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Research Article

Artemisinin Yield and Cyto/Genotoxic Properties of Naturally Grown *Artemisia Annua* L. In Uludag, Turkey

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Abstract

Artemisia annua L. known as sweet wormwood in ancient literature is an essential oil bearing and medicinal aromatic plant and belongs to Asteraceae family. It has been used for many years in traditional eastern medicine especially in the treatment of malaria and some parasitic diseases, and it gives successful results. Recently, it has been addressed with its potential effect against coronavirus. In this study, naturally growing *Artemisia annua* L. sample in Uludag, in Bursa province of Turkey, was used as raw drug. The aim of this study is to determine the yields of artemisinin in three different solvent phase extracts using High Performance Liquid Chromatography (HPLC) and examine cytotoxic/genotoxic effect of total ethanol extract on epithelial cells isolated from human bronchial epithelium. The highest amount of artemisinin was found as 0.19 mg/g in ethanol extract. As a result of cell toxicity assay, no cytotoxicity or genotoxicity effect was observed at doses less than 500 µg/mL.

Keywords: medicinal aromatic plants, *Artemisia annua* L., chromatography, phytotherapy, cell toxicity, covid19

1. Introduction

Essential oils and extracts of aromatic plants have been used for many purposes such as food, spices, medicine and cosmetics etc. since ancient times. Some of these plants are professionally grown and cultivated. However, a significant portion of these herbs is wild-collected and used in old folk medicine. The most prominent and researched properties of essential oil bearing plants are their usage for therapeutic purposes [1]. Herbal therapy is practiced in many countries around the world under different names such as traditional therapy, complementary therapy, natural therapy, and so on. The first records of the usage of aromatic plants were found in Mesopotamia around 5000 BC and nearly 250 herbal medicines were identified [2]. The demand for medicinal aromatic plants shows significant increases in both developed and developing countries thanks to its naturalness, low cost, lack of side effects, and low toxicity [3-4].

Artemisia annua L., belongs to the Asteraceae family [5]. It is an annual herbaceous plant and has a small yellow-greenish flowers [6]. It is also known as “sweet wormwood” especially in European countries. It is perceived with its characteristic and perpetual odor and has a slightly bitter and refreshing aroma. It is reported that different cultures used it in multifarious ways for health problems due to its widespread on different geographies.

Artemisia annua L. is a well-known medicinal plant in traditional folk medicine and often involved in ethno-pharmacological applications [7]. It has been used worldwide since 2000 BC for the treatment of fever, parasitic infections and painful conditions [8–10]. It is known that *Artemisia annua* L. has antiviral properties and has been used generally in Chinese folk medicine in the treatment of high fever and malaria since old times [11–13]. According to Guideline on Good Agricultural and Collection Practice (GACP), its aerial parts and leaves are used in the treatment of tuberculosis, jaundice and fever. It is rumored that, in ancient times, Artemisia was placed inside the tombs for protection. Expanded ethnobotanical research has been conducted on this subject, and a book written by Ge Hong (AD 281-340) was found during the archaeological excavation. It has been learned from this book that *Artemisia annua* L. was used as fresh herbal tea for resistant febrile diseases [14]. Thanks to the ancient medical book, Dr. Tu Youyou did an important research in the laboratory and isolated the antimalarial active substance Artemisinin and its derivatives in 1972. In the early phase of isolation experiments, it was determined that annua extracts had positive effects on infected mice, but the active ingredient isolation could not be achieved with a effective method [15]. It is thought that the structure of artemisinin could be degraded due to heat application during extraction process. At this stage of experiment, based on a recommendation in Ge Hong's book, it has been suggested to extract the plant at low temperature. Then, it has been reported that extracts obtained with modified method are non-toxic and 100% effective on infected mice. Eventually, she was awarded the Nobel Prize in 2015 with improved extraction and Artemisinin isolation process [16].

