different locations in Lebanon[25].

Thymol has been used in traditional medicine for treatment of diseases for many years. Valuable information have been revealed antioxidant, antiinflammatory, antifungal, wound healing, antimutagenic properties[26,27]. Thymol (64.97 %) present in the essential oil of *T. sintenisii* showed bacteriostatic activity against most of the Gram-positive and negative bacteria. The activity of TSEE

**TABLE 1: COMPONENTS OF ETHANOL EXTRACT OF *T. SINTENISII***

| Compound | Rt[a] (min) | %[b] | Name |
|---|---|---|---|
| 1 | 8.892 | 0.53 | ß-Linalool |
| 2 | 11.241 | 0.19 | Cyclohexanecarboxylic acid, 2-hydroxy-, ethyl ester |
| 3 | 11.879 | 2.25 | Borneol |
| 4 | 14.261 | 0.86 | 7-Ethyl-4-decen-6-one |
| 5 | 15.821 | 64.97 | Thymol |
| 6 | 16.081 | 0.43 | 6-Methyl-cyclodec-5-enol |
| 7 | 16.979 | 0.21 | 1-Butyn-3-one, 1-(6,6-dimethyl-1,2-epoxycyclohexyl)- |
| 8 | 17.189 | 1.49 | 2,6-Di-tert-butylhydroquinone |
| 9 | 17.516 | 0.77 | ??? |
| 10 | 18.329 | 11.99 | p-tert-Butyl catechol |
| 11 | 18.992 | 0.44 | 5-Hepten-3-yn-2-ol, 6-methyl-5-(1-methylethyl)- |
| 12 | 19.135 | 0.15 | 1-Buten-3-one, 1-(2-carboxy-4,4-dimethylcyclobutenyl)- |
| 13 | 19.261 | 0.60 | 4-tert-Butyl-O-phenylene diacetate |
| 14 | 19.873 | 0.94 | Phenol, 4-methoxy-2,3,6-trimethyl- |
| 15 | 20.359 | 0.54 | Spathulenol |
| 16 | 20.477 | 1.66 | Caryophyllene oxide |
| 17 | 21.198 | 0.95 | Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl- |
| 18 | 21.475 | 0.71 | Isoaromadendrene epoxide |
| 19 | 21.668 | 0.59 | Longipinocarveol |
| 20 | 23.295 | 0.20 | 1-Nonadecene |
| 21 | 24.185 | 0.32 | Syringic acid |
| 22 | 24.386 | 0.19 | 3,4,5-Trimethoxyphenylacetic acid |
| 23 | 24.529 | 0.15 | 1,7-Octadiene, 2,5-bis-(cis)-(2,2-dimethyl-3-carboxycyclopropyl)- |
| 24 | 24.68 | 0.17 | Cyclohexanol, 4-(1,1-dimethylethyl)-1-(2-propenyl)- |
| 25 | 25.703 | 0.22 | 4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol |
| 26 | 26.768 | 1.03 | Naphthalene, 1,2,3,4,4a,5,6,7-octahydro-4a-methyl- |
| 27 | 27.423 | 1.32 | Hexadecanoic acid |
| 28 | 30.854 | 0.15 | 4-(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-butan-2-one |
| 29 | 32.154 | 1.18 | Androstan-17-one, 3-ethyl-3-hydroxy |
| 30 | 32.229 | 0.63 | Retinoic acid |
| 31 | 32.439 | 0.33 | ??? |
| 32 | 32.498 | 0.65 | Norethindrone |
| 33 | 33.093 | 0.17 | 5-Pregnen-3ß-ol-20-one, methyl ether |
| 34 | 33.387 | 0.32 | 1,4,7-Androstatrien-3,17-dione |
| 35 | 34.402 | 0.51 | Pregn-5-ene-3,20-dione |
| 36 | 35.878 | 0.35 | 5-Pregnen-3ß-ol-20-one, propionate |
| 37 | 36.063 | 0.22 | 16-Methyl-20-oxopregn-5-en-3-yl acetate |
| 38 | 36.44 | 0.66 | 5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-penta-2,4-dien-1-ol |
| 39 | 36.885 | 0.14 | Bufotalin |
| 40 | 37.539 | 0.38 | Cycloeucalenyl acetate |
| 41 | 38.294 | 0.16 | Naphtho[2,3-b]furan-2-one, 3-[[(benzo[1,3]dioxol-5-ylmethyl)amino] |
| 42 | 42.69 | 0.16 | ??? |
| 43 | 59.249 | 0.15 | 5ß-Cholestan-26-oic acid, 3α,7α,12α-trihydroxy- |

