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A study on the antioxidant, antimicrobial and cytotoxic activity of *Thymbra spicata* L. var. *spicata* ethanol extract

Thymbra spicata L. var. spicata etanol ekstresinin antioksidan, antimikrobiyal ve sitotoksik etkisinin araştırılması

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SUMMARY

Objective: Thymbra species are widely used in Turkish folk medicine as salad or herbal tea for antioxidant, antiseptic, antitussive, carminative, anti-inflammatory and antimicrobial activities. The present study was aimed to determine antioxidant, antimicrobial and cytotoxic properties of ethanol crude extract prepared from Thymbra spicata L.var. spicata.
Method: The antioxidant properties of ethanol extract of Thymbra spicata L. var. spicata was investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) radical scavenging activity, total flavonoid and total phenolic content analysis. Antimicrobial activity of the plant extract were determined using the microdilution method, while proliferative activity were evaluated by MTT assay on MCF-7 breast cancer and PC3 prostate cancer cell line.
Results: Total phenol and total flavonoid content were 216.75 ± 0.017 (equivalent of Gallic acid mg/g dry extract) and 78.17 ± 0.063 (equivalent of Quercetin mg/g), IC50 value for DPPH and ABTS scavenging activity was 16.91 µg/mL and 5.2 ± 0.009 µg/mL, respectively. Antimicrobial activity observed for the extract ranging between 1.25 and 2.5 mg/mL as MIC value against tested microorganism. MTT assay was employed for evaluation of cytotoxic activity, ethanol extract demonstrated growth inhibitory effect against MCF-7 breast cancer cells with IC50 value 340 µg/mL.
Conclusions: According to the obtained results, antioxidant, antimicrobial and cytotoxic activity of Thymbra spicata L.var. spicata is scientifically support the widespread use of this plant among the people.
Keywords: Thymbra spicata L.var. spicata, antioxidant, antimicrobial, cytotoxicity

ÖZET

Amaç: Tymbra türleri halk arasında antioxidant, antiseptic, antitussive, karminatif, antienflamatuvar ve antimikrobiyal özelliklerinden dolayı salata ve bitkisel çay olarak yaygın kullanılmaktadır. Çalışmamızın amacı, Thymbra spicata L. var. spicata.'dan hazırlanan ham etanol özütünün antioksidan, antimikrobiyal ve sitotoksik etkisini araştırmaktır.
Yöntem: T.spicata L.var. spicata etanol ekstresinin antioksidan aktivitesi DPPH ve ABTS serbest radikal süpürücü etki, total fenol ve total flavonoid miktar tayini yöntemleri kullanılarak değerlendirildi. Antimikrobiyal aktivitesi ise mikrodilüsyon metodu ile, sitotoksisite aktivitesi prostat kanseri PC3 hücre hattı kullanılarak MTT metoduyla değerlendirildi.
Bulgular: Total fenol ve total flavonoit miktarı kuru ekstre bazında 216.75 ± 0.017 (gallik asite eşdeğer mg/g) ve 78.17 ± 0.063 (kersetine eşdeğer mg/g) olarak bulundu. DPPH ve ABTS radikal süpürücü etki IC50 değeri sırasıyla 16.91 µg/mL and 5.2 ± 0.009 µg/mL olarak bulundu. Etanol ekstresinin test edilen mikroorganizmalar karşı minimum inhibe edici konsantrasonu 1.25-2.5 mg/mL aralığında tespit edildi. Sitotoksik aktivitenin değerlendirilmesi için MTT testi kullanıldı, etanol ekstraktı MCF-7 meme kanseri hücrelerine karşı IC50 değeri 340 µg / mL olan büyümeyi önleyici bir etki gösterdi.

