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Antioxidant, Anti-urease Activities and Genotoxic Effects of *Terfezia Claveryi* Methanol Extracts on Human Lymphocytes

Terfezia Claveryi Metanol Ekstrelerinin Antioksidan, Anti-üreaz Aktiviteleri ve İnsan Lenfosit Hücreleri Üzerindeki Genotoksik Etkileri

Turgut Taşkın[1], Tuğçe Yeşil Devecioğlu[2], İsmail Şenkardeş[3]

[1] Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, Turkey
[2] Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Turkey
[3] Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Turkey

ORCID IDw of the authors:

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ABSTRACT

Objective: *Terfezia claveryi* is a dark brown color truffle and has been used extensively in human diet in Turkey. Therefore; it is an important issue to determine their biological activities and toxicity. This study aims to investigate the antioxidant and anti-urease effects of *T. claveryi* methanol extracts and to evaluate the genotoxicity of each extract.

Methods: The antioxidant activities of methanol extracts were examined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) and ferric reducing/antioxidant power (FRAP) methods. The amount of total phenolic compounds contained in the extracts was determined using the Folin-Ciocalteu reagent (FCR) method. Anti-urease activities of methanol extracts were evaluated by indophenol method. In addition, potential DNA damaging effects of *T. claveryi* extracts on human lymphocytes were determined by the alkaline comet assay.

Results: UM extract exhibited higher DPPH radical scavenging activity than MM extract [the concentration of the substance which provides the inhibition of 50% of the radical (IC_{50}):0.77±0.01 mg/mL, 1.17±0.02 mg/mL, respectively, p<0.0001] however, lower activity than standard (p<0.0001). Both extracts in different concentrations of *T. claveryi* caused DNA damage (p<0.0001) compared with solvent control DMSO. Also, total comet scores was higher in cells which had been incubated with MM extract (p<0.05). UM extract exhibited higher ferric reducing and anti-urease activity than MM extract but not found statistically different; also activities of both extract were found lower than positive controls of each methods (p<0.0001).

Conclusion: *T. claveryi* methanol extracts induced genotoxicity on lymphocytes and showed very low antioxidant activity compared to standards. This preliminary study should be confirmed with further studies and in different cell lines.

Keywords: *T. claveryi*, genotoxic, antioxidant, anti-urease, maceration, ultrasonic bath

ÖZ

Amaç: *Terfezia claveryi* koyu kahve renkli bir trüf mantarı olup, Türkiye'de gıda olarak kullanımı oldukça yaygındır. Bu yüzden, mantarın biyolojik aktivitelerinin ve toksitesinin incelemesi önemli bir husustur. Bu çalışma ile *T. claveryi* metanol ekstrelerinin antioksidan, anti-üreaz aktivitelerinin araştırılması ve her bir ekstrenin genotoksisitesinin değerlendirilmesi amaçlanmıştır.

Yöntemler: Metanol ekstrelerinin antioksidan aktiviteleri 2,2-difenil-1-pikrilhidrazil (DPPH), 2,2'-azinobis-(3-etilbenzotiazolin-6-sulfonik asit) (ABTS) ve demir (III) iyonu indirgeme antioksidan gücü (FRAP) yöntemleri ile incelendi. Ekstrelerin toplam fenolik bileşik içeriği Folin-Ciocalteu reaktifi (FCR) ile tayin edildi. Metanol ekstrelerinin anti-üreaz aktiviteleri indofenol yöntemi ile değerlendirildi. Ayrıca, *T. claveryi* ektrelerinin DNA hasarı üzerindeki etkisi alkali comet tekniği ile insan lenfosit hücrelerinde incelendi.