Artemisinin was first found in China and was named after being associated with the Artemisia species. Its original name is Qinghaosu. Artemisinin is pure white and in powder form and is generally unsoluble in water and soluble in organic solvents. Due to its chemical structure, it is included in the sesquiterpene lactone class. It contains active lactone, peroxy and acetal groups. Since it gives a very weak peak at near UV, it is characterized with derivatization by various reactions of functional groups in its structure. It is usually analyzed by converting it into a compound called Q260. From 1976, semi-synthetic derivatives have been produced in order to improve the physical properties of artemisinin and increase its effectiveness [17]. Dihydroartemisinin, Artesunate, Artemether and Artemisinic acid can be given as examples of the most known artemisinin derivates [18].

Researchers have different opinions about the activation and action mechanism of artemisinin [19]. According to the common opinion on this issue, this effect is based on the distinctive endoperoxide bridge in the structure of artemisinin [20]. This bridge splits in the infected red blood cell called erythrocytes. Reactive carbon centered radicals are supervened upon after cleavage [16, 21]. This degrades the essential proteins of the parasites and causes the mass death of the parasites. Although the unclear details of this mechanism still exist, the antiparasitic effect of artemisinin and its derivatives can be directly explained with a strategical iron (inside erythrocytes) cataliysted endoperoxide mechanism [19, 22]. Furthermore research shows that Artemisinin is not the only medically critical ingredient in *Artemisia annua* L. [23]. Extracts and infusions prepared from *Artemisia annua* L. can be used for HIV and treatment-resistant cancer [24, 25]. Efferth et al. compared the use of Artemisinin alone and Scopoletin and 1-8 Cineol (also existing in *Artemisia annua* L. extract) in cancer treatment as cytotoxic [26]. They proved that when artemisinin alone is used, cancer cells can develop resistance, but when used together with 1-8 Cineol, it kills cancer cells which resistant to Artemisinin. Here, artemisinin comes into prominence as having a integrative effect with other components inside extracts rather than its individual effect [23, 26].

Nowadays, antimalarial and antiviral drugs have been used for the treatment of the disease in the coronavirus pandemic [27-29]. It caused Artemisinin to reawaken and become a part of cure for Covid-19 [30]. Gendrot et al. reported that one of the reasons for the emergence of the COVID-19 pandemic in Africa long afterwards from America and Europe was the frequent use of artemisinin-based antimalarial drugs [31]. *Artemisia annua* L. is thought to inhibit the enzymatic activity of CLPro (chymotrypsin-like protease), an enzyme produced by SARS-CoV-2 during COVID-19 infection [30]. Additionally, an artemisinin-based liquid extract was prepared by Madagascar to fight with COVID-19 but this product has been qualified as an unproven study by the World Health Organization [32]. Hence, the use of *Artemisia annua* L. as a treatment agent for the new type of coronavirus should be examined with more extensive research.

Although it is well known in eastern medicine, there are limited studies on the chemical and phytotherapeutic properties of *Artemisia annua* L. in Turkey. Previous studies have generally focused on the yield of artemisinin, but its cell toxicity properties have not been examined. The aim of this study is to examine naturally grown *Artemisia annua* L. sample specified in Uludağ in Bursa province of Turkey and to determine (a) the amount of artemisinin in three different solvent phase with High Pressure Liquid Chromatography (b) cytotoxic and genotoxic effect of total ethanol extract on epithelial cells isolated from normal human bronchial epithelium.

2. Material and Methods

Artemisia annua L. plant was used as raw material and its leaves and flowering branches of aerial parts were collected by Yasam Pharma from (40.178915, 29.141146) coordinates of Uludağ in Bursa during the semi-flowering period in the end of summer 2020.

Ethanol (Merck, USA), Methanol (Merck, USA), n-Hexane (VWR Chemicals, USA), Sodiumhidroxide (IsoLab, Germany) and Acetic acid (IsoLab, Germany) were used in maceration and derivatization reactions and selected as analytical grade purity. Artemisinin chromatography grade standard (CAS: 63968-64-9) was purchased from Sigma-Aldrich, USA.