Sonuç: Elde edilen bulgulara göre, Thymbra spicata L.var. spicata bitkisinin antioxidant, antimikrobiyal ve sitotoksik aktivitesi bu bitkinin halk arasındakı yaygın kullanımını bilimsel yönden desteklemektedir.
Anahtar sözcükler: Thymbra spicata L.var. spicata, antioksidan, antimikrobiyal, sitotoksisite

INTRODUCTION

Cancer is one of causes of death and being a major concern in the worldwide. Plants play very important role in drug discovery and development of cancer chemotherapeutic agents due to their multiple phytochemical compounds and great effectiveness and toxicity tolerance. In fact, most of the plant drived toxic molecules are widely used in chemotherapy. Nowadays, there is a recrudescence of interest for the natural alternative such as medicinal plants and dietary[1]. Reactive oxygen species (ROS), produced from extracellular and intracellular sources in excessive amounts, reacts with DNA, lipids, and cellular proteins can causes oxidative stress[2] Previouse reports suggests that there is a strong relationgship between ROS and some chronic diseases such as cancer, neurologic disorder, cardiovascular disease and age related disease[3]. Antioxidants are the biochemical constituents produced in the body, serve as the biological defense system, in case of their inefficient, dietary antioxidants should be take in order to prevent oxidative stress from further damaging the biological system. a plethora of studies demonstrated that utilization of plant-origin antioxidants can be reduce the risk of occurrence of numerous human diseases narrated to the oxidative stress[4-5] Extensive use of antibiotics and the problem of emerging infectious dissseases which are resistance to currently available antibiotics, which made it ineveitable to search for new antimicrobials of plant origin[6].

Thymbra spicata L. is represented by four taxa and two species: *T. spicata* var. *spicata* and *T. spicata* var. *intricata* in Turkey. *T. spicata* is naturally found in Southeastern Anatolia, coastal areas of Thrace, Aegean and Mediterranean region of Turkey[7-8]. *T. spicata*, is a thyme-like plant, is known as "Zahter" and "Karabas kekik" in the Southeastern Anatolia Region of Turkey due to contains a variety of aromatic compounds mainly are carvacrol and thymol. It is used in salads and tea either dry or fresh leaves and flowers. Dried aerial parts of the plant are used in the form of herbal teas, spice and to treat colic, bronchitis, asthma, coughs, diarrhoea and rheumatism in folk medicine in various regions of Turkey[9]. *Thymbra* has been reported to be used as raw material in pharmaceutical formulations due to its significant pharmacological properties in many countries[10]. However, there is no available report on the pharmacological activities of extract excepet from essential oil. Therefore in the present study, the plant material was obtained from herbal market in Hatay province of Turkey and prepared ethanol extract by maceration method to study their antioxidant, antimicrobial and cytotoxic properties.

MATERIAL AND METHODS

Chemicals

1,1-Dipheny l-2-picryl-hydrazyl (DPPH), 2,2-azino-bis (3-ethylbenz- thiazoline-6-sulfonic acid (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quercetin, and gallic acid were obtained from Sigma Aldrich Co., St. Louis, USA. All other chemicals used were of analytical grade.

Plant Materials

The plant material were obtained from herbal market of Hatay province.and identificated by botanist Dr. Mehmet Tekin, Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Botany.

Preparation of the extract

The dried herbs of *T. spicata* L.var. *spicata* were pulverized using a grinder and 100g of them was macerated in 1000 mL of 80% ethanol (Ethanol : water = 80 : 20) for 48h with intermittent shaking at room temperature. The crude aqueous ethanol extract was filtered through Whatman filter paper No.1. and concentrated with rotary evaporator (Buchi R-100 equipped with Vacuum Pump V-300 and Control unit I-300) at 40°C to constant dryness, and stored at -20ºC for further use.

Antioxidant Activities

DPPH free radical scavenging activity

The scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical was evaluated by the method of Blois[11]. Sample solutions were prepared as a series of concentrations (1, 10, 50, 100, 250, 500, and 1000 µg/mL) by diluting 2mg/ml of extract stok solution dissolved in %10 DMSO of methanol). Ascorbic acid was used as standard in 1-100 µg/mL solution. 0.1mM of DPPH was prepared in methanol and mixed with sample solution in a 1:3 volume to volume ratio in test tubes separately in triplicates.