[a]Retention time, [b]relative percentage obtained from peak area

was found to be 0.5 mg/ml against *S. boydii* and *P. aeruginosa* (clinical isolate) strains, whereas ≥2 mg/ml MIC value was identified against other bacterial strains. Extract showed a quite strong activity against *C. tropicalis* with 0.06 mg/ml (Table 2). Antimicrobial activity level of *T. sintenisii* ethanol extract was compared in terms of MIC values, it has been found that it had the strongest effect against *C. tropicalis*, then against *S. boydii* and *P. aeruginosa* strains. The antimicrobial effect against other tested strains is found to be weaker. Olasupo *et al.* revealed antimicrobial effect of thymol[28]. Another study, thymol exhibited antimicrobial activity against *S. aureus* and *Escherichia coli*[29]. Thymol inhibits

**TABLE 2: ANTIMICROBIAL ACTIVITIES OF *T. SINTENISII* EXTRACT**

| Gram-negative bacteria | Zone of inhibition[z] | | MIC (mg/ml) | |
|---|---|---|---|---|
| | Ext | Cont[a] | Ext | Cont[b] |
| *Klebsiella pneumoniae* | 7.3±0.5 | 26.6±2.0 | >2 | 0.03 |
| *Shigella boydii* | 18.6±1.1 | 25.0±1.7 | 0.5 | <0.03 |
| *Pseudomonas aeruginosa* (clinical isolate) | 7.4±0.4 | 25.4±3.1 | 0.5 | <0.03 |
| *Pseudomonas aeruginosa* | 7.6±0.5 | 24.6±1.1 | >2 | <0.03 |
| *Proteus vulgaris* | 9.6±1.1 | 28.6±1.1 | 2 | <0.03 |
| *Acinetobacter baumannii* (clinical isolate) | – | – | – | – |
| **Gram-positive bacteria** | | | | |
| *Staphylococcus aureus* (clinical isolate) | 7.8±0.9 | 21.9±1.4 | >2 | 0.03 |
| *Staphylococcus aureus* | 8.3±0.5 | 19.6±1.5 | >2 | 0.03 |
| *Bacillus cereus* | 12.3±0.5 | 32.3±0.5 | 1 | <0.03 |
| **Fungi** | **Ext** | **Cont[c]** | **Ext** | **Cont[d]** |
| *Candida tropicalis* | 11.8±1.4 | 13.1±0.8 | 0.06 | 0.03 |

Ext: ethanol extract of *T. sintenisii*, Cont: control, '[a]' cefoperazone/sulbactam (2:1), '[b]' piperacillin/tazobactam (8:1), '[c]' fluconazole disc (25 µg), '[d]' fluconazole, MIC: minimum inhibitory concentration, '[z]' values are mean±SD (mm, n=3)

growth of *S. aureus*, *E. coli* and *Salmonella typhimurium*[30]. As mentioned in the literature, thymol exhibited high antibacterial and antifungal activities[25,31].

In this study radical scavenging activity of TSEE was investigated and $IC_{50}$ value was found to be 12.5 µg/ml. $IC_{50}$ value of the standard ascorbic acid was 5.02 µg/ml (fig. 1). Bozin *et al.* have reported that the antioxidant activity of Lamiaceae types was high[32]. These authors reported $IC_{50}$ of DPPH assay as 0.39 µg/ml. Saidi *et al.*[24] have studied the antioxidant and antimicrobial properties of *T. spicata* L. and reported that the antioxidant and antimicrobial properties of this plant were remarkable. $IC_{50}$ value of *T. spicata* L. was found to be 1.28 µl/ml. In another study, essential oil of *T. capitata* was reported to exhibit potent antioxidant activity[33]. Strong antioxidant activity observed in this investigation was consistent with the results of other studies.

The premier objective is to destroy cancer cells in oncologic research, but to alleviate side effects of anticancer agents it is essential that the newer agents interfere with fewer biochemical pathways. Cytotoxic activity was determined by XTT method after incubating the cells for 24 h with the compound at increased concentrations. The extract was tested on MCF-7 and MG63 cancer cell lines to find out its cytotoxicity activity. Healthy L929 mouse fibroblast cell line was used as control. Extract was found to have cytotoxic effects on both cell lines at 40 µg/ml concentrations and above (fig. 2). $IC_{50}$ values were found to be against MG63- 37.28 µg/ml, MCF-7- 44.40 µg/ml and L929- 44.84 µg/ml at 24 h (fig. 2). Delgado-Adámez *et al*. reported that cytotoxic

