

# Evaluation of the Cytotoxic Effects of Ultrasonic Extracts of *Tribulus Terrestris* L. on MCF-7 Cell Line by MTT Assay

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## ABSTRACT

People, especially those living in rural environments and those who have recently been dissatisfied with traditional medicine, use medicinal plants for their therapeutic effects. In this study, ethanol, ethyl acetate and methanol extracts of *Tribulus terrestris* L. plant, which is used by local people as one of these medicinal plants in Kars province (Turkey), were obtained by ultrasound assisted extraction method. The cytotoxic effects of the obtained extracts on MCF-7 breast cancer cells in the concentration range of 10-1000 ppm at 24 h of exposure were investigated by the MTT method, which is a colorimetric method. *T. terrestris* L. ethanol extracts at 1000 ppm caused a moderate cytotoxic effect on MCF-7 breast cancer cells. It was determined that ethanolic extracts in the concentration range of 10-500 ppm and methanol and ethyl acetate extracts in the concentration of 10-1000 ppm caused cell proliferation.

### Keywords:

*Tribulus Terrestris* L.; MCF-7; Ultrasound assisted extraction; Cytotoxicity; MTT Assay

### Article History:

Received: 2021/07/16

Accepted: 2021/11/20

Online: 2021/12/31

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## INTRODUCTION

It is known that human beings used only natural medicines until 1800 in the treatment of diseases. Compounds obtained through isolation from natural sources since the beginning of 1800 have been synthesized in the laboratory environment since 1830 [1]. In recent years, the belief that natural remedies are safer than synthetic drugs have led to an increase in people's use of natural products such as herbs, phytotherapeutics and phytopharmaceuticals. Therefore, researchers conduct various studies on the biological significance of plant extracts [2, 3]. However, there is no regulatory system that determines the safety and required dose of natural products, or even commercially sells them. The most widely used of these natural products are herbs. Unfortunately, most of those who use these herbs therapeutically do not have adequate knowledge or training in the safe use of the products. For these reasons, many studies are required to standardize natural plant products and evaluate possible risks such as undesirable side effects, overdose and toxicity [4].

*Tribulus terrestris* L. is known in Turkey as “deve çökerten”, “çoban çökerten” and “çarık diken”. These names were given to the plant because its fruit has horn-shaped spikes (Figure 1). The plant is of medicinal

and pharmaceutical interest due to its steroidal saponin content [5, 6]. *Tribulus terrestris* L., in addition to its hypolipidemic and hypoglycemic effects, is also used for the treatment of vitiligo, urological infections, prostatic hypertrophy, eye, abdomen and cardiovascular system diseases [6-8]. There are limited in vitro studies on the effect of *T. terrestris* L. extracts [7], which have low cytotoxicity on normal cells, on different cancer cells [7, 10-12]. In this context, in this study, the cytotoxic effects of ethanol, ethyl acetate and methanol extracts of *Tribulus terrestris* L. on MCF-7 human breast cancer cell line for 24 hours were investigated by MTT Assay.

## MATERIAL AND METHODS

### Collection of Chemicals and Plant Material

Ethanol, ethyl acetate, methanol, DMSO, MTT, DMEM, Penicillin-Streptomycin, Fetal Bovine Serum (FBS), Phosphate Salt Buffer (PBS) and Trypsin EDTA (Sigma Aldrich, Germany) were commercially available. *Tribulus terrestris* L. aerial parts (leaf, fruit and stem) were collected from Kars, Turkey at the altitude of 1410 m at 40°29'46" North and 43°33'40" East coordinates. Map views related to these coordinates were obtained from Google Earth online and were gi-



**Figure 1.** *Tribulus terrestris* L. plant

ven in Figure 2. The collected plant was described by Prof. Dr. Fatma Güneş. The collected plant samples were dried in the dark and ground using a grinder.



**Figure 2.** Map view of 40°29'46" North, 43°33'40" East coordinates where *Tribulus terrestris* L. samples were collected

### Instruments

In this study, Bandelin Sonorex RK 106 at 35 kHz frequency, Panasonic MCO-170AICUVH-PE CO<sub>2</sub> Incubator, Hed Lab X BIO MSC CLASS II Biosafety cabinet, Thermo Fisher EVOS FL Inverted Microscope and BioTek Epoch UV-Vis Spectrophotometer were used.