Bulgular: UM ekstresi, MM ekstresinden daha güçlü DPPH radikal süpürücü aktivite gösterdi [radikalin % 50'sinin inhibisyonunu sağlayan madde konsantrasyonu (IC50): 0.77±0.01 mg/mL, 1.17±0.02 mg/mL, sırasıyla p<0.0001]. Fakat her iki ekstrenin standarda göre daha düşük radikal süpürücü aktivite gösterdiği tespit edildi (p<0.0001). Her iki ekstrenin farklı konsantrasyonlarının çözücü DMSO ile kıyaslandığında DNA hasarına yol açtığı (p<0.0001) ve MM ekstresi ile inkübe edilen hücrelerde total comet skorunun yüksek bulunduğu saptandı (p<0.05). UM ekstresi, MM ekstresinden daha yüksek demir (III) iyonu indirgeyici ve anti-üreaz aktivite göstermekle beraber fark istatistiksel anlamlı bulunmadı ve her iki yöntemde de ekstrelerin pozitif kontrollerden daha düşük aktivite gösterdiği saptandı (p<0.0001).

Sonuç: *T. claveryi* metanol ekstreleri lenfositler üzerinde genotoksisiteye neden olmaktadır ve her iki ekstre standartlara göre düşük antioksidan aktivite göstermektedir. Bu ön çalışmanın sonuçlarını ileri çalışmalarda farklı hücre dizilerinde doğrulanması gerekmektedir.

Anahtar Kelimeler: *T. claveryi*, genotoksik, antioksidan, anti-üreaz, maserasyon, ultrasonik banyo

Correspondence Author/Sorumlu Yazar: Turgut Taşkın **E-mail/E-posta:** turguttaskin@marmara.edu.tr
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INTRODUCTION

Edible mushrooms have been used for nourishment, as functional foods to prevent ailments, and as a source of medicine to treat diseases. Edible mushrooms are used for lowering cholesterol, lowering blood pressure, strengthening the immune system against diseases, fighting tumors and improving liver function (1).

Desert truffle (Ascomycetes) is a kind of edible mushroom that grow in arid and semiarid areas on every continent other than Antarctica. It has been shown that Desert truffle contain rich fiber, protein, amino acids, fatty acids, minerals, vitamins, carbohydrates, potassium, phosphate, ascorbic acid, anthocyanins, esterified phenols, free phenolics, flavonoids, and carotenoids (2).

Terfezia and *Picoa* are two of the most common edible desert truffles in the world. *Terfezia claveryi* Chatin (Family, Terfeziaceae) is the dark brown color truffle and the juice of these mushrooms have been used in the Middle East to treat eye and skin diseases. *T. claveryi* contains saturated fatty acid, linoleic acid, protein, and carbohydrate compounds (3).

Turkey is a rich country in mushroom diversity and medicinal plants. Turkish people have a tradition of using various types of mushrooms for food, instead of using them for the treatment of various ailments (2). In consideration of the extensive use of mushrooms in human diet, it is an important issue to determine their bio-potential and toxicity. Therefore, the aim of this study was to comparatively reveal the antioxidant and anti-urease effects of *T. claveryi* methanol extracts obtained by using two extraction method followed by to evalute the genotoxicity of each extract.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate), ascorbic acid, 2,2-diphenyl-1-picryl-hydrazyl, Folin Ciocalteu's phenol reagent, gallic acid and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt were obtained from Fluka (Sigma-Aldrich; Buchs, Switzerland). All other reagents were of analytical grade.

Collection and identification of material

Terfezia claveryi Chatin were collected during the edible season from Batman-Sason, Turkey. The taxonomic identity of the material was confirmed by Assist. Prof. Dr. Ismail Senkardes. The voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Marmara University; herbarium numbers: MARE-18813.

Preparation of extracts

The fresh mushroom materials were washed carefully with water. Then, this parts were cut into small pieces. Two different extraction methods were followed to prepare crude extracts from this mushroom materials

***Maceration*:** 94 g materials was extracted with methanol for 7 days at 25 °C.

***Ultrasonic bath extraction*:** Mushroom materials (94 g) was extracted with 300 mL methanol for 30 min. in ultrasonic bath set at 30 °C.