These solution mixtures shanked vigorously, then were allowed to stand at dark for 30 min and optical density of reduced DPPH. was measured at 517 nm. Methanol with DPPH solution was used as blank. Scavenging DPPH. activity of plant extracts by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations. The % inhibition was calculated by the formula given below:

Percent (%) inhibition of DPPH activity = (Absorbans of Blank-Absorbans of Test) / Absorbans of Blank × 100

ABTS radical cation decolorization assay

The scavenging activity of ABTS free radical was determined according to the method described by Re et al[12].ABTS radical cations ($ABTS^{\bullet+}$) was generated by reacting 2.45 mM potassium persulphate with a 7 mM aqueous ABTS stok solutions. The ABTS cation working solution was obtained by mixing the two stok solutions in equal volumes and incubate them to react for 16 h at 25°C in the dark. Before using, this solution was dilute with methanol to give the absorbance of 0.70 ± 0.02 at 734nm and equilibrated at 30°C. Ascorbic acid used as references standard. 1mL of sample was reacted with 1 mL of fresh $ABTS^{\bullet+}$ radical cation solution in test tubes, and absorbance is read (at 734 nm) after 7 min incubation in the dark. ABTS radical scavenging capacity was expressed as Trolox equivalents (in µM).per gram of dry extract.

Determination of total phenolics contents

The determination of total phenolic content was performed through Folin-Ciocalteu method with slight modifications[13]. Briefly, 50 µL of 1 mg/mL crude extract was mixed with 450 µL of distilled water and 2.5 mL of 1N Folin-Ciocalteu reagent. After 5 min, 2 mL of %7.5 sodium carbonate was added. The absorbance of the solution was read at 730 nm in spectrophotometer after 30 min of incubation at 37ºC. The total phenolics content was expressed as milligrams gallic acid (GAE) equivalents per gram of dried extract.

Determination of total flavonoids contents

The determination of flavonoids was performed according to the method developed by Misbah et al[14]. 500 µL of crude aqueous ethanol extract prepared with methanol as 1 mg/mL were mixed with 1.5 mL of %95 ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of deionized water. After incubation at room temperature for 30min, the absorbance of reaction mixture was determined by spectrophotometer at 415 nm. Ethanol was used as a blank. The content of flavonoids was established as quercetin mg/g dry extract. The experiments were conducted in triplicate.

Antimicrobial Activities

Aqueous ethanol extract of *T.spicata* L.var. *spicata* was determined for antimicrobial potential by micro-well dilution assay as previously reported[15] against bacterial and fungal strains: Gram positive (*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)), Gram-negative (*Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922)) and fungal strain (*Candida albicans* (ATCC 10231)). Plant extracts were dissolved in 8% DMSO to prepared 20 mg/mL of stok solution. 50 µL sterile distilled water was added in each well of 96-well microtiter plate. 50 µL of plant extract was added into the first well and a serial two-fold dilution was performed by transferring 50 µL of the suspension to the subsequent wells up till the 9th well; the final 50 µL of the suspension was discarded. 10th well was added Gentamicin for bacteria and Flucanazole for Candida used as drug positive control while 11th well was added 50 µL 2% DMSO used as negative control and 12th well was added 50 µL Mueller Hinton Broth (sterility control). Concentration of plant extract in wells ranged from 5.00 to 0.02 µg/mL. Final inoculum size was 5 $x10^5$ CFU/mL at bacteria and 0.5-2.5 $x10^3$ CFU/mL at Candida in each well. Mueller Hinton Broth and Saboraud Dekstroz Broth was used for dilution bacteria and Candida culture's, respectively. Microtiter plates were incubated at 37 °C for bacteria and 35 °C for *Candida* between 16-24 hours. Afterwards, 50 µL of freshly prepared 2 mg/mL 2,3,5-Triphenyltetrazolium chloride (TTC) (Meck, Germany) was added to each well of the plate to indicate microbial growth. It was incubated at 37 °C for a further 2 h. Reduction in density of formazan's red color after incubation was accepted MIC value. The experiment was performed in duplicate and the standard deviation was zero.

