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DETERMINATION OF CLOMIPRAMINE HYDROCHLORIDE FROM ITS COMMERCIAL DRUG FORM BY VOLTAMMETRY

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ABSTRACT

In this study, electroanalytical technique was developed for the quantitative analysis of clomipramine hydrochloride from its commercial tablet dosage forms based on its oxidation behavior. The electrochemical determination of clomipramine hydrochloride was easily carried out on glassy carbon electrode (GCE) by two voltammetric techniques. The electrochemical measurements were carried out on GCE surface in different buffer solutions in the pH range from 2.00 to 12.00 by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques. The effect of pH on the anodic peak current and peak potential was investigated. Phosphate buffer (pH 6.50) was selected for analytical purposes. The diffusion-controlled nature of the peak was obtained. A linear calibration plot for DPV analysis was constructed in the clozapine concentration range from $5x10^{-6}$ mol L^{-1} to $3x10^{-5}$ mol L^{-1}. Limit of detection (LOD) and limit of quantification (LOQ) were obtained as $2.8x10^{-7}$ mol L^{-1} and $8.4x10^{-7}$ mol L^{-1} respectively.

1. INTRODUCTION

As an effective and important antipsychotic drug, clomipramine hydrochloride (Figure 1) is widely used in the treatment of psychiatric disorders. As an effective and important antipsychotic drug, clomipramine hydrochloride (Figure 1) is widely used in the treatment of psychiatric disorders. Many analytical methods have been developed to investigate its characteristics, such as spectrophotometer chemiluminescence, high-performance liquid chromatography, capillary zone electrophoresis, etc. (Huang *et al.*,2008) Nevertheless, there are few reports about clomipramine hydrochloride studied by means of electrochemical methods for its low redox activity under normal conditions. Among the few examples, rotating gold and platinum electrodes were used to study its electrochemical mechanism in sulfuric acid (Bioship and Hussein,1984). The highly boron-doped diamond electrode in HPLC used to determine clomipramine hydrochloride and got satisfactory results, but the preparation of the electrode was complicated and time-consuming (Ivandidi *et al.*,2002). A novel ion-selective

was fabricated electrode based on poly (vinyl chloride) membranes for clomipramine hydrochloride, the electrode had high stability and responded rapidly, while the detection limit of $4x10^{-7}$ mol L^{-1} seemed somewhat unsatisfactory (Ortuno *et al*.,2006).

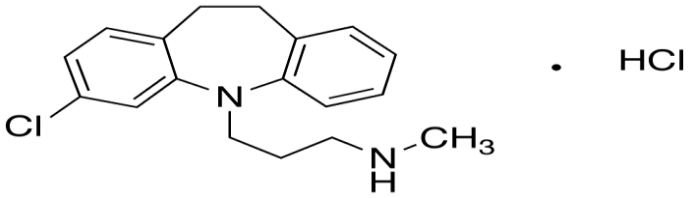

Figure 1. Chemical structure of clomipramine hydrochloride

The purpose of this study is to suggest a rapid and precise voltammetric technique for analyzing the substance in drug dosage forms.

2. MATERIAL AND METHODS

2.1. Apparatus

Electrochemical measurements were carried out using a Model Metrohm 757 VA Trace Analyzer (Herisau, Switzerland) with a three-electrode system consisting of GCE as working electrode (GCE; φ = 3 mm, Metrohm), a platinum wire auxiliary electrode and Ag/AgCl (KCl 3 M, Metrohm) reference electrode. The GCE was polished with alumina solutions (prepared from φ = 0.01μm aluminum oxide) on alumina polish pad before each measurement and then, rinsed with ultra-pure deionized water and ethanol. The firstly, the deoxygenation process of the supporting electrolyte solutions were carried out with argon gas for 5 min before all measurement. Firstly, the deoxygenation process of the supporting electrolyte solutions were carried out with argon gas for 5 min before all measurement. Then, the argon gas was passed from the solutions for 60 s after the addition of each sample solution in the measurements. In each new experiment, a new bare electrode surface was used. In each new experiment, a new bare electrode surface was used. Metrohm 744 pH meter (Herisau, Switzerland) was used for pH measurements. All measurements were carried out at ambient temperature of the laboratory (15-20°C). The following parameters were optimized: pulse amplitude 50 mV; pulse time 0.04 s, voltage step 0.009 V, voltage step time 0.04, potential step 10 mV (DPV); the scan rate in the range 10-1000 mVs^{-1} (CV).

2.2. Reagents and materials for analysis

Clomipramine hydrochloride and Anafranil were kindly supplied by (TEOFARMA, Istanbul, Turkey). A stock solution of $1.0x10^{-2}$ mol L^{-1} of clomipramine hydrochloride was prepared by dissolving an accurate mass of this active material in an appropriate volume of ultra-pure-deionized water and kept in the refrigerator of laboratory. The standard working solutions were prepared by dilution stock solution. All solutions were protected from light and were used within 24 h to avoid decomposition. 0.067 mol L^{-1} phosphate buffer; pH:4.50-8.00 0.04 mol L^{-1} Britton Robinson buffer; pH:2.02-12.00 (acetic acid: Riedel, Seelze, Germany, 100 m/m %; boric acid; Merck, Darmstadt, Germany, and phosphoric acid, Carlo Erba, Rodeno, France, 85 m/m %) were used to the supporting electrolyte solutions. Ultra-pure-deionized (0.055 μS/cm) water obtained from TKA Smart 2 model was used to prepare supporting electrolytes. Other chemicals, all of the analytical-reagent grade (Merck) were used without purification.

2.3. Calibration plot for quantitative analysis of clomipramine hydrochloride

The stock solution of clomipramine hydrochloride was diluted with ultra-pure--deionized water to obtain various clomipramine hydrochloride concentrations (changed concentrated to dilute). Under the optimum conditions described in the experimental section, a linear calibration plot was constructed in the clomipramine hydrochloride concentration range $5x10^{-6}$-$3x10^{-5}$ mol $L^{-1.}$ The repeatability, accuracy, and precision were determined.

2.4. Analysis of clomipramine hydrochloride from spiked Anafranil tablet dosage forms

Ten tablets were weighed and ground to a fine powder. $1x10^{-2}$ mol L^{-1} clormipramine solution is prepared and centrifuged 20 min at 4000 rpm to complete dissolution and then diluted to volume with the same solvent. Appropriate solutions were prepared by taking suitable aliquots of the clear supernatant liquor and diluting with selected buffer solution as supporting electrolyte. Each solution was transferred to the measurement cell.

3. RESULTS AND DISCUSSION

3.1. Electrochemical Oxidation Behavior of Clomipramine Hydrochloride

The electrochemical determination of clomipramine hydrochloride based on its oxidative behavior at surface of GCE were firstly carried out by CV and DPV techniques. CV measurements performed with clomipramine hydrochloride $1x10^{-4}$ M at various scan rates between 10 – 1000 mVs^{-1} at surface at GCE in 0.067 mol L^{-1} phosphate buffer (pH 6.50) are given in Figure 2.