The maceration methanol (MM) and Ultrasonic bath methanol (UM) extracts were filtered through filter paper and concentrated by rotary vacuum evaporator (Heidolph Hei-Vap; Schwabach, Germany). All the extracts obtained were stored at 4 °C for future analysis.

After extraction, studied concentrations were determined by preliminary experiments accordingly results of activities.

Extract yield percentage

The extraction yield is a measure of the solvent's efficiency to extract specific components from the original material (4). The percentage yield was obtained using this formula A_2-$A_1/A_0 \times 100$. Where A_2 is the weight of the extract and the container, A_1 is the weight of the container alone and A_0 the weight of the initial dried sample.

Quantification of total phenolic contents

Extracts prepared at different concentrations were taken in 0.1 mL tubes and 4.5 mL of water was added to them. Then Folin-Ciocalteu reagent (diluted 1/3 with distilled water) and 0.3 mL of 2 % sodium carbonate solution were added to the mixture. The mixture was allowed to stand at room conditions for 2 hours, and then absorbance was measured at 760 nm against the reference. The total phenolic compounds contained in the extracts were given as mg gallic acid equivalents / mg extract (5).

In vitro evaluation of antioxidant assays

2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging activity: DPPH solution (0.1 mM, 3.9 mL) was added to extracts (0.1 mL) prepared at different final concentrations (20 µg/mL, 50 µg/mL, 100 µg/mL). Then the mixture was allowed to stand at room temperature for 30 min. The absorbance of the mixture was measured against the reference using a spectrophotometer (Shimadzu Corporation; Japan) at 517 nm. The experiment was repeated three times and the averages of the values and the standard deviation were calculated. The results obtained in the experiment were given as the concentration of the substance which provides the inhibition of 50 % of the radical (IC_{50}) = mg / mL (6).

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation scavenging assay: 40 µL of extracts prepared at different final concentrations (20 µg/mL, 50 µg/mL, 100 µg/mL), 3960 µL of $ABTS^{\bullet+}$ working solution were combined. The absorbance of the mixture was measured against the reference at 734 nm for 6 min. The data obtained in this study were expressed as mM trolox / mg extract (7).

Ferric reducing/antioxidant power (FRAP) assay: The ferric reducing ability of different extracts were estimated by the method

of Benzie and Strain (1996) (8). The FRAP reagent [300 mM acetate buffer (pH 3.6), TPTZ solution and 20 mM $FeCl_3.6H_2O$] was kept at 37 °C for 30 min. in incubator device (Nuve; Ankara, Turkey). 3.8 mL of FRAP reagent was mixed with 0.2 mL of extract and after 4 min. the absorbance of the mixture was measured against the reference at 593 nm. FRAP values of the extracts were expressed as mM Fe^{2+}/ mg extract.

In vitro urease inhibitory activity

The indophenol method with some modifications was used to measure the urease inhibitory activity of the extracts (9). 5 mg /mL stock solutions from different extracts were prepared and working solutions were prepared at 2 mg/mL concentrations by diluting these solutions. 500 µL of urease enzyme was added to 100 µL of the working solutions and incubated at 37 °C for 30 min. in the incubator (Nuve; Ankara, Turkey). Then, 1100 µL of urea was placed on this mixture and the mixture was incubated for 30 min. at 37 °C in the incubator. R_1 (1 % phenol, 0.005 % sodium nitroprusside) and R_2 (0.5 % NaOH, 0.1 % sodium hypochlorite) reagents were added respectively to the mixture. The mixture was incubated for 2 hours in the incubator (37 °C) (Nuve; Ankara, Turkey) and then the absorbance of mixture was read at 635 nm.

The % inhibition of urease enzyme was calculated by the formula:

% enzyme inhibition = $[(A_0 – A_1) / A_0] \times 100]$.

A_0 : The absorbance of the control solution

A_1 :Absorbance of plant extracts and standard solutions.