Beas-2B (Epithelial cells isolated from normal human bronchial epithelium, ATCC® CRL-9609™) was used for in vitro cell culture study in scope of cytotoxic and genotoxic analysis. Culture medium was prepared with RPMI 1640 medium (PAN Biotech, Germany), 10% Fetal Calf serum (PAN Biotech, Germany), 1 mM Sodium Pyruvate (PAN Biotech, Germany), 1 mM L-Glutamine (Sigma Aldrich, USA), 100 U/ml Penicillin - 100 µg/ ml Streptomycin (Biochrom AG, Germany).

3. Experiment

3.1. Extraction

The plant sample (g) / solvent (mL) ratio was designated as 1/50. Dry plant sample (5 g) was placed in a lid-glass bottle and the solvent was added directly on it. The mixture was kept away from sunlight and shaken per 4 hours at room temperature. Maceration time and solvent type (Table 1) were selected by inspiring from previously published methods [33].

Table 1. Solvent type and maceration time

Sample	Solvent	Concentration (%)	Maceration Time (h)
1	Ethanol	80	72
2	Methanol	80	72
3	n-Hexane	100	96

3.2. Derivatization

It is recommended that the extract was pre-derivated prior to HPLC analysis due to low peaks of Artemisinin at UV region [34-37]. The crude extract was treated with 2% NaOH. Upper phase was separated, neutralized and evaporated, respectively. The residue was solved 95% ethanol and it was treated with 0.2% NaOH solution at 50 °C for 30 minutes (reaction ratio 1/4). Ultimately, reaction product (Q260) was neutralized with 0.08 M acetic acid [14].

3.3. HPLC Analysis

3.3.1. HPLC Analysis Test Conditions

The Agilent 1260 Infinity Quaternary LC brand/model chromatography instrument was used for HPLC analysis. 0.2% Formic Acid and Acetonitrile (50:50) eluents were used as mobile phases in the analysis carried out in the column ACE C18 (5 µm; 250 x 4.6 mm). The flow rate of the mobile phase was optimized to 1 mL/min, the injection volume to 50 µL and the column temperature was set at 30 ºC. The wavelength at which the samples show maximum absorbance on the photodiode array detector system was specified as 254 nm and the analysis time was set to 10 minutes.

3.4. Lyophilization

Solvent was removed from the macerate under vacuum. The remaining aqueous mixture was firstly kept in the freezer (-18 ºC) for 24 hours and then dried at -40 ºC with a freeze-drying device (Lyophilizer, Labconco Freezone1). The obtained lyophilized extracts were used in cytotoxic and genotoxic tests.

3.5. Cytotoxicity Test

The cytotoxic activity of *Artemisia annua* L. extract was investigated using XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) and Clonogenic assay.

3.5.1. XTT Analysis

The cultured cells were suspended by trypsinisation after covering 80% of the T75 flasks. Cell viability was determined with Trypan Blue (Sigma Aldrich, USA) solution. Cells were seeded in 96-well plates as $8x10^3$ live cells per each well and left to incubate for 24 hours. Then, the cells were exposed to different concentrations of *Artemisia annua* L. extract (75, 100, 200, 300, 400, 500, 750, 1000, 2000, 3000 µg / mL) for 24 hours. After the treatment, cell viability was determined with XTT Cell proliferation Kit (BI; Biological Industries, Kibbutz Beit Haemek). Absorbance values at 450 nm in each well were measured using a microplate reader (Biotek ELx800, Biotek Instruments Inc.). The viability percent of the cells were calculated from absorbance values using Equation 1 [38, 39].

$$\%Cell\ viability = \frac{\text{Absorbance}_{sample\ group}}{\text{Absorbance}_{control}\ \text{group}}\ x\ 100 \qquad (1)$$

The XTT method was applied as three independent replicates and the mean of the IC50 (concentration that inhibits 50% of the cells) results was calculated. H_2O_2 was used as positive control in the method.