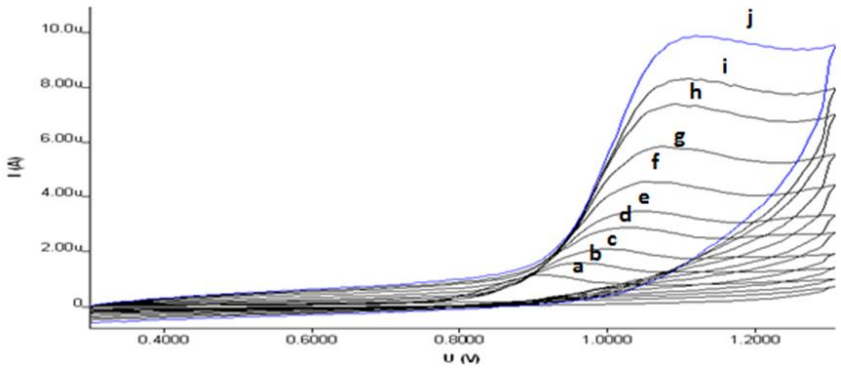

Figure 2. The cyclic voltammograms of $1x10^{-4}$ mol L^{-1} clomipramine hydrochloride in 0.067 M phosphate buffer (pH 6.50) on GCE. Scan rate, mV s^{-1} a) 10, b) 25, c) 50, d) 100, e) 150, f) 250, g) 400, h) 600, i) 750, j) 1000

The best linear relationship existing between peak current and the square root of the scan rate between 10-1000 mV s^{-1} ($(Ip(\mu A) = 0.1354v^{1/2} + 0.1141)$ with correlation coefficient 0.9977 were observed. This result is shown that the oxidation reaction is predominantly diffusion controled. In addition, ideal reaction of solution on surface electrode is determined by the result of correlation coefficient and slope of the peak (logarithm of peak current versus the logarithm of scan rate) 0.9993 and 0.46, respectively (Yılmaz *et al*., 2013; Eker *et al*., 2017).

CV voltammogram of clomipramine hydrochloride gave one anodic peak without reverse scan indicates the irresible properties of electrode reaction. (Figure 2) (Yılmaz *et al*., 2013; Eker *et al*., 2017).

3.2. pH Effect of Peak Current for Clomipramine Hydochloride

The effect of pH on the peak current was investigated in Britton-Robinson, acetate and phosphate buffer solutions. The results showed that the analyses were strongly pH dependent. The DPV peak current of the oxidation was inversely proportional to pH (Fig.3a-c). The convenient pH for the electroanalytical determination of the clomipramine was found as 6.50 in the0.067 mol L^{-1} phosphate buffer.

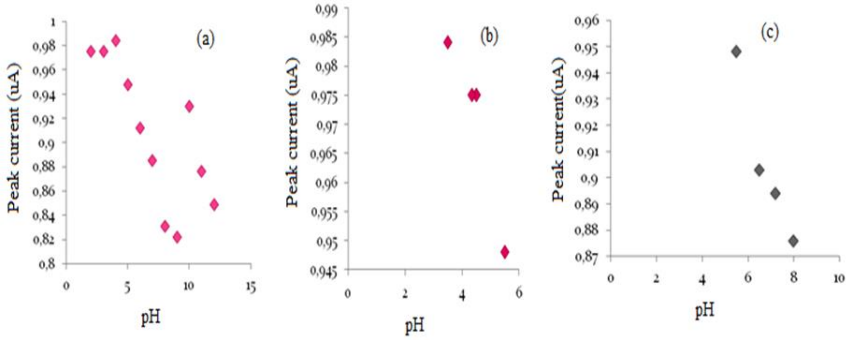

Figure 3. pH changes on the peak current of $5x10^{-5}$ mol L^{-1} clomipramine hydrochloride in a) 0.04 mol L^{-1} Britton-Robinson Buffer (BRT) , b) 0.2 mol L^{-1} acetate buffer, c) 0.067 mol L^{-1} phosphate buffer as supporting electrolyte by DPV.

3.3. Calibration Plots for The Determination of Clomipramine Hyrochloride

DPV technique was used for quantitate determination of the drug in pharmaceutical formulation. Under the optimized experimental conditions, the linearity value is high in the concentration range of $5x10^{-6}$-$3x10^{-5}$ mol L^{-1} Figure 4.

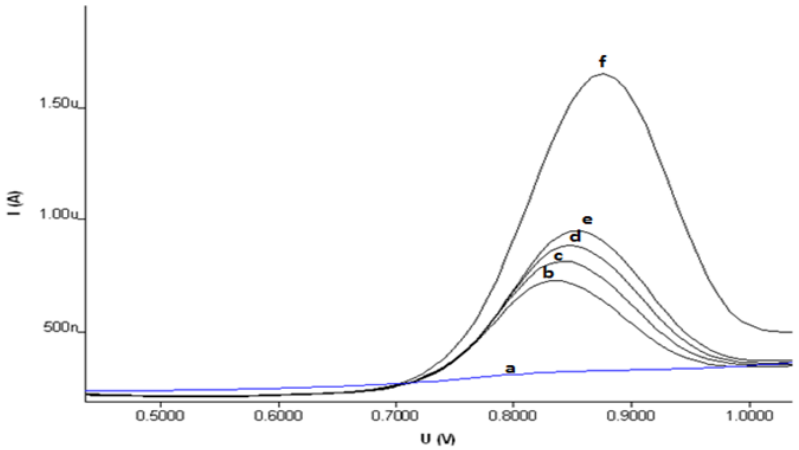

Figure 4. The calibration voltammograms at different concentrations of clomipramine hydrochloride in 0.067 mol L^{-1} phosphate buffer (pH 6.50) on GCE by DPV. a) supporting electrolyte, b) $5x10^{-6}$ c) $7x10^{-6}$ d) $9x10^{-6}$ e) $1x10^{-5}$ f) $3x10^{-5}$ (mol L^{-1}).

Applied voltammetric technique was validated for determination of the clozapine with evaluation of the limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and reproducibility) in Table 1, accuracy (bias) and recovery values in Table 2. Table 1 and Table 2 is showed the linearity values. Slope and r of the slope is related with

lineartiy. It will be better to put linearity knowledge in here (Çıtak *et al.*, 2007; Skrzypek *et al*., 2005; Yılmaz *et al*., 2013; Yagmur *et al*., 2017; Eker *et al*.,2017).

The values of LOD and LOQ were calculated as $2.8x10^{-7}$ and $8.4x10^{-7}$ mol L^{-1} respectively. A good repeatability and reproducibility of the anodic peak current and potential were calculated from five independent measurements for 1 $x10^{-5}$ mol L^{-1} clomipramine hydrochloride (Skrzypek *et al*., 2005; Çıtak *et al.*, 2007; Yılmaz *et al*., 2013; Yagmur *et al*., 2017; Eker *et al*., 2017). Repetability peak current and peak potential were found as 1.79 and 0.76 respectively. The RSD values for the reproducibility were found as 0.90 and 0.01 respectively.