Determination of genotoxic activity

Fresh venous blood sample (~30 mL) was obtained from a volunteer 30 years old non-smoking women and collected in lithium heparin tubes (Vacuette®). Lymphocytes were isolated from peripheral blood by centrifugation in a density gradient of Histopaque 1077 (Sigma Diagnostics, St. Louis, USA) (40 min, 400 g, 21°C). The viability of the cells was measured after isolation by the trypan blue exclusion assay and was found to be about 99 %. The cells were incubated for 30 minutes at 37°C with MM and UM extracts of *Terfezia claveryi* at 20, 50 and 100 µg/mL final concentration. The viability of the cells was checked concurrently by trypan blue exclusion assay in all experiments at all tested concentrations of MM and UM extracts of *T. claveryi*. An equal volume of 0.4 % trypan blue reagent was added to the 40 µl cell suspension and the percentage of viable cells was evaluated under a microscope.

The alkaline Comet assay (10) with some modifications was performed to determine the genotoxic properties of *T. claveryi* extracts. MM and UM extracts of *T. claveryi* was added to the suspension of the cells to give final concentrations of 20, 50 and 100 µg/mL. Lymphocytes were incubated with extracts for 30 minutes at 37°C. The experiment included 100 µg /mL final concentration of DMSO as a solvent control since it was used to solved extracts and it was applied for 30 minutes at 37°C. Also, hydrogen peroxide at 100 µM applied for 7 min at 4°C was included as a positive control. After treatment with extracts, the cells were washed and ready to spread onto microscope slide. Each experiment was carried out in triplicate.

Fully frosted microscope slides were covered with 1 % high melting agarose (HMA). Lymphocytes were mixed with 1% low melting point agarose (LMA) at the end of incubation and was spread onto covered microscope slides. After solidification of low-melting agarose, slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 and 10% DMSO) for at least 1h at +4ºC. After the lysis, the slides were left in the cold electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) for 30 min to allow the unwinding of the DNA. And then, slides were placed in a horizontal electrophoresis tank that was filled with the same cold buffer and electrophoresed for 30 min at 300 mA and 15 V. Slides were washed three times with neutralization solution (0.4 M Tris buffer, pH 7.5). At the end of all this steps; slides were treated with % 50, % 75 and % 100 cold Ethanol for 5 min, respectively for fixation of cells and then were left in room temperature to dry. The slides stained with 50 µl ethidium bromide (EtBr – 20 µg/mL) were visualized under a microscope with a fluorescence attachment (Olympus BX51; Tokyo, Japan) and were examined by eye. One hundred cells 50 per replicate were scored and total comet score was calculated according to following formula (11).

Arbitrary Unit: 0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration. Figure 1 presents microscope pattern of cells undamaged cells to damaged cells with appearance of comet.

Statistical Analysis

The normal distribution of continuous variables was tested by Shapiro–Wilk's test. The test statistic is defined by the two-independent sample t-test between two group. The statistical analysis was performed using a one-way analysis of variance (ANOVA), with a Tukey multiple comparison, post hoc test for comparisons of different treatments versus the respective controls. Results were expressed as mean ± standard deviation of the mean (SD), with n= 3. A difference at $p<0.05$ was considered statistically significant (SPSS, an IBM Company; USA).

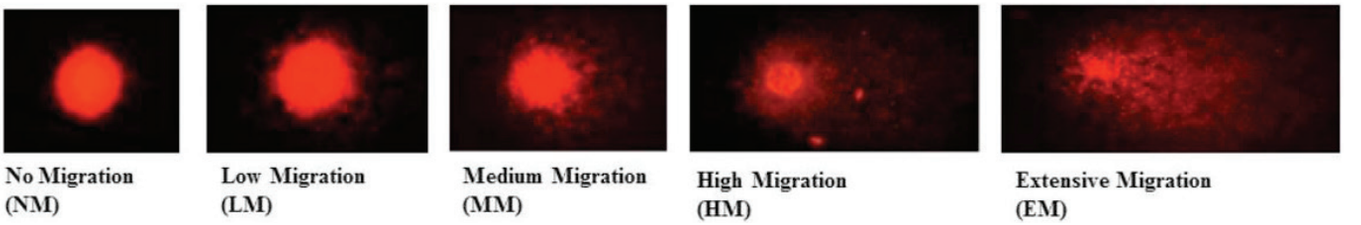

Figure 1. Comet pattern of undamaged cells to damaged cells.