Cytotoxicity assay

The *In vitro* antiproliferative activity was carried out by the colorimetric MTT assay[16]. Exponential growing breast cancer MCF-7 cells and prostate cancer PC-3 cells and control cells L929 mouse fibroblast cell line were plated in 96-well micropates at a density of 5×10^3 cells per well in 100 µL of 10% FBS contained RPMI-1640 culture medium and were allowed to adhere for 16 h, cultured at 37 °C in a humidified atmosphere of 5% CO_2 before treatment. Increasing concentrations of extract (1–1000 µg/mL) in their

respective extraction solvent were then added. The final concentration of ethanol in the culture medium was maintained at 0.5% (v/v). The cells were incubated for 24 h in the presence or absence of extract. After incubation, 100 µL of MTT solution [5 mg/ml in PBS: medium (1:3)] was added per well, and the plate incubated for 4 h to allow reaction of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved with 100 mL of dimethyl sulfoxide (DMSO). The absorbance of purple formazan, proportional to the number of viable cells, was measured at 595 nm using a microplate reader (Epoch, USA). The experiments were carried out in triplicate. The cytotoxicity of extract was presented as IC_{50} value, defined as the concentration that caused a 50% of cell death. The IC_{50} value was calculated from extract concentration – cell growth inhibition curve by Graphpad prism 7.0 software.

RESULTS AND DISCUSSION

The yields of crude ethanol extract was 16.97 % calculated according to dry weight basis. Scavenging activity for free radicals of DPPH and ABTS have been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. Free radical scavenging activity of ethanolic extract *T. spicata* was quantitatively determined using DPPH assay. Figure 1 shows the results of DPPH radical scavenging assay. When the DPPH radical scavenging activity of the *T.spicata* extract examined, it was found that the extract have potent radical scavenging activity as quercetin in Fenton reagent environment[17]. Previous study on the antioxidant activities of other species of the genus *Thymus* measured by DPPH, showed an IC_{50} value of 16.15 µg/mL for methanol extract of *T.spathulifolius*[18], 38.2 and 44.5 µg/mL for methanol and hexane fractions of *T.capitatus*[19].

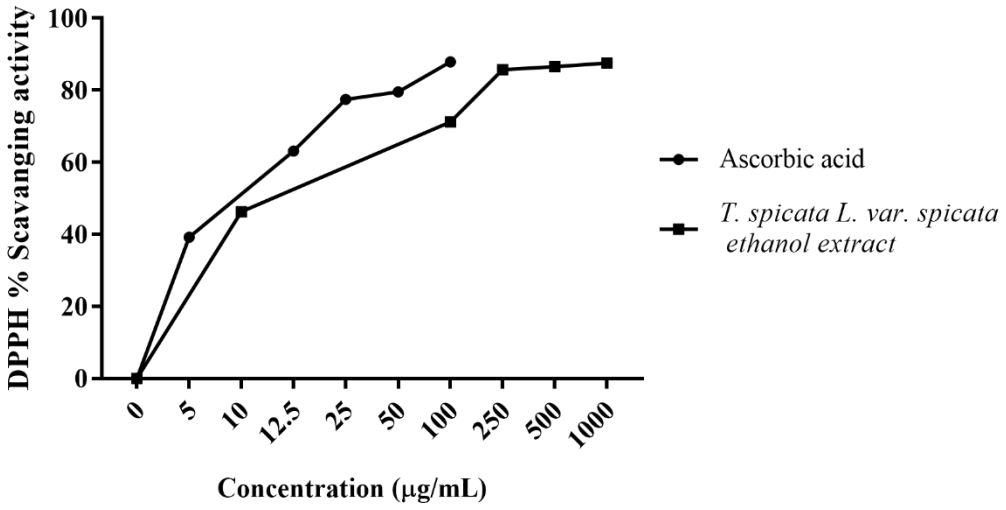

Figure 1. DPPH free radical scavenging activity of ethanol extracts of *T.spicata* L.var. *spicata*

The ABTS radical scavenging activity of the extract was given in Figure 2. When compared to DPPH, the extract have potential ABTS radical scavenging activity in lower concentration. Therefore, the extract may be contain polar constituent which are more potent in radical scavenging activity on ABTS than DPPH, because the ABTS is water soluble radical while DPPH is water in-soluble radical.