3.5.2. Clonogenic Assay

Clonogenic assay is based on the ability of a cellgeneration to reproduce a large colony or clone and to proliferate indefinitely. Cells could not form a sufficient level of colonies are considered as dead even if provide DNA and protein synthesis and pass 1-2 mitosis. Therefore, only containing at least 50 cells colonies were considered for colony counting [40]. The cultured cells were suspended by trypsinisation after covering 80% of the T75 flasks. Cell viability was determined with Trypan Blue (Sigma Aldrich, USA) solution. Cells were seeded as 50000 live cells in T25 flasks and left to incubate for 24 hours. Different concentrations of *Artemisia annua* L. extract (75, 100, 200 ,300, 400, 500 µg / mL) were used in the clonogenic assay according to IC50 concentration determined by the XTT method. The viability percent of the cells were calculated according to Equation 2. Here, L and N represent average number of colonies per petri-dish for control and sample group, respectively.

$$\%\ \text{Cell viability} = \frac{\text{N}}{\text{L}}\ x\ 100 \qquad (2)$$

3.6. Genotoxicity Test

Comet assay was used to determine the genotoxic activity. The basic principle of the method is based on the migration of single cell DNA in the electrophoretic field in agarose gel. This method allows to seperation of fragmented and non-fragmented DNA. The cultured cells were treated with *Artemisia annua* L. extract in T25 flasks as mentioned at clonogenic method. The cells were washed and then suspended with low melting agarose (LMA). Cell samples were removed from this suspension and spread on slides previously coated with agarose. After LMA was allowed to freeze, slides were treated with lysis buffer solution (100mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO) and kept at +4 ℃ for a night. At the end of the period, the slides were placed in the electrophoresis tank and the running buffer solution (0.3 M NaOH, 1mM EDTA, pH 13) was filled in the tank to cover the slides and kept for 20 minutes. The

power-supply source was set at 25 V and 300 mA, samples were treated for 25 minutes and kept in the dark at +4 ° C neutralization buffer (400 nm Tris, pH 7.5) for 5 minutes. Then, the slides were washed and allowed to air dry. Ultimately, slides were left to dry again by fixing in cold ethanol for 5 minutes. Then, slides were stained with Ethidium Bromide for 1 minute and comet photographs were taken at 40X magnification under fluorescent microscope. 50 comet images were analyzed for each replicate of experimental groups. The evaluation of the comets was carried out using Kameram21 software programme. The three parameters Olive Tail Moment (OTM), Tail DNA (Tail % DNA) and Tail Length) accepted in the international comet workshop held in Germany in 2001 and recommended to be evaluated in comet studies were used to evaluation of assay [41]. H_2O_2 was used as a positive control and mean values were reported.

3.7. Statistical analysis

Cytotoxic and genotoxic tests were analyzed via SPSS 21.0 software programme. Comparison of significant differences was made using the Kruskal–Wallis method ($p < 0.05$).

4. Results And Discussion

4.1. HPLC Analysis

4.1.1. Linearity

The regression line equation of Artemisinin[1] was y=4.1996x+3.4628 with a correlation coefficient as 0.9997. The regression line equation of Artemisinin[2] was y=0.1872x+0.1518 with a correlation coefficient as 0.9989 and the regression line equation of Artemisinin[3] was y=2.6225x-5.6966 with a correlation coefficient as 0.9981. Outstanding linearity has been observed between peak areas and concentrations of 5-100 mg/L.

4.1.2. Analysis of Artemisia Annua L. Extracts

HPLC chromatograms of the artemisinin contents in *Artemisia annua* L. extracts prepared in n-hexane, ethanol and methanol solvent phases were given in Figure 1, respectively. Moreover, the artemisinin amount in n-hexane, ethanol and methanol solvent phases were presented in Table 2.