The equation of the linear regression plots was Ip(μA)= $3.1996x10^4$ C (mol L^{-1}) +0.293 found with correlation coefficient, r=0.999 (n=5 repeat measurements). Standard deviations for intercept and slope of the calibration plot are given in Table 1.

Table 1. Regression analysis of the calibration plot for the analysis of clomipramine hydrochloride. The calibration plots were obtanied in 0.067 mol L^{-1} phosphate buffer (pH 6.50) on surface of GCE by applied DPV technique.

Parameter	**Results**
Measured potential (V)	0.903
Linear concentration range (mol L^{-1})	$5x10^{-6}$-$3x10^{-5}$
Slope (μA mol L^{-1})	$3.1996x10^4$
SD of slope	1372
Intercept (nA)	0.293
SD of intercept	0.070
Correlation coefficient, r	0.999
Number of measurements, n	5
LOD (mol L^{-1})	$2.8 x10^{-7}$
LOQ (mol L^{-1})	$8.4x10^{-6}$
Repeatability of peak current (R.S.D %)	1.79 for $1x10^{-5}$ mol L^{-1}
Repeatability of peak potential (R.S.D %)	0.76 for $1x10^{-5}$ mol L^{-1}
Reproducibility of peak current (R.S.D %)	0.90 for $1x10^{-5}$ mol L^{-1}
Reproducibility of peak potential (R.S.D %)	0.01 for $5x10^{-5}$ mol L^{-1}

3.4. Analysis of Clomipramine Hydrochloride in Anafranil® Tablets by Voltammeric Techniques

The labeled value of clomipramine hydrochloride in Anafranil commercial tablets was calculated from its calibration values (Table 2). The accuracy of the applied methods were evaluated by recovery tests after the addition of a certain amount of active drug to pre-analyzed formulations of clomipramine hydrochloride to determine whether excipients in the tablets interfered with the analysis, Table 2). The method was validated. The linear range, detection and quantification limits reported for non-electrochemical method and electrochemical methods are given in Table 3.

Table 2. The analysis of clomipramine hydrochloride in Anafranil tablets and mean recoveries on surface of GCE by DPV.

Parameter	Results
Labeled clozapine (mg)	25.00
Amount Found (mg)	24.50
Relative Standard deviation, R.S.D.%	0.96
Bias %	2.00
Added clozapine (mg)	2.50
Found clozapine (mg)	2.25
Number of measurement, n	5
recovery (%)	99.09
Relative standard deviation of recovery, R.S.D. %	0.30
Bias %	0.01

Table 3. Comparison of linear range and detection limits for clomipramine hydrochloride to different known methods.

Linear range	Limit of detection *(LOD)*	Limit of quantification *(LOQ)*	Method	Reference
5×10^{-5} - 1×10^{-6} mol L^{-1}	6×10^{-9} mol L^{-1}	_	voltammetry	Huang *et al.* (2008)
1×10^{-6}-1×10^{7} mol L^{-1}	1×10^{-9} mol L^{-1}	_	UV	Guiying *et al.* (2008)
$4x10^{-6}$-$4x10^{-5}$ mol L^{-1}	$1x10^{-9}$ mol L^{-1}	_	UV	Guiying *et al.* (2008)
$5x10^{-6}$-$3x10^{-5}$ mol L^{-1}	$2.8x10^{-7}$ mol L^{-1}	$8.4x10^{-7}$ mol L^{-1}	voltammetry	**This study**

The important advantage of the applied electrochemical technique over the other techniques such as spectrometry and chromatography is that it can be applied directly to the analysis of pharmaceutical dosage form without the need for extensive sample preparation since there was no interference from the excipients and endogenous substances. Another advantage is that the developed DPV technique is fast, requiring about 5-10 min to run any sample and involves no sample preparing other than dissolving, diluting, precipitating, centrifuging and transferring an aliquot to the supporting electrolyte.

4. CONCLUSIONS

In this study, a simple, low-cost, sensitive and selective DPV technique for the quantitative analysis of clomipramine hydrochloride based on the electrochemical oxidation at surface of GCE was realized. The changing of current with pH, it is understood that electrode reaction process pH dependent. Only oxidation peak was observed. There is no reduction peak indicate that electrode proses is irreversible. Clomipramine hydrochloride was successfully determined in 0.067 mol L^{-1} pH:6.50 phosphate buffer in tablets dosage by DPV technique.

Conflict of Interest Statement

The authors declare no conflict of financial, academic, commercial, political, or personal interests.

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BIOMONITORING OF HEAVY METALS DEPOSITION WITH PSEUDEVERNIA FURFURACEA (L.) ZOPF IN ÇORUM CITY, TURKEY

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ABSTRACT

Heavy metal air pollution is an important environmental problem. One of the methods used to monitor pollution in air is the method of transplanting lichen samples by the "bag technique". In this study, Pseudevernia furfuracea was used as a bioindicator to determine the heavy metal level in the air of Çorum and to generate an air pollution map of the city. The lichen samples were collected from the Yapraklı Mountains in Çankırı in 2002 and transplanted to 8 different stations in Çorum. Lichen samples were retrieved at two different periods in three month intervals. Inductively Coupled Plasma (ICP) spectrometry (Varian Liberty ICP-OES Sequential) was used to identify the heavy metals, such as copper (Cu), cadmium (Cd), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) in the lichen samples. The chlorophyll a and b contents were determined by using the modified DMSO (dimethyl sulfoxide) method. With these values chlorophyll a+b, a/b and b/a were also calculated. According to the results of the heavy metal analysis by the use of P. furfuracea, air pollution levels in Çorum was detected. The reasons of pollution can be stated as heavy traffic, industrial activities and heating processes in the city. P. furfuracea can be used as a bioindicator for pollution studies.

1. INTRODUCTION

Lichens are symbiotic organisms that occur in almost all terrestrial ecosystems and by virtue of their ability to tolerate long periods of drought may even colonise areas with extreme environmental conditions. Because of their high surface: volume ratio, the simple anatomy and absence of a cuticle, they accumulate heavy metals, concentrating them in thalli. Due to this ability, they may show an elemental composition which reflects, over the long term, the

dissolved gases, particulate matter and metal ions of the atmosphere, and can be considered important biomonitors of environmental pollution (Yıldız *et al.* 2011). Air pollution has received attention due to the fact that it is detrimental for human health and the environment (Abdunnasır *et al.* 1994, Karademir and Toker 1998, Çayır *et al.* 2008).

Lichens and mosses have been widely used for more than 20 years for assessing the atmospheric deposition of heavy metals and radionuclides in urban areas (Adamo *et al.* 2003). Lichens occurring naturally in the area as well as those transplanted have been used as biomonitoring organisms in a large number of studies (Bargagli 1998, Brown 1984, Conti and Cecchetti 2001, Henderson 1994, Nimis 1996, Richardson 1992, Tyler 1990, Yıldız *et al.* 2008, Yıldız *et al.* 2011).