### Plant Extraction

For ultrasound assisted extraction, 10 g from the milled plant sample was weighed and placed in the round-bot-

tom flask, then added in a 30 mL the extraction solvent (ethanol, ethyl acetate and methanol). Extraction was continued 60 min. Temperature was measured at 15 minutes intervals. The obtained extracts were filtered with blue band filter paper and the liquid part was dried in a rotary evaporator. For the MTT test, a stock solution of 1000 ppm was prepared and other solutions (500, 250, 100, 10 ppm) were prepared from this stock solution.

### MTT Assay

100 µL (~5000 cells/well) of MCF-7 cell suspension prepared according to protocols [13] was placed in each well of 96-well plates and incubated for 24 h. After incubation, 100 µL aliquots from prepared solution (1000, 500, 250, 100 and 10 ppm) were added to the wells (100 µL of medium + 100 µL of cell suspension was added to the control wells) and allowed to incubate again for 24 h. Then, 10 µL of MTT (5 mg/mL) solution prepared in PBS was added to each well and incubated for 4 h. In order to dissolve the formed purple colored formazan crystals, 100 µL of DMSO was added to all wells and incubated at 18 h. After this period, absorbances were measured at a wavelength of 570 nm with a microplate spectrophotometer. Cell viability percentages were calculated with the following equation 1. All experiments were performed in triplicate.

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of test well}}{\text{Absorbance of control well}} \times 100 \quad (1)$$

## RESULTS AND DISCUSSION

### Extraction

Ultrasound assisted extraction is an important technology for green chemistry. The extraction process, which takes hours or days with traditional methods, can be completed in a short time thanks to ultrasound wave-assisted extraction. The most important advantages of this extraction method are short duration, high reproducibility, low solvent consumption and high purity of the final product [14, 15]. The temperatures were recorded as 24 °C, 29 °C, 34 °C and 38 °C (for ethanol); 25 °C, 36 °C, 42 °C and 44 °C (for ethyl acetate) and 26 °C, 32 °C, 38 °C and 39 °C (for methanol) at 15, 30, 45 and 60 minutes, respectively. The yields of extraction were found to be 45.25 % for ethanol, 52.39 % for ethyl acetate and 62.64 % for methanol.

### FT-IR Spectroscopy

In many studies, it has been reported that ethanol, ethyl acetate and methanol extracts of the *Tribulus terrestris*

L. plant are quite rich in terms of phenolic acids, flavonoids and saponins [16, 17]. The biological activity of the plant is also attributed to these components. Dinchev et al. reported that there are saponins such as protodioscin, prototribestin, pseudoprotodioscin, dioscin, tribestin, tribulosin, rutin in the ethanolic extracts of the TT plant collected from Turkey [17]. Li et al also determined that the plant contains tigogenin, gitogenin, hecogenin and neohecogenin [18]. Reshma et al. attributed the antioxidant activity of ethyl acetate extracts of the plant to the total phenolic substance content and reported that it contains caffeic acid, chlorogenic and 4-hydroxybenzoic acid phenolic acids [19]. Phenolic compounds such as  $\alpha$ -Amyrin as the major component and 3,7,11,1-tetramethyl 2-hexadecen 1-01, n-hexadecanoic acid, hexadecanoic acid, ethyl ester, phytol, 1,2-octadecadienoic acid, 9,12,15-octadecanoic acid, benzenedicarboxylic acid and diisooctyl ester as the minor component have been reported in the methanolic extracts of the plant [20]. In another study, it was determined that the methanolic extracts of the plant contain flavanoids such as naringin, rutin, hyperoside, quercetrin, quercetin, naringenin, hesperetin, campferol and apigenin, as well as phenolic acids such as pyrogallol, gallic acid, protocatechuic acid, catechin, catechol, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, vanillic acid, ferulic acid, salicylic acid, ellagic acid, coumaric acid and cinnamic acid [21]. These compounds generally have functional groups such as O-H, C-H (aromatic), C-H (aliphatic), C=O, C=C, C-H bending and C-H belonging to substituted benzene rings vibrations. When the FT-IR spectrums of the obtained extracts are examined, the vibrations of the O-H groups are seen in the range of 3500-3200  $\text{cm}^{-1}$ . C-H stretching (aromatic), C-H bending (aliphatic) and C-H bending (substitue benzenes) vibrations are observed in the 3100-2900  $\text{cm}^{-1}$ , 2900-2800  $\text{cm}^{-1}$  and 850-700  $\text{cm}^{-1}$  regions, respectively. The vibrations of the C=O group are observed at 1750-1650  $\text{cm}^{-1}$  (Figure 3-5).