According to analysis result, the highest amount of artemisinin was found as 194,091±8,671 ppm (0.19 mg/g) in ethanol extract (Table 2). This expected result can be attributed to the fact that ethanol is a polar and intense solvent. Due to these properties, it is known that aqueous ethanol is frequently used in the extraction of medicinal and aromatic plants [42]. Subsequently, the following highest yield was obtained with n-hexane and methanol extracts, respectively.

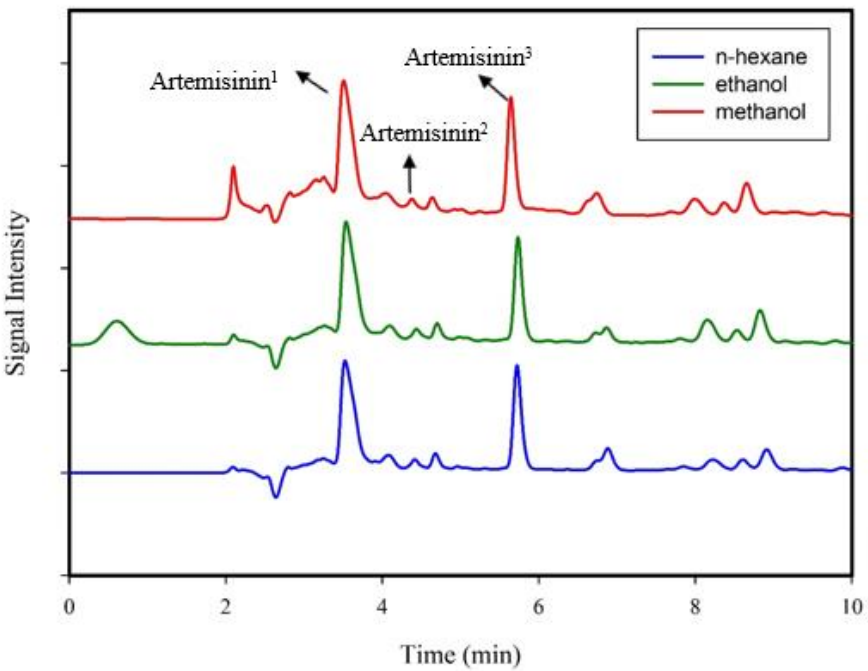


Figure 1. HPLC chromatogram of *Artemisia annua* L. extract in n-hexane, ethanol and methanol solutions.

Table 2. Artemisinin contents of *Artemisia annua* L. extracts in n-hexane, ethanol and methanol solutions.

ID	Solvent Phase	Retention Time (min)	Peak Area	Concentration (mg/L)
Artemisinin[1]	n-hexane	3.524	264.051	56,547±4,769
	ethanol	3.537	288.063	60,847±5,997
	methanol	3.507	286.076	60,876±5,565
Artemisinin[2]	n-hexane	4.415	13.076	66,433±5,278
	ethanol	4.436	15.265	74,859±6,215
	methanol	4.379	10.558	55,791±0,775
Artemisinin[3]	n-hexane	5.722	154.755	60,199±0,878
	ethanol	5.734	149.546	58,385±0,772
	methanol	5.643	155.860	62,110±0,459
Total Artemisinin	n-hexane			183,179±7,167
	ethanol	-	-	194,091±8,671
	methanol			178,777±5,637

The percentage of artemisinin in the dried drug was determined approximately 0.02%. A previous research in Turkey [43] reported that artemisinin amount of culvitated plant was calculated as 6.32 mg100g^{-1}. It is obvious that the obtained results are consistent with the literature [44]. While the results are close to the yield in relatively similar climates such as Argentina, Spain and Iran [45-47], it has been observed that it is even close to the yield of cultivated plants in some European countries such as Germany [48].