In urban areas, where lichens are often scarce or even absent, the ‘‘bags technique’’ has been set up and developed in order to monitor air pollution (Goodman and Roberts 1971). Bags consist of a mesh or grid, generally made of nylon, containing lichens. This technique has the following advantages: uniformity of entrapment surface and exposure period, flexibility both in site selection and in the number of stations that can be chosen, known original concentrations of contaminants in the biomonitors and greater collection efficiency for most elements. In addition, the bag techniques eliminate the possibility of contamination via root uptake and, in comparison with dust fall jars or bulk samplers, offer lower cost and higher efficiency (Adamo *et al.* 2003). The duration of exposure is another critical aspect of biomonitoring by bags (Bargagli 1998). Biomonitors may reach a saturation point for the uptake of an element and biomonitoring performance may also be altered by climatic and environmental conditions (Garty *et al.* 1993). The comparability of results obtained with different cryptogamic organisms is another problem associated with biomonitoring studies (Schmid-Grob *et al.* 1992) as a consequence of differences in ecophysiology and mechanisms of metal bioaccumulation. In areas with widespread geochemical natural and anthropogenic sources of metals, epiphytic lichens seem more reliable biomonitors of atmospheric deposition of trace elements (Bargagli and Mikhailova 2002).

It is known that the chlorophyll content of the lichen decrease with increasing pollution which is the physiological effect of pollution on lichens (Yıldız *et al*. 2011). This may be due to the inhibition of “the novo synthesis” and (or) an increase in amino acid degradation (Godzik and Linskens 1974). It has been shown that air pollutants ultimately reduce both photosynthetic and respiration rates in lichens (Pearson and Skye 1965, Puckett *et al.* 1974, Beekley and Hoffman 1981).

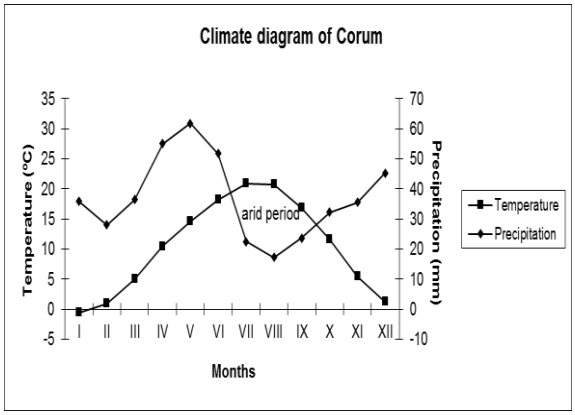
The purpose of this study is to determine Pb, Cu, Cd, Mn, Ni and Zn concentrations and chlorophyll a and b contents in *Pseudevernia furfuracea* (L.) Zopf. For this purpose, lichens from an unpolluted area were transplanted to the selected locations in Çorum.

2. MATERIAL AND METHODS

2.1. Study Area

The study was performed in the urban area of Çorum. Çorum is located at Central Anatolia (Figure 3) with a population of 221,699 inhabitants (according to the 2000 census) (Anonymous 2000). The total number of vehicles registered was 82,487 in 2002 (Anonymous 2003). The main air pollution sources of Çorum are urban motorway, industrial and domestic heating. The climate of the city is semi-arid continental climate with cold winters (Akman 1999) with mean annual temperature of 8,5 ^{o}C. The climate diagram of Çorum is given in (Figure 1). The prevailing winds are from ENE **(**Anonymous 2008) (Figure 2). Eight exposure sites were

selected in the city of Çorum as monitoring stations (Table 1). All the stations in Çorum are at the city centre and the pollution sources are urban motorway traffic, industrial and domestic heating.



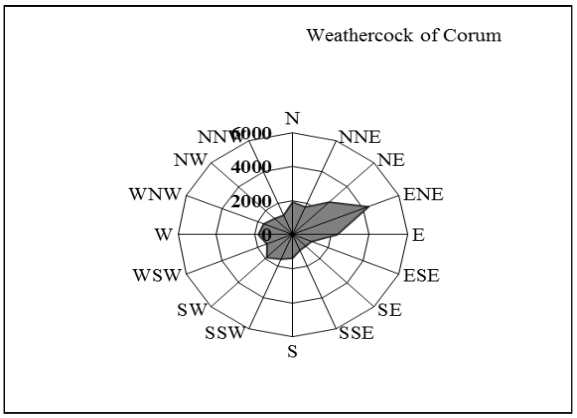


Figure 1. Climate diagram of Çorum **Figure 2. Weathercock of Çorum**

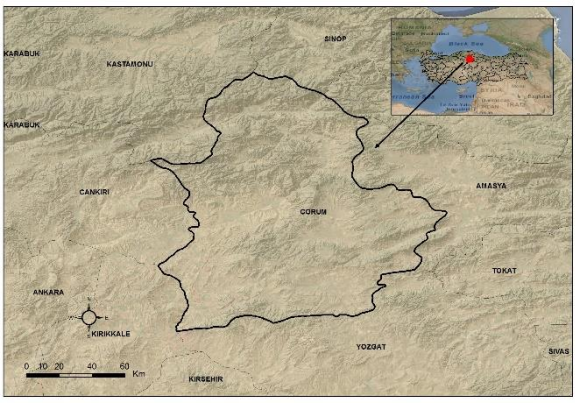


Figure 3. Map of study area

2.2. Biological Material

The thalli of *P. furfuracea* lichen samples were collected from a forest near Yapraklı Büyük Yayla Forest Çankırı. This region is far from the pollution sources and thought to be unpolluted as compared to the selected city centre stations. About 20 g of fresh material was packed loosely in a fine nylon net. Each lichen bag included several thalli. At each monitoring stations two of these bags was tied on a nylon rope and hanged on two different trees above 3 meters from the ground. All the lichen samples were exposed to air pollution for two periods of 3 months (totally 6 months) from 04 July 2002 to 09 January 2003. Hanging date was 04-05 July 2002, first collection date was 05 October 2002 and the last collection date was 09 January 2003.