RESULTS

Extract yield percentage

The yield percentages of *T. claveryi* was shown in Figure 2. According to in this study, MM extract (3.96 %) was found to have higher recovery than UM extract (2.57 %).

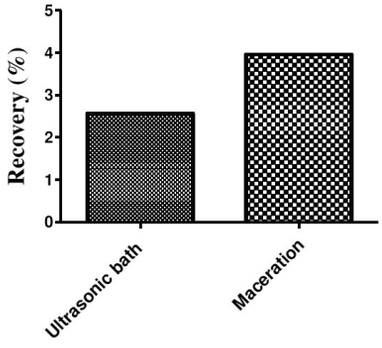

Figure 2. Extract yield percentage of methanol extracts from *T. claveryi*

Antioxidant activity and total phenolic contents of *T. claveryi* extracts

Antioxidant activities of methanol extracts obtained from *T. claveryi* were given in Table 1. In the DPPH radical scavenging activity assay, it was found that the UM extract (IC_{50}: 0.77 mg/mL) has stronger free radical scavenging activity than MM extract (p<0.001). When the activity results of both two the extracts were compared with the standard, it was found that these extracts had lower activity than standard compounds (BHA and ascorbic acid) (p<0.001).

In this study, It was found that the methanol extract (4.09 mM trolox/mg extract) obtained from maceration method exhibited stronger ABTS radical cation scavenging activity than ultrasonic bath methanol extract (p>0.05). According to results, it was found that both extracts had lower activity than the BHA compound used as standard (p<0.001).

According to the results obtained from the FRAP assay, UM extract (2.02 mM Fe^{2+}/mg extract) has stronger ferric reducing antioxidant power activity than MM extract (p>0.05). The methanol extracts obtained from two different extraction methods showed lower ferric reducing antioxidant power activity than the BHA compound (p<0.001).

When the amounts of the phenolic compounds in the extracts were compared, it was found that the MM extract (0.0097±0.0002 mgGAE/mg extract) contained more phenolic contents than the UM extract (p<0.05) (Table 2).

According to the three antioxidant activity tests, UM extract exhibited stronger free radical scavenging and ferric reducing antioxidant power activity than MM extract but the results were not found statistically significant except DPPH method. In addition, MM extract had stronger ABTS radical cation scavenging activity than UM extract however statistically not significant. It was also found that MM extract had higher total phenolic contents than other extract. Therefore, a linear relationship was found between ABTS radical cation scavenging

Table 1. Effects of extracting methods on the antioxidant activity of *T. claveryi* extracts

Samples	DPPH (IC_{50}: mg/mL)		ABTS (mM trolox/mg extract)		FRAP assay (mM Fe^{2+}/mg extract)	
	Ultrasonic bath	Maceration	Ultrasonic bath	Maceration	Ultrasonic bath	Maceration
Methanol	0.77±0.01[a,b]	1.17±0.02[a,b]	3.71±0.03[b]	4.09±0.03[b]	2.02±0.05[b]	1.92±0.06[b]
BHA	0.006±0.6		52.63±0.08		16.91±0.02	
Ascorbic acid	0.004±0.9					

Values are mean of triplicate determination (n = 3) ± standard deviation

[a] Significancy of *T. claveryi* extracts doses compared with ascorbic acid at p<0.001

[b]: Significancy of *T. claveryi* extracts doses compared with BHA at p<0.001

DPPH: 2,2-diphenyl-1-picryl-hydrazyl

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)

FRAP:ferric reducing/antioxidant power

BHA:Butylated hyroxyanisole

Table 2. Total phenolic contents and urease inhibitory activity of methanol extracts from *T. claveryi*.