4.2. Cytotoxic and Genotoxic Properties

Cytotoxic and genotoxic test results were given below under each subheadings and eventually, all results were evaluated together with the examples from literature.

4.2.1. Cytotoxicity Results

As results of XTT experiments, cell viability percentage and mean results are shown in Figure 2. The average IC50 value calculated from each repetition was determined as 432,841 ± 7,757 µg/mL (r^2= 0,9421 ± 0,010). According to results, a significant data difference in terms of toxicity was found starting from 500 µg/mL compared to the control group. No cytotoxicity effect was observed at doses of 400 µg/mL and less.

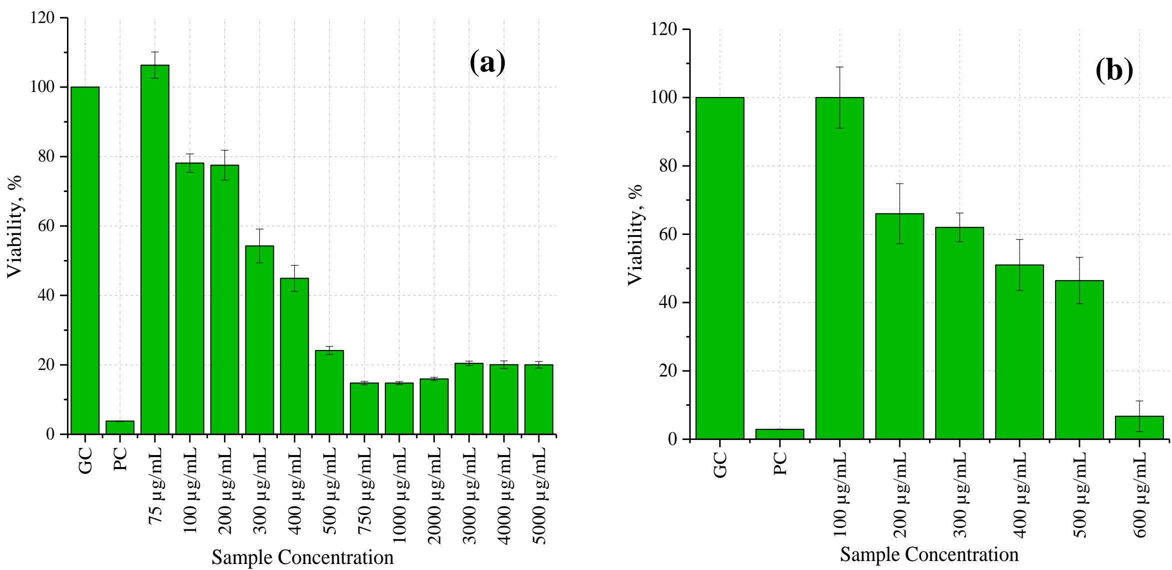
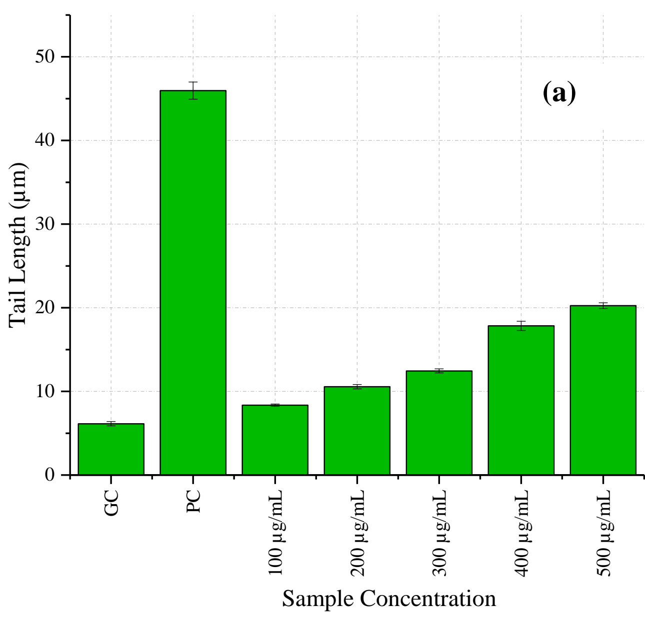