Table1. Locations of the stations

Station no	Station	Substrate of the specimen	Altitude of the station (GPS)(m)	Coordinates
C1	Çankırı-Yapraklı, Yapraklı Büyük Plateau, Dikilitas area (control group)	*Pinus sylvestris*	1750 m	N 40º 47' 600" E 33º 46' 818"
C2	Çankırı-Yapraklı, Yapraklı Büyük Plateau, Dikilitas area (control group)	*Pinus sylvestris*	1750 m	N 40º 47' 600" E 33º 46' 818"
1	Çorum - Centrum, Hurriyet Park	*Pinus nigra* subsp. *pallasiana*	801 m	N 40º 33' 005" E 34º 57' 287"
2	Çorum- Velidedeoglu Park, Samsun Road, Inonu Street, Gazi Street.	*Pinus brutia*	800 m	N 40º 33' 275" E 34º 57' 995"
3	Çorum- Samsun road, İnönü street, Gazi Street, in front of Cement Factory	*Fraxinus* sp.	820 m	N 40º 33' 857" E 34º 58' 796"
4	Çorum- Binevler vicinity, In front of Old Governor Hall	*Fraxinus* sp.	840 m	N 40º 34' 482" E 34º 58' 032"
5	Çorum- Iskilip cros road , Garden of Eser tile manufactory	*Salix alba*	774 m	N 40º 33' 226" E 34º 55' 675"
6	Çorum- Samsun Highway, Burun Farm Vicinity, Emiroğlu Flour Mill	*Salix* sp.	743 m	N 40º 31' 839" E 34º 55' 114"
7	Çorum- Yenikent, Mimar Sinan Square, Mimar Sinan Mosque	*Populus alba*	805 m	N 40º 31' 907" E 34º 56' 900"
8	Çorum- İpekli Baglari, 19th Street, No:7, House garden	*Malus* sp.	874 m	N 40º 32' 326" E 34º 58' 721"

2.3. Sample Preparation and Heavy Metal Determination

After the collection of the transplanted lichen samples, they were first washed with tap water and distilled water twice to remove any dirty substances. Specimens were dried in paper bags at 80°C for 24 hours to protect them against microbial decomposition and to provide reference values for dry weight. The dried lichen samples were ground into powder using mortar and pestle.

All the glass, plastic and porcelain equipment was put in water with detergent and left over night, washed with tap water and then put into a solution of 20% nitric acid and left

overnight again. After these steps the glassware were washed with double-distilled water and dried at 60°C before use. For the preparation of all standards solutions of 65% w/w nitric acid and aqua regia 35% w/w HCl were used. All the steps of standard and solution preparations and also for dilutions, double distilled water was used. HNO_3 was used for dissolving specimen parts, which is very common in such processes (Halıcı *et al.* 2005). About 1g of the dried lichen sample was put into a porcelain crucible and burned at 460°C for 24 hours in an oven. Samples turned into ash and were put into a 100 mL beaker and then a 65% solution of 10 mL HNO_3 added. Beakers were heated in a sand bath in order to evaporate the excess HNO_3. Just before all the HNO_3 evaporated, the beakers were taken from the sand bath and left to cool at room temperature. After evaporation, the remaining part was placed into centrifuge tubes and the volume adjusted to 15 mL with 1% HNO_3. Samples were centrifuged at 3000 rpm (3000 rpm= 1157 g relative centrifuge acceleration) for 20 min. After centrifugation the supernatant was transferred into 25 mL volumetric flask and the volume was adjusted to 25 mL with 1% HNO_3. Heavy metal contents were determined by using ICP (Varian Liberty ICP-OES Sequential) f(Halıcı *et al.* 2005).

2.4 Chlorophyll Measurement

Chlorophyll was extracted from 20 mg of the airdried lichen material using pure DMSO (Dimethylsulphoxide (for synthesis) 99% purity, Merck 8.02912). Then 5 mL of DMSO was added to the thalli for extraction. Tubes with DMSO and lichen samples were incubated at 65°C for 40 min in the dark and then allowed to cool to room temperature. The extracts were filtered through a Whatman no 3 filter paper. The spectrophotometer (Varian Liberty ICP-OES Sequential) was calibrated at 750 nm with DMSO. Absorbance of the extracts was read at 665 and 648 nm. Calculations were done according to the following equations;

$C_a = 14.85A^{665} - 5.14A^{648}$

$C_b = 25.48A^{648} - 7.36A^{665}$

$C_{a+b} = 7.49A^{665} + 20.34A^{648}$

Chlorophyll extractions were done according to method describe by Barnes *et al.* (1992).

3. RESULTS AND DISCUSSION

The heavy metals, Cu, Cd, Ni, Pb, Mn and Zn and chlorophyll a and chlorophyll b contents of *P. furfuracea* specimens which were hanged at 8 stations in Çorum stations and 2 stations in Çankırı as a control were determined (Table 1). The specimens were collected two times in time intervals of 3 months. According to the analysis, there was an increase in heavy metal accumulation of the Cu, Ni, Zn, Mn and decrease in Chlorophyll a content of the specimens in two following periods (Table 2). It was known that the chlorophyll content of a plant decreases with increasing pollution. If the pollution rates increase, chlorophyll b degradation starts and rises up considerably. Any considerable change was observed in chlorophyll b content at different time intervals.

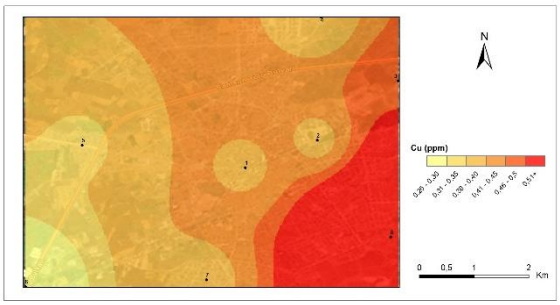
Table 2. Results of lichen material analysis (Values for Cu, Cd, Ni, Pb, Mn and Zn are in µg.g^{-1}. Chlorophyll a and chlorophyll b are in µg chl.mg air-dry wt thallus^{-1})

Elements	Periods	Cu	Cd	Ni	Pb	Mn	Zn	Chlorophyll a	Chlorophyll b	Chlorophyll a+b	Chlorophyll a/b	Chlorophyll b/a
1	1	0,28423	0,02621	0,27508	0,51637	1,89763	0,15076	7,7827	1,945	9,7277	5,0007	0,312
	2	0,38909	0,02757	0,28306	0,55338	1,94752	0,57671	9,252	3,013	12,265	4,5167	0,3337
C2	1	0,25191	0,03153	0,20229	0,52883	1,91850	0,18884	4,9797	1,109	6,0887	5,7143	0,2017
	2	0,34413	0,02832	0,31485	0,56882	1,98790	0,58973	4,8937	1,036	5,9297	5,9523	0,1983
1	1	0,38453	0,02075	0,26365	0,48536	1,73946	0,18607	2,184	0,499	2,683	4,377	0,228
	2	0,49511	0,02657	0,26200	0,50564	1,73805	0,26309	3,541	0,997	4,538	3,552	0,282
2	1	0,37696	0,02140	0,28538	0,52764	1,66436	0,15476	6,615	1,331	7,946	4,976	0,201
	2	0,46015	0,02205	0,30775	0,53300	1,80570	0,17035	0,711	0,519	1,23	1,37	0,730
3	1	0,49033	0,02142	0,39869	0,59078	1,88175	0,14193	4,006	0,743	4,749	5,392	0,185
	2	0,68991	0,02411	0,45526	0,69311	2,40114	0,26192	0,185	0,206	0,391	0,898	1,114
4	1	0,35153	0,02894	0,51852	0,61448	2,17770	0,17050	2,849	0,605	3,504	4,792	0,209
	2	0,48507	0,02527	0,67293	0,55186	2,31232	0,42495	2,809	0,717	3,526	3,918	0,255
5	1	0,34388	0,03312	0,86758	0,53409	3,22150	0,26841	1,243	0,453	1,696	2,744	0,364
	2	0,48547	0,02465	0,96167	0,49614	3,26384	0,59104	0,113	0,104	0,217	1,087	0,920
6	1	0,20476	0,02328	0,19107	0,35280	1,29833	0,60275	4,465	0,653	5,118	6,838	2,916
	2	0,42504	0,02971	1,23956	0,61766	2,83451	0,15157	1,889	0,508	2,397	3,719	0,269
7	1	0,34959	0,03057	0,40533	0,46533	2,25299	0,18544	2,189	0,613	2,802	3,571	0,280
	2	0,33478	0,02234	0,39414	0,41925	2,04386	0,28176	0,582	0,491	1,073	1,185	0,844
8	1	1,30756	0,02268	0,75375	0,59376	2,82091	0,22735	4,332	0,788	5,12	5,497	0,182
	2	2,09217	0,02473	0,40454	0,54071	2,17521	0,85283	1,839	0,556	2,395	3,308	0,302