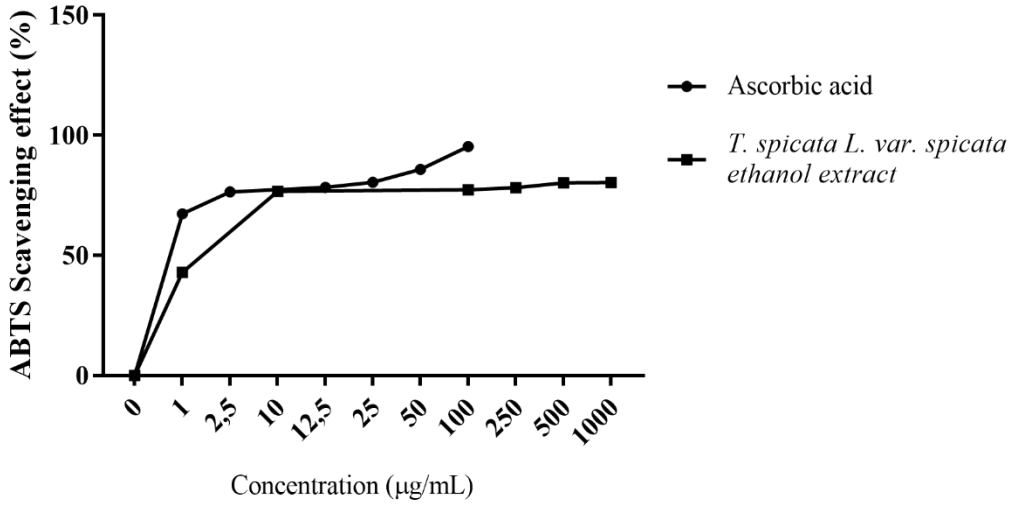

Figure 2. ABTS radical scavenging activity of ethanol extracts of *T.spicata* L.var. *spicata*

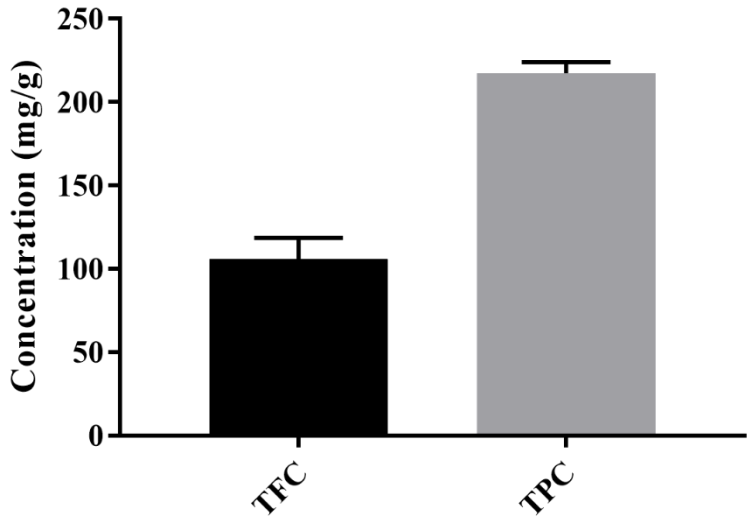

Figure 3. Total phenolic (TPC) and flavonoid (TFC) content of ethanol extracts of *T.spicata* L.var. *spicata*

The total phenolic and total flavonoid contents of ethanol extracts of *T.spicata* L.var. *spicata* revealed significant amounts of TPC and TFC as given in Figure 3. Total phenolic content in the extract 217.37 mg/g GAE and total flavonoid content was 105.97 mg quercetin equivalent flavonoid in g dry weight of extracts. Though the bioactivity of flavonoids appears to be mediated through a variety of mechanisms, particular attention has been focused on their direct and indirect antioxidant actions.

Antimicrobial activity

MIC values of the ethanol extract of *T.spicata* L.var. *spicata* was detected with broth microdilution assay. As can be seen from the Table 1, plant extracts showed different antimicrobial activity against the test microorganisms. MIC values of the extracts in the range between 1.25–2.5 mg/mL. According to the results, *E.faecalis* was the less sensitive against the extract of *T.spicata* L.var. *spicata* then other microorganisms which have the lowest MIC values 2.5 mg/mL. In previous studies, it was reported that the *T.spicata*

extracts was potentiated the antimicrobial properties of antibiotics from 8 to 128 fold against multridrug resistant strains when combinated with *T.spicata* extract [20]. In current study, the MIC value for all the tested strains was lower than the reports as 6.25-100 mg/mL for ethanol, aqueous and petroleum ether extract, which reflects a good antibacterial effect against studied strains.