Figure 2. (a) XTT analysis (b) Clonogenic assay results (GC: Growing Control, PC: Positive Control H_2O_2)

Based on XTT analysis results, cell viability was calculated again with the Clonogenic assay. Clonogenic experiments were fulfilled in triplicate and calculated cell viability percentage and mean results are presented in Figure 2(b). The average IC50 value calculated from each repetition was determined as 447,542 ± 14,817 µg/mL (r^2= 0,9963 ± 0,004) As a result of clonogenic assay which shows the ability of cells to form clones, there is no cytotoxic effect was observed at doses less than 400 µg/mL.

4.2.2. Comet Assay

In the light of the findings obtained from XTT and Clonogenic test, the Comet assay was carried out in triplicate. Tail length, Tail % DNA amount and OTM (Olive Tail Moment) values were evaluated as mean of these repeats and results were given at Figure 3 and Figure 4, respectively.



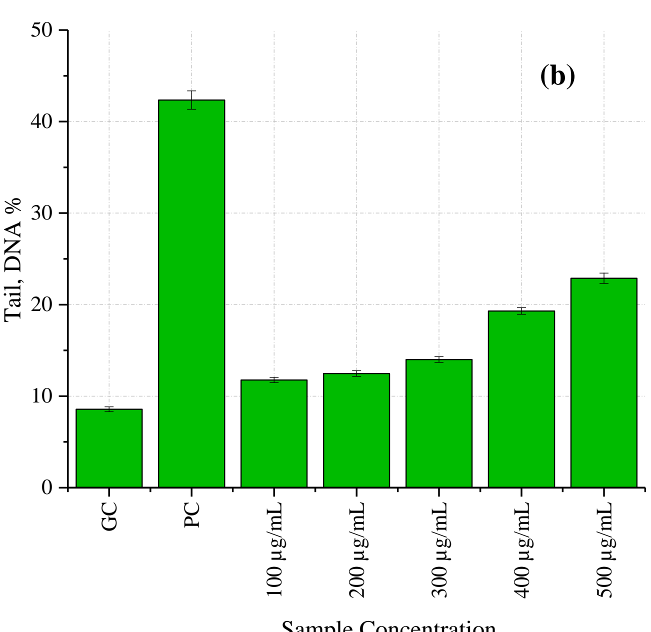


Figure 3. (a) Comet assay tail length (b) Tail DNA% results (GC: Growing Control, PC: Positive Control H_2O_2)

Statistical results of the comet assay showed that there was no in vitro genotoxic effect for doses less than 400 µg/mL. In view of DNA effect, comet assay indicated that the damage dose starts at approximately higher than 500 µg/mL and so doses less than 350 µg/mL could be considered as safe. Figure 4 (b) presents the comet images taken at different concentrations.