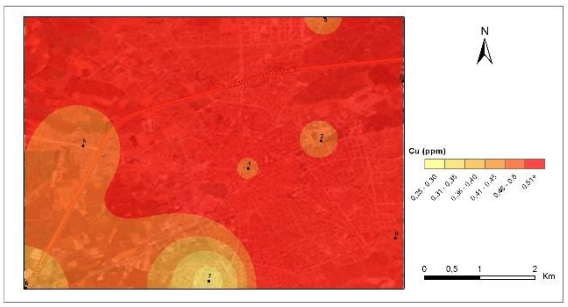
In the view of heavy metal analysis, it can be said that *P. furfuracea* accumulated heavy metals and worked well as a bioindicator organism for biomonitoring. With respect to the maps, the pollution status of the city can be easily observed and compared. The changes in the first and second period can be examined from these maps (Figure 4, Figure 5). The examination of the Cu maps showed that there were differences between the first and the second periods in all 8 stations. Based on the Cu analysis results it can be said that there is a continuous high level of Cu around 8th station and the difference occurred at the other stations could be explained with the climatic circumstances. Station 8 was a house garden but it was so close to the main street. The reason of high Cu accumulation might be the main street traffic and crowd.

Figure-4 Pollution maps of Çorum according to the heavy metals Cu, Cd, Mn, Ni, Pb and Zn