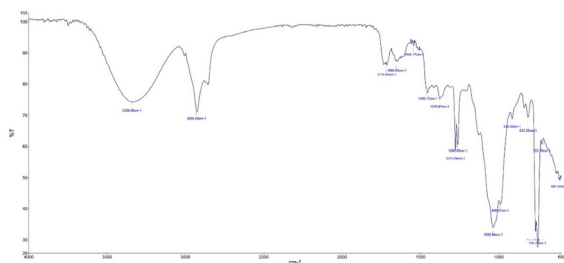


Figure 3. FT-IR spectra of ethanol extract of *Tribulus Terrestris* L.

### MTT Assay

In this study, compared with the cell control group, it was determined that *Tribulus terrestris* L.'s methanol extracts caused proliferation of MCF-7 cells at all concentrations.

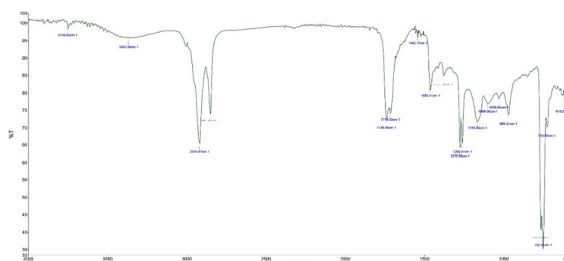


Figure 4. FT-IR spectra of ethyl acetate extract of *Tribulus Terrestris* L.

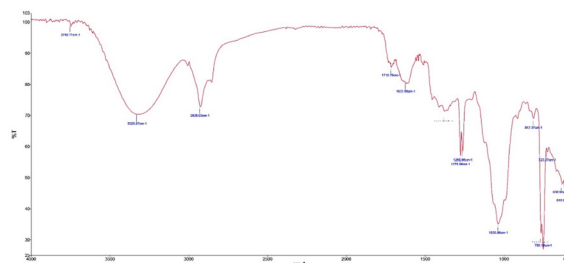


Figure 5. FT-IR spectra of methanol extract of *Tribulus Terrestris* L.

It was determined that cell proliferation increased with decreasing concentration. While 12 % cell proliferation occurred at 1000 ppm concentration, cell viability doubled (210 %) at 10 ppm concentration. Methanol used as a solvent caused 5 % cytotoxicity on MCF-7 cells (Figure 6).

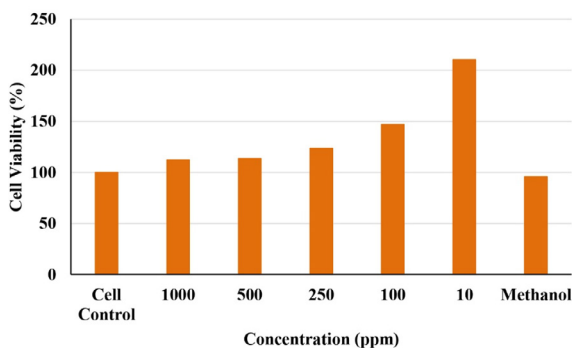
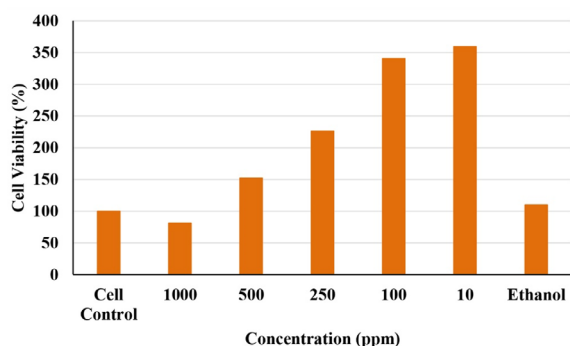


Figure 6. Effect of *Tribulus terrestris* L.'s methanol extract on MCF-7 cell line