Table 1. Minimum inhibitory concentrations of ethanol extrat of *T.spicata L. var. spicata*

	Ethanol extract	mg/mL
S/No.	Microorganisms	
1	*E.coli*	1.25
2	*S.aureus*	1.25
3	*P.aeruginosa*	1.25
4	*E.faecalis*	2.5
5	*C.albicans*	1.25

Cytotoxicity assay

The proliferative effect of plant extracts on cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan by the action of mitochondrial enzyme succinate dehydrogenase in viable cells. The results indicate that ethanol extract have antiproliferative activity aganist MCF-7 cancer cells with IC_{50} of 340 µg/mL, while not effecient on PC-3 human prostate cancer cells (Figure 4). The hydro-alcoholic extract of *T.spicata* on lung cancer cell line SK-Mes-1 exhibited dose-dependent cytotoxic effect and IC_{50} was determined as 110 µg/mL[21]. The antiproliferative activity attributed to the chemical composition of the extract or essential oil. It has been studied that thymol and carvacrol are the major constituents of the plant either in essential oil and extract[22]. The main compounds also have been investigated for their biological activities. Thymol and carvacrol were found to be more potent against MCF-7 cancer cell lines than other compounds presented in the essential oil of *S.thymbra*[23]. Carvacrol has been studied against various cancer cell lines and showed moderate to strong antiproliferative activity[24–27]. When these data taking into consideration, it is possible that the amounts of the thymol and carvacrol in the extract or essential oil determine the degree of antiproliferative effect or other biological activities.

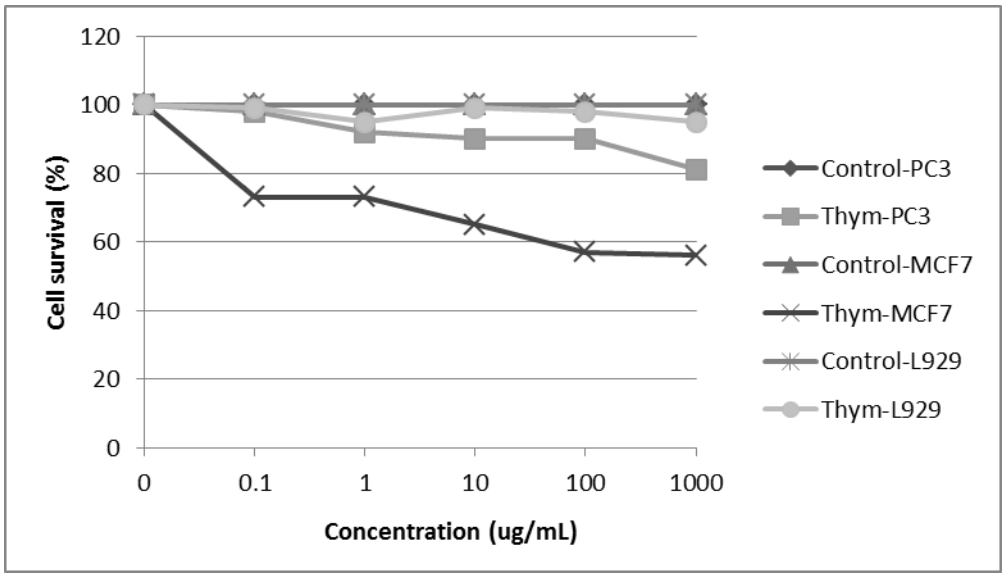

Figure 4. Cytotoxicity activity of ethanol extracts of *T.spicata* L.var. *spicata*

CONCLUSION

In the light of our results of investigations, it can be conclude that the ethanol extract of *T.spicata* herein exhibited strong antioxidant, antimicrobial and antiproliferative potential, it may be related with the presence of higher content of phenolic and flavonoid compounds. These findings confirm the use of the studied plants in Turkish ethnomedicine. This is the first study as far as we are aware that the antiproliferative activity of the ethanol extract of *T.spicata* has been studied. The results of the present study show that the ethanol extract *T.spicata* contained a high total phenolics level, and can be a promising source of antioxidant, antibacterial as well as health-promoting agents; therefore, it can be considered potentially useful for medicinal application. However, more detailed *in vivo* studies are required to make firm the safety, bioavailability and quality control of *T.spicata* L.var. *spicata* as well as and further research linked to identify the active molecule via bioassay-guided isolation.

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