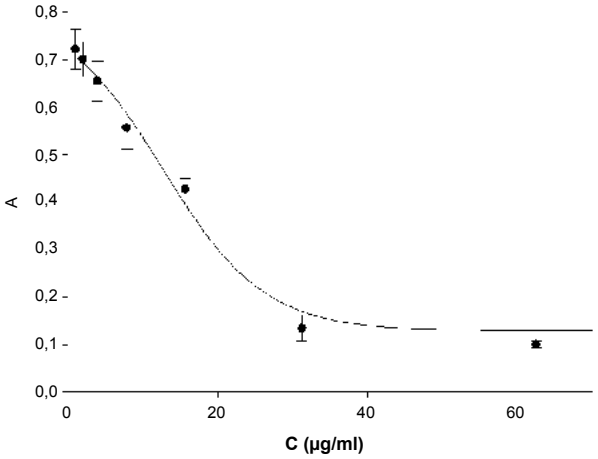


**Fig. 1: DPPH graph of TSEE**
**A: Absorbance, C: concentration of TSEE (*T. sintensii* ethanol extract)**

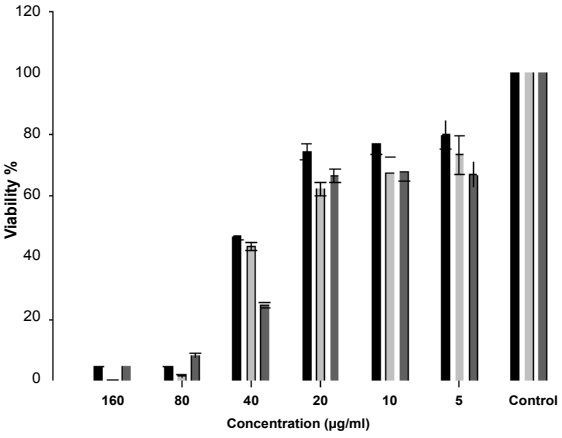


**Fig. 2: Cell culture treatment with TSEE**
**$IC_{50}$ values for TSEE (*T. sintenisii* ethanol extract) at 24 h against MG63- 37.28 µg/ml, MCF-7- 44.40 µg/ml and L929- 44.84 µg/ml. p<0.05 vs control. ■ L929; ■ MCF-7; ■ MG63**

activities of essential oil of *T. capitata* and *Thymbra* species on human epithelioid cervix carcinoma and leukaemia cell lines[34]. Thymol was reported to be a

major component of thyme. Yeh *et al.* demonstrated that thymol decreased cell viability on human prostate cancer cells[35]. Thymol stimulated cytotoxic activity in MCF-7 with $IC_{50}$ of 2.5 µg/ml[36]. Chang *et al.* reported that thymol (400 µg/ml) trigger cytotoxic activity in MG63[37].

Wound is result of disruption of normal anatomical structure and functional integrity. Recently, many studies reported about wound healing, but these have not been at the desired level yet. TNF-α is a pro-inflammatory, angiogenic cytokine and growth factor. Therefore, determination of TNF-α level would be an important determinant for wound healing[38]. In the present study, the effect of TSEE was determined and compared to the control, which was found to be at the 7/30 ratio, indicating that the extract possessed at least four times better healing activity compared to untreated (fig. 3). The extract significantly accelerated wound healing process in the treated rats. It has been observed that TNF-α level of the treated group was decreased about 30 % in 7 d (fig. 4).

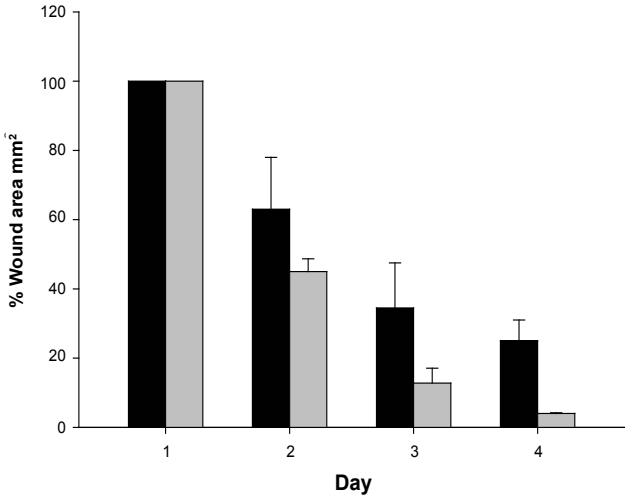


**Fig. 3: Wound area**
**1: Day 0 of the wound, 2: day 3 of the wound, 3: day 5 of the wound, 4: day 7 of the wound (■) control, (■) treatment group**

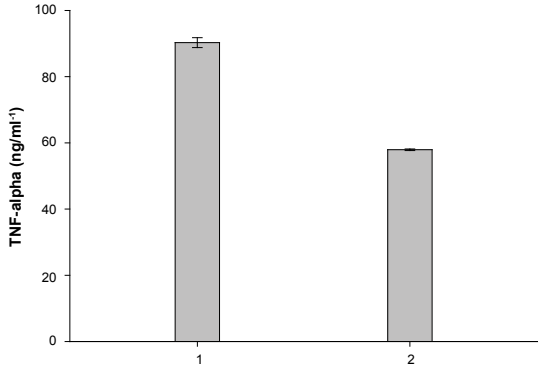


**Fig. 4: TNF-alpha levels**
**1: Control, 2: extract**

As a result, in this study it was observed that antimicrobial, antioxidant, cytotoxic activities and wound healing effect of *T. sintenisii* were quite remarkable. These results indicated that this plant could be used as a natural source for developing newer therapeutic agents. Further studies are needed to better understand the molecular mechanisms underlying these effects of *T. sintenisii* extract.

## Conflict of interest:

The authors report that they have no conflicts interest


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Nil.


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