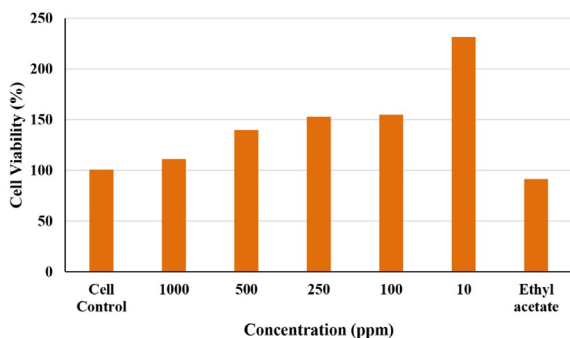
The ethanolic extract of *Tribulus terrestris* L. at 1000 ppm concentration caused 19.9 % cytotoxicity. *Tribulus terrestris* L. extracts at 500 ppm, 250 ppm, 100 ppm and 10 ppm concentrations caused proliferation in MCF-7 cells. While proliferation increased with decreasing concentration, more cell viability was found from the methanolic extracts of the plant. Ethanol used as a solvent also increased cell viability by 10 % (Figure 7).

The effects of *Tribulus terrestris* ethyl acetate extracts on MCF-7 are similar to its methanol extracts. That is, the cell viability percentages are similar. 10 % cell viability was found at 1000 ppm concentration and 230 % cell viability at 10 ppm concentration. Ethyl acetate, which was used as a solvent, caused 9.3% moderately cytotoxic [33] on MCF-7 cells (Figure 8). Comparison of effect of the all extracts on

MCF-7 cell viability was given in Figure 9.

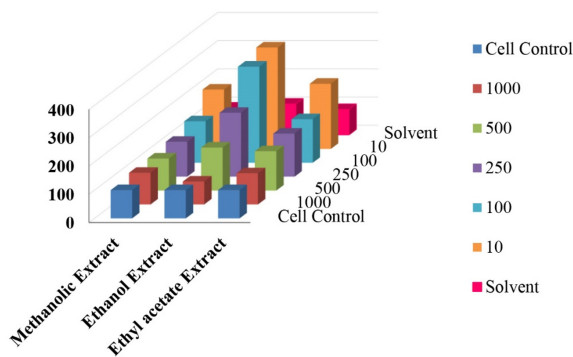


**Figure 7.** Effect of *Tribulus terrestris* L.'s ethanol extract on MCF-7 cell line



**Figure 8.** Effect of *Tribulus terrestris* L.'s ethyl acetate extract on MCF-7 cell line

Ammar et al (2018) reported that methanol and petroleum ether extracts of *T. terrestris* L. were cytotoxic to human hepatocellular carcinoma cells (HepG2) at 16.1  $\mu\text{g}\cdot\text{mL}^{-1}$  and 21.5  $\mu\text{g}\cdot\text{mL}^{-1}$  concentrations [21]. Angelova et al. (2013) examined the cytotoxic effects of *T. terrestris* extract on cell viability in human breast cancer (MCF-7) and normal (MCF10A) cell lines. It was determined that this plant extract inhibited the viability of breast cancer cells in a concentration-dependent manner ( $\text{IC}_{50}$  =15 mg/mL) [7]. Similarly, Neychev et al. (2007) found that the obtained saponins from *T. terrestris* exhibited low toxicity to normal human fibroblasts than some cancer cells [9]. Sun et al. reported that saponins isolated from the of *T. terrestris* L.'s ethanolic extract inhibited the proliferation of Bcap-37 breast cancer cell line and showed a cytotoxic effect on BEL-7402 cell line by causing apoptosis [10, 12]. Wang et al. reported that steroidal saponins isolated from the *T. Terrestris* L.'s fruits exhibited antitumor property on NCI-H460, SF-268, MCF-7 and HepG2 tumor cells [22]. Abudayyak et al. examined the cytotoxicity of NRK-52E cells exposed to *T. terrestris*'s methanol extracts for 24 hours by MTT assay. It was found that cell viability was 68.5 % at 500 mg/mL concentration. The  $\text{IC}_{50}$  value was determined as 160 mg/mL. It was determined that water and chloroform extracts of this plant did not show cytotoxic effects in the concentration range of 62.5-500 ppm [23]. Chauhan et al. obtained the extracts of *T. Terrestis* fruits by mixing method in ethanol:water (1:1) solvent