Pollution maps for Cu

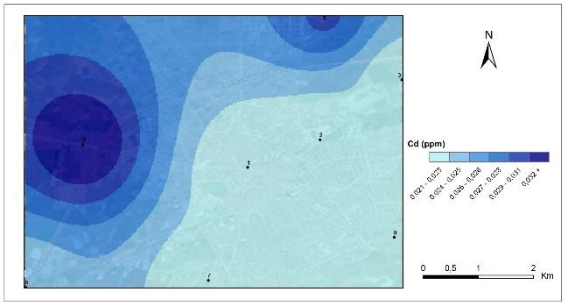


First period

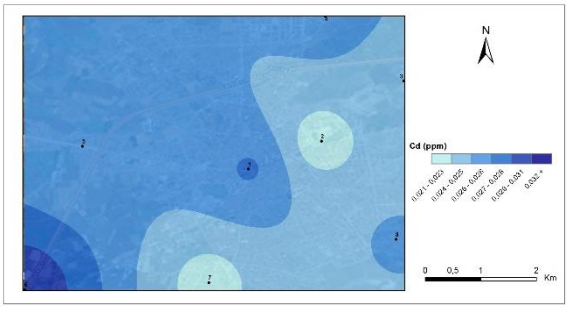


Second period

Pollution maps for Cd

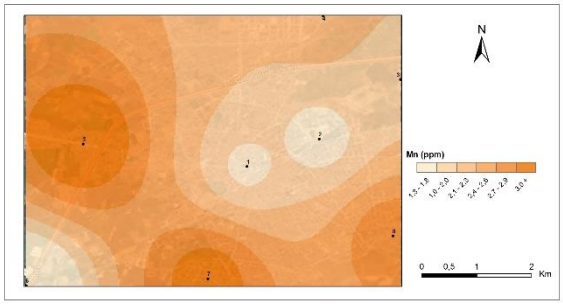


First period

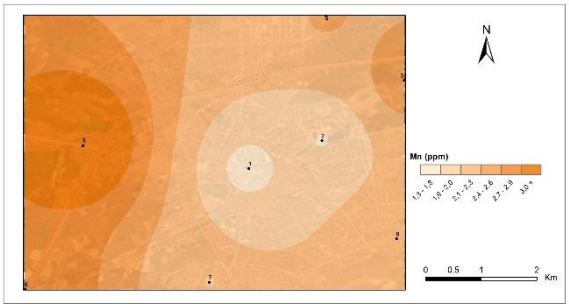


Second period

Pollution maps for Mn

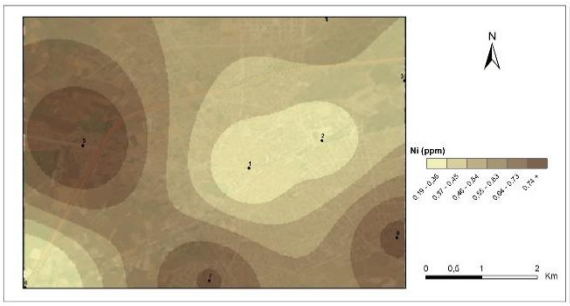


First period

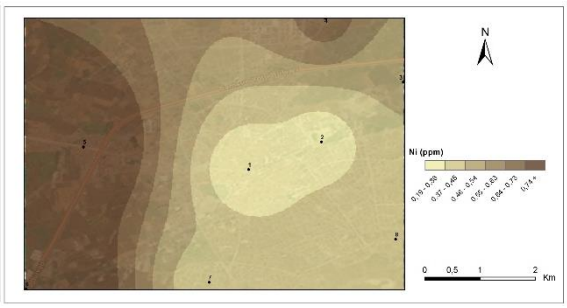


Second period

Pollution maps for Ni

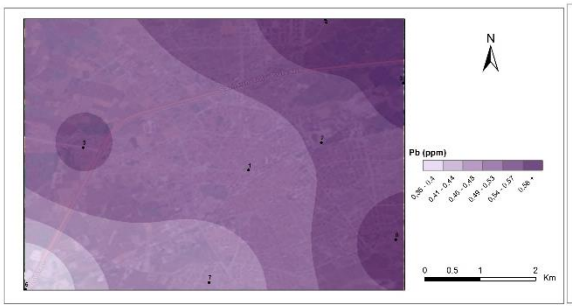


First period

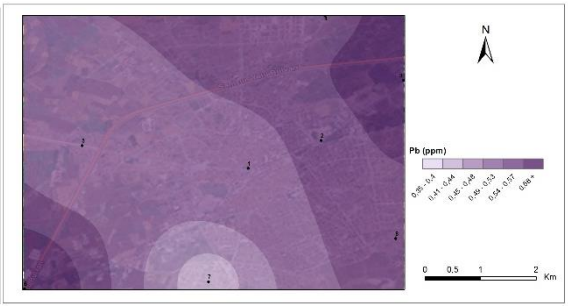


Second period

Pollution maps for Pb

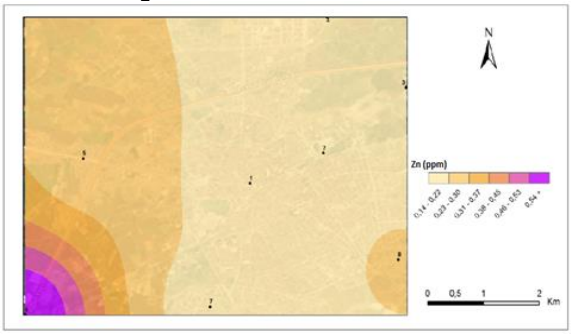


First period

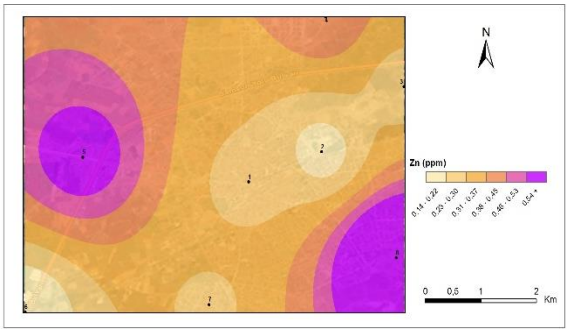


Second period

Pollution maps for Zn

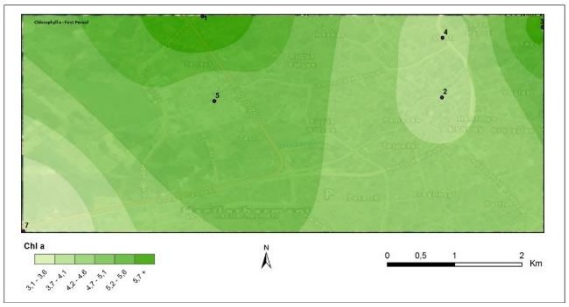


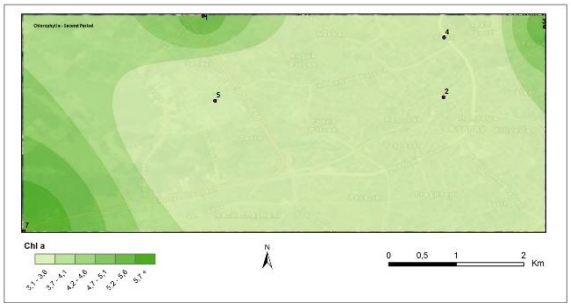
First period



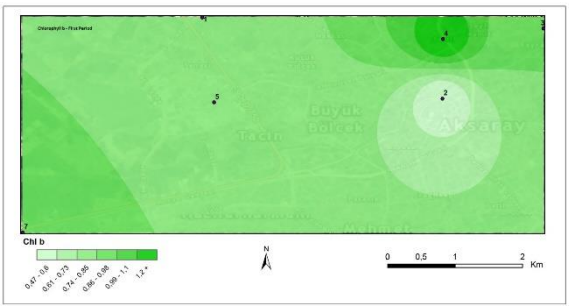
Second period

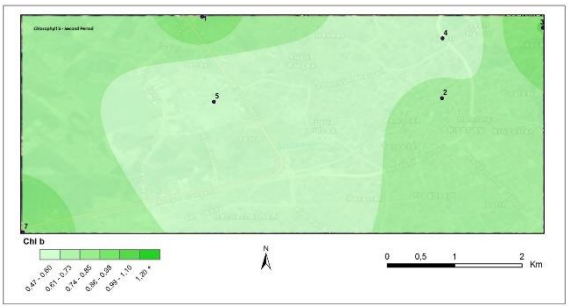
Figure-5 Pollution maps of Çorum according to Chlorophyll a and b degradation



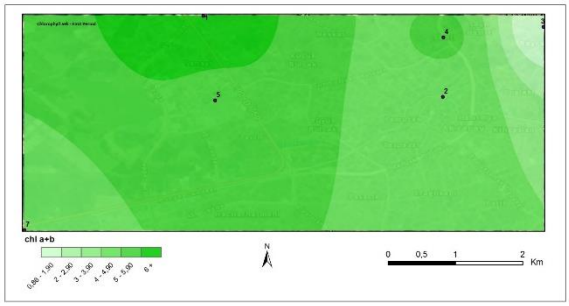


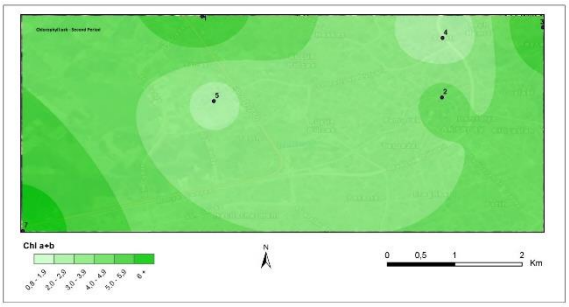
First period **Second period**



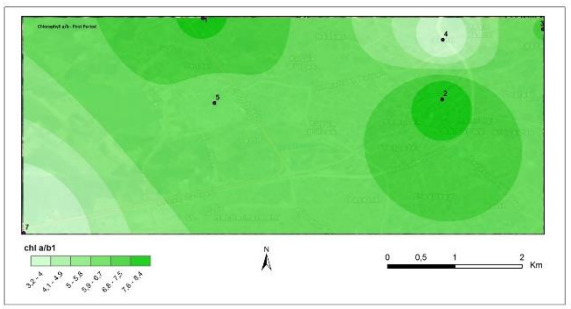


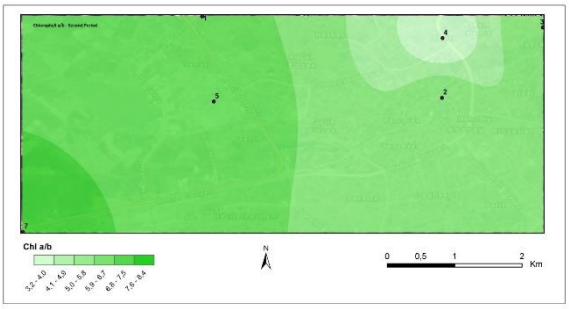
First period **Second period**



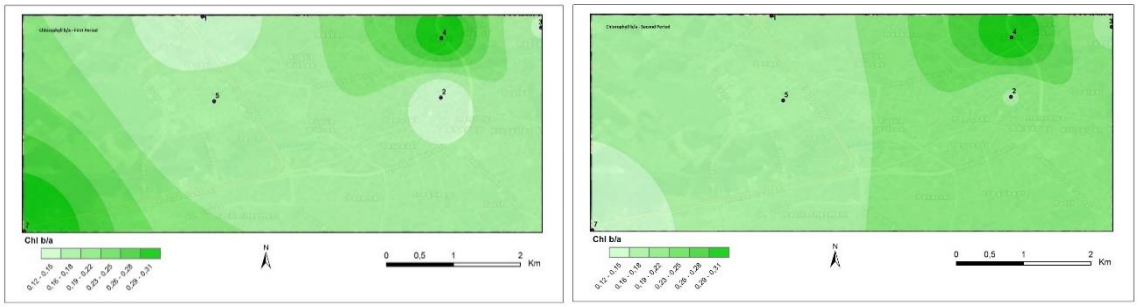


First period **Second period**





First period **Second period**



First period **Second period**

As figured on the maps, it can be said that the pollution level of Zn decreased in the second period, although at first period there were some high values observed.