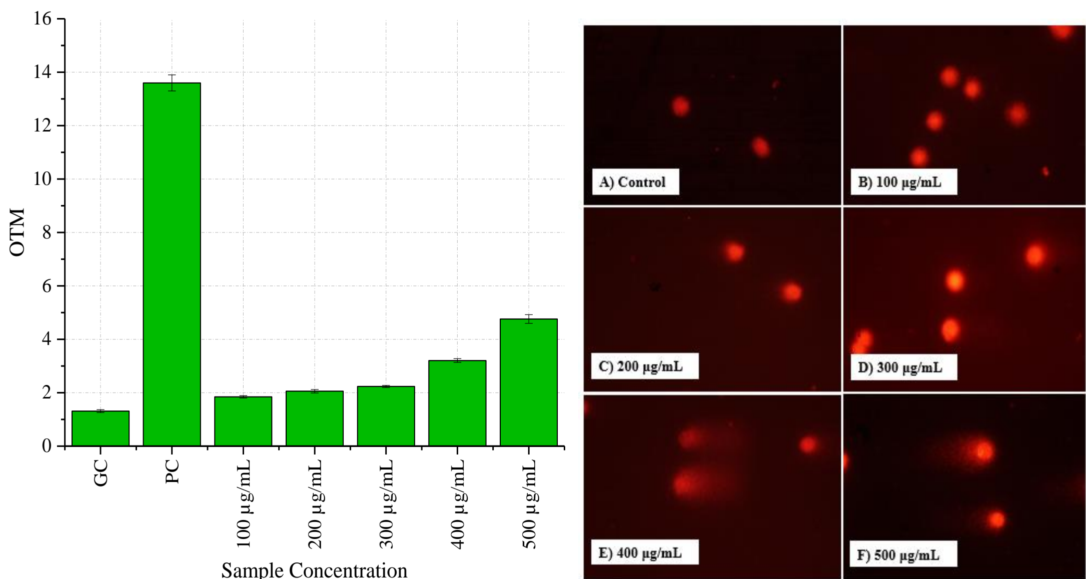


Figure 4. (a) Comet assay OTM values (b) Comet images (OTM: Olive Tail Moment, GC: Growing Control, PC: Positive Control H_2O_2)

As a result of 24-hour exposured analyzes, no cytotoxicity or genotoxicity effect was observed at doses less than 500 µg/mL on Beas-2B human healthy bronchial epithelial cells. Moreover, it was determined that high concentrations (approximately 500 µg / mL) of the extract sample had in vitro cytotoxic and genotoxic activity. Similarly, a previous research argued that in a comet test performed with the liver cell line regarding the genotoxic effects of artemisinin and artesunate on human cells, a dose-dependent increase in cytotoxicity and genotoxicity was shown [49]. In a good agreement with literature, a research examining mitotic activity in Allium cepa root meristem cells with increasing doses of *Artemisia annua* L. methanol extract growing in the Black Sea region, mitotic index inhibition was observed at high doses and it was stated that doses less than 450 mg/mL were safe [50]. Furthermore, some studies showed that the cytotoxic and genotoxic effects of *Artemisia annua* L. are effective not only on healthy cells but also on cancer cells. As a good example, a study where tumor cells and healthy cells were examined together, it has been stated that tumor cells are significantly more affected by *Artemisia annua* L. extract [26]. In another remarkable case conducted on different cancer cell lines, it was shown that the methanol extract isolated from *Artemisia annua* L. caused a significant decrease in cancer cell proliferation and had less cytotoxicity on normal cells [51].

Moreover, it was determined that total hydroalcoholic extract showed stronger cytotoxic activity than pure artemisinin in a conspicuous research based on compare the cytotoxicity and antiproliferative effect of extract obtained from *Artemisia annua* L. and pure artemisinin. All these findings reveal the potential importance of this study and its compatibility with the literature.

5. Conclusion

In this study, the amount of artemisinin in three different solvent phase was investigated on naturally grown *Artemisia annua* L. sample specified in Uludağ, Bursa, Turkey. The highest amount of artemisinin was found as 194,091 ppm (0.19 mg/g) in ethanol extract of plant. Furthermore, cytotoxic and genotoxic effects of total ethanol extract were tested on epithelial cells isolated from normal human bronchial epithelium. In the light of the analysis results and previous

studies, the dose range of 300-350 µg/mL and less had no in vitro toxicity activity could be accepted as safe, and it could be considered as a preliminary dose for further studies. In future studies, it is necessary to compare the extracts of *Artemisia annua* L. which grows in different regions and harvest periods. Hence, the use of *Artemisia annua* L. as a treatment agent for viral diseases especially for the new type of coronavirus should be examined with more extensive research.

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