Samples	Total phenolics (mgGAE/mg extract) (n=3)		Urease inhibition (%) (25 µg/mL) (n=2)	
	Ultrasonic bath	Maceration	Ultrasonic bath	Maceration
Methanol	0.0084±0.0003	0.0097±0.0002	31.012±2.6[b]	27.125±1.9[b]
	p=0.005*			
Thiourea				96.85±0.9

GAE–Gallic acid equivalents

[b] Significancy of *T. claveryi* extracts doses compared with Thiourea at p<0.001

* The test statistic is defined by the two independent sample t-test (p<0.05)

activity and phenolic compounds in this study. However, a linear relationship was not observed between total phenolic contents and antioxidant activity (DPPH, FRAP assay) of maceration methanol extract.

Urease inhibitory activity

The results for the assessment of urease inhibitory activity of *T. claveryi* methanol extracts (25 µg/mL) obtained through different extraction methods were shown in Table 2. The UM extract (31.012 %) exhibited stronger anti-urease activity than MM extract (27.125 %) (p>0.05). In the present study, the results of two different extraction methods showed that ultrasonic bath method were the most suitable method to get the strongest anti-urease activity. According to this study, both extracts from two extraction methods exhibited lower anti-urease activity than thiourea (96.85 %) used as standard (p<0.001). A linear relationship wasn't found between anti-urease activity and total phenolic contents in this study.

Genotoxic activities of *T. claveryi* extracts

Lymphocytes were exposed two methanolic extracts that extracted by different method at three concentration (20 µg/mL, 50 µg/mL and 100 µg/mL) for 30 min at 37°C. The comet assay was performed to determine the DNA damaging activity of the extracts as it is a sensitive method. Our results revealed that extracts of *T. claveryi* induced DNA damage in lymphocytes (compared with DMSO solvent control, p<0.001) and total comet scores was higher in cells which had been incubated with MM extracts (p<0.05). Table 3 shows total comet scores in lymphocytes after exposures of different methanolic extracts of *T. claveryi* and the mean total comet score distributions of different methanol extracts at different concentrations are shown in Figure 3.

Table 3. Total comet scores in lymphocytes after exposures of methanol-maceration and methanol-ultrasonic bath extracts of *T. claveryi* at 20, 50 and 100 µ/mL final concentration for 30 min at 37˚C.

Sample	Concentration	Total Comet Score [a] (mean±SD)
Negative control	0	31.67±10.50
Methanol-maceration extract of *T. claveryi*	20 µg/mL	343±20.66***
	50 µg/mL	349±5.2***
	100 µg/mL	344.66±13.32***
Methanol-ultrasonic bath extract of *T. claveryi*	20 µg/mL	303.66±13.43***
	50 µg/mL	311±7***
	100 µg/mL	325.33±10.79***
Solvent control DMSO	100 µg/mL	45.33±10.02
Positive Control H_2O_2	100 µM	355±5.03

Total comet score: 0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration (EM). [a] Significancy of *T. claveryi* extracts doses compared with DMSO solvent control at * p< 0.05; ** p<0.005; *** p<0.001