**Figure 9.** Comparison of effect of the all extracts on MCF-7 cell viability

mixture for 24 h. Researchers examined the effects of the extracts on HCT-15 cells in the concentration range of 10-70 ppm and found that the cells proliferated at 10 and 20 ppm. At 40-70 ppm, the cell viability was found as 25 %. They determined that cytotoxicity increased with increasing concentration [24]. In another study, the water:methanol (1:4) extracts of the fruits of the plant were obtained by the Soxhlet method. The effects of plant extracts on colon cancer cells (HT29) and prostate cancer cells (LNCaP-FGC-10) cells were investigated by MTT method. While colon cancer cell viability was 10 % at 0.5 ppm, no cytotoxicity was observed on prostate cancer. At 12 ppm, the viability of prostate and cancer cells was reported as 10 % and 30 %, respectively [25]. In addition, the aqueous extracts of this plant obtained by ultrasonic wave assisted extraction and these extract's cytotoxicity was investigated on Hepa 1c1c7 and Ovar 3 cells. Apart from our study, this study is the only study in which ultrasonic wave assisted extraction method was used. Plant samples were purchased commercially and water was used as the extraction solvent. In these samples, only one caused severe cytotoxicity at 250 ppm and 500 ppm concentrations, while notable cytotoxicity was observed in the others [26]. Naz et al. evaluated the cytotoxicity of extracts of leaves and fruits of *Tribulus Terrestris* on brine shrimps. They used methanol as the extraction solvent. Leaf and fruit extracts at 1000 ppm concentrations exhibited mortality of 3.50 % and 5.20 %, respectively. Mortality percentages at 10 ppm were reported as 1.55 % and 2.65 %, respectively [27]. In a study by Menon et al., the effects of methanolic extracts of *Tribulus Terrestris* on DLA and EAC cells in the concentration range of 100-500 ppm were investigated by trypan blue exclusion method. Cell viability rates decreased below 50 % at 380 and 420 ppm [28]. Gacche et al. obtained extracts of *Tribulus Terrestris* fruits with different solvents by Soxhlet extraction. Water extract, ethanol extract and chloroform extract caused cytotoxicity of 50 %, 45 % and 25 %, respectively, at 1000 ppm concentrations on HeLa cells [29]. The cell line we used in our study is MCF-7 breast cancer cell line. Patel et al. first extracted the plant with n-hexane and then re-extracted the defatted extracts with ethanol. It was determined that the seeds of the plant caused 47.02 % cell vi-

ability at 100 ppm concentration and 80.54% cell viability at 12.50 ppm on MCF-7 cell line. It had been reported that the leaves of the plant cause 98.46 % and 69.51 % cell viability on MCF-7 cells at 100 ppm and 12.50 ppm concentrations, respectively [30]. The biological activity of a plant sample varies according to many conditions such as the solvent used in the isolation of its phenolic compounds, extraction technique, climate, coordinates, altitude, soil quality and fertilizer used [31, 32]. The results of previous studies are also very different from each other as they are heavily influenced by these factors.

## CONCLUSION

In conclusion, according to the findings of previous studies, it was determined that saponins isolated from methanol and petroleum ether extracts and ethanolic extracts of *T. terrestris* L. cause cytotoxicity. In this study, the effects of the extracts of the aerial parts of the plant obtained by ultrasound assisted extraction using methanol, ethanol and ethyl acetate solvents on the viability of MCF-7 cells were investigated by MTT method. It was determined that only 1000 ppm ethanol extract from the obtained extracts caused moderate cytotoxicity and cell viability was found approximately 80 %. In general, it was determined that *T. terrestris* L. ethanol, ethyl acetate methanol and extracts caused cell proliferation at all other concentrations. Also, breast cancer cells are strong cells compared to normal cells. The fact that the plant extract used in this study did not caused cytotoxicity is attributed to these reasons. Therefore, the use of *Tribulus Terrestris* L. plant collected for this study as an anticarcinogenic agent against breast cancer is not recommended. In addition, it is recommended that more studies be carried out for the use of plants for alternative treatment, especially in patients.

## ACKNOWLEDGEMENT

The authors are thanked to Yıldırım Öztürkkan and Kenan Kara for their help in the collection of plant samples, and to Prof. Dr. Fatma Güneş for their support in plant identification, to Prof. Dr. Bingür Sönmez and Prof. Dr. Fikretin Şahin for their support in the supply of cell lines, and to Prof. Dr. Hacı Necipoğlu, Dr. Cem Öziç, Dr. Yunus Ensari and Didem Yıldırım for their technical support.

## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## AUTHOR CONTRIBUTION

Giray Bugra Akbaba: Performing cytotoxicity experiments and Evaluation of Results; Füreya Elif Ozturkkan: Planning of the study, Performing all experiments Writing-Reviewing and Editing and Evaluation of Results; Mustafa Sertcelik: Performing extraction experiments

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