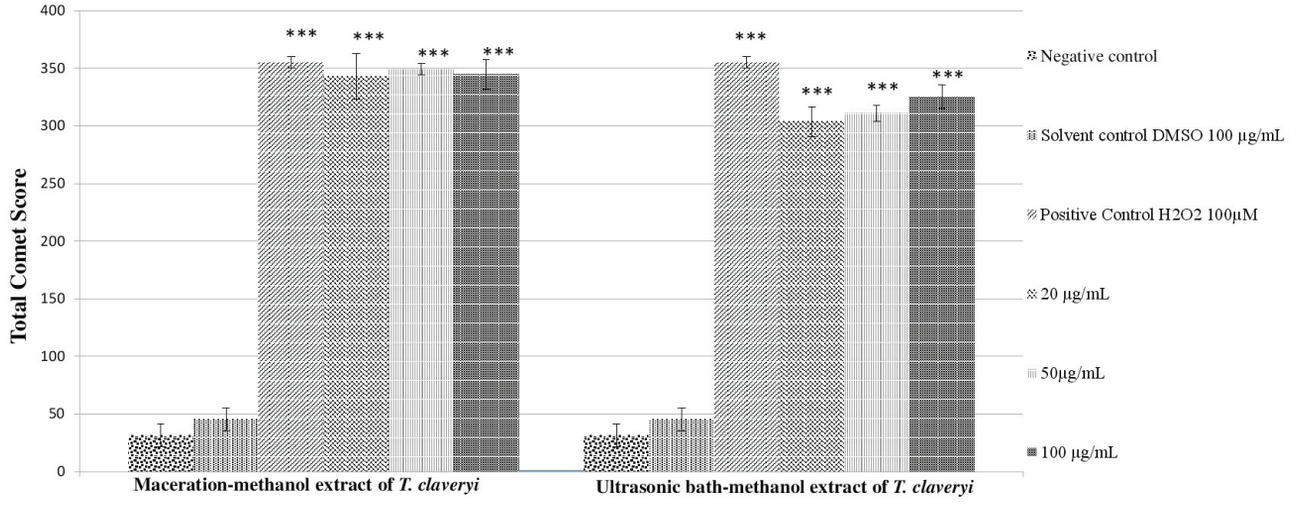

Figure3. DNA damage levels in lymphocytes after exposures of maceration-methanol and ultrasonic bath-methanol extracts of *T. claveryi* at 20, 50 and 100 µg/mL final concentration for 30 min (*** p<0.001).

DISCUSSION

According to the literature review, there are some studies on the antioxidant activity of different extracts from *T. claveryi*. The antioxidant activity of methanol extract from *T. claveryi* powder have been reported before. That study have reported that methanol extract (100 µg/µL) had stronger DPPH radical scavenging activity (7.31 %) (2). In another study, antioxidant activity of hexane, ethyl acetate, ethanol, methanol and water extracts obtained from hot maceration method were examined. According to results, ethanol extract exhibited stronger DPPH (IC_{50}:52.10 µg/mL) and ABTS (IC_{50}:64.76 µg/mL) radical scavenging activity than other extracts. In addition, hexane extract (105.8 µmol Fe^{2+}/mg) showed the highest ferric reducing antioxidant power activity (3). According to the work of Neggaz and co-worker, methanol extract (IC_{50} 8.56 mg/mL) obtained from Soxhlet method showed stronger DPPH radical scavenging activity than chloroform and ethyl acetate extracts (12).

Unlike above discussed studies, in current study, the antioxidant activities of methanol extracts obtained from two different extraction methods were examined using by the three different methods (DPPH, ABTS, FRAP). In addition, the amounts of phenolic contents contain in the extracts and the effects of extraction methods on antioxidant activity were examined. Our study showed that UM extract had higher free radical scavenging and ferric reducing antioxidant power activity than MM extract. In addition, MM extract exhibited higher total phenolic contents and ABTS radical scavenging activity than UM extract. Each extracts exhibited lower antioxidant activity than standards.

Although the bio-potential of *T. claveryi* were investigated in many studies (2, 3), the toxicity of this species are still poorly explored. To the best of our knowledge, this research is the first study that investigates the genotoxic activity of *T. claveryi*. According to the our results, each methanolic extracts have a potential genotoxic activity in lymphocytes and extract of maceration exhibited higher DNA damage than extract of ultrasonic bath.

CONCLUSION

In this study, DPPH free radical scavenging activity of UM extract was found to be stronger than MM extract however, activity of both of extracts found lower than standards. It was also found that UM extract had a lower genotoxic effect than the MM extract. This findings may be related to the recovery of the extraction methods as well as to the different composition of the extracts. This study is a preliminary study and it is aimed to analyze the composition of these different extracts in further study. In addition, the genotoxic activity of this species should be confirmed with further studies and in different cell lines.

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