For the heavy metal Mn, there were fluctuations throughout the sampling period. But it was obvious that stations 3, 4, 5,6,7,8 illustrated high Mn levels. These stations were close to the main roads and the pollution around these stations was mainly caused by heavy traffic and factories established. Also station 3 was closed to an industrial area. The area was the only cement factory in the city. At the second periods it has shown obvious pollution values for almost all heavy metal types.

The Pb accumulation did not show a considerable change in samplings. The Zn pollution which was also originated from vehicles (Markert 1993) were found higher around station 8, it was under the influence of heavy traffic. Heavy metal accumulation in plant tissues results in degradation of chlorophylls (Garty *et al.* 1985, Ra *et al.* 2005). At the maps of chlorophyll concentration, there was a decrease in the chlorophyll a and b contents of the samples. The ratio of chlorophyll b to a (Chlorophyll b/a) is the sign of decreasing rates in photosynthesis pathways. Because of the small changes in the values of chlorophyll b, the changes in chlorophyll b/a were dependent on chlorophyll a and the decrease in chlorophyll a results in increase in chlorophyll b/a. The highest increase in chlorophyll b/a was observed around the stations 3 and 6. It was expected that, high pollution results in decrease in chlorophyll a (Backor *et al.* 2003). So the chlorophyll a/b content of the samples decreased and these changes can be observed from the maps. All changes in chlorophyll a and b contents and the ratios could be explained by the environmental stress like pollution but it is hard to say that the only reason of these changes were the pollution, also climatic conditions, seasons, strength of the light and the lichen itself effective on these changes. The chlorophyll a+b content of the samples shows similarities with the maps of chlorophyll a and chlorophyll b as expected. Chlorophyll a+b content of the tissues depended mostly on chlorophyll a content because of the great change in time. But the small decrease in chlorophyll b content was also effective. The results are also supported by the maps for chlorophyll b/a. Analysing all the maps overall it can be said that the lichen species, *P. furfuracea*, accumulated the heavy metals and it worked well as a biomonitor organism.

4. CONCLUSION

1. Atomic absorption method used in this study provides remarkable results on bioaccumulation caused by heavy metal pollution.
2. The results of ICP method also provide an early warning system with a higher sensitivity than the other techniques. The system is not only sensitive but also economic.

3. Lichens can be used as biomonitors of heavy metal pollution.

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ATTITUDES OF MEDICAL STUDENTS TO VIOLENT DISCIPLINARY METHODS, SOCIAL GENDER ROLES AND CHILDREN'S RIGHTS: A CROSS-SECTIONAL RESEARCH

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ABSTRACT

The use of all types of violent disciplinary methods degrading the child including physical punishment is a common violation of children's rights. As a result, the aim of this study is to investigate the attitudes of medical students related to "violent disciplinary methods, social gender roles and children's rights" and to examine the correlation between these attitudes. Based on the United Nations Convention of the Rights of the Child and the child abuse literature, a survey developed by the researcher aiming to measure attitudes and containing 5-point Likert type questions was applied to medical students. The correlations between attitude questions were analyzed with Kendall's Tau Correlation. The survey was voluntarily completed by medical students in years 1 to 5. Of students 54.1% were female and the mean age was 21.3±2.7 years. There was a statistically significant positive correlation between attitudes that "children may be punished physically" and "the use of some behavior with the aim of demeaning children as a disciplinary method" with attitudes "supporting traditional social gender roles" ($p<0.05$). There was a statistically significant negative correlation between these violent disciplinary methods and attitudes supporting stereotypical gender roles with "rights of the child" ($p<0.05$). Students gave the answer "definitely disagree" at a rate of 32.8% in answer to the statement "some harmful traditional applications may be carefully used with the aim of increasing children's stamina". Medical students' attitudes supporting "violent disciplinary methods" and "traditional social gender roles" are an important factor causing attitudes that prevent protection and provision of children's rights. This study reveals the need for educational interventions aiming to change attitudes of medical students in terms of selective preventive studies.

1. INTRODUCTION

Punishment is a negative stimulus applied to reduce or end a behavior (Orhon *et al*., 2006). It is used quite commonly in society as a type of discipline method for children and typically encompasses verbal and/or physical forms of punishment (Şimşek *et al*., 2004; Orhon *et al*., 2006). Verbal punishment is a common type of parental discipline and includes behaviors like scolding, shouting or humiliating (Berlin *et al.*, 2009). Corporal punishment involves the use of physical force and is defined as behavior with the aim of causing pain to the child, no matter how slight, or making the child feel uncomfortable (United Nations Committee on the

Rights of the Child General Comment 8, 2006). According to World Health Organization (WHO) data, ¾ of children aged from 2 to 4 years in the world in general are exposed to disciplinary behavior involving violence regularly by carers (WHO, 2009). Many parents believe that the use of these types of punishment involving physical or psychological violence is appropriate/acceptable while raising children (Dawes *et al*., 2004; Admassu *et al*., 2006; WHO, 2009; Gebrehiwot, 2015;). The use of all these disciplinary methods involving violence is a common violation of children's rights (WHO, 2009; Gebrehiwot, 2015). Cultural and social norms are very effective in shaping individual behavior including the use of violence (WHO, 2009). These norms encompass parental child relationships and social gender roles (Turla et al., 2010) and may encourage violence (WHO, 2009). An important factor supporting attitudes related to violence is having traditional attitudes related to social gender roles (Çetinkaya, 2013; Uçtu and Karahan, 2016). Acceptance of violence culturally as a conflict resolution method or as a normal part of raising children is a risk factor for all types of interpersonal violence (World report on violence and health, 2002). For example, hitting children as a means of discipline (i.e., spanking or corporal punishment) is a strong risk factor for physical abuse (Taylor *et al*., 2017).

There are very few studies about the attitude and behavior relating to corporal punishment and punishing disciplinary methods used by parents in Turkey (Bilir *et al*., 1991; Şimşek et al.,2004; Orhon *et al*., 2006; Biçer *et al*., 2017). According to research by Bilir *et al*. in 1991, 62.4% of female children aged from 4 to 12 years and 62.9% of male children were exposed to physical punishment (Bilir *et al*., 1991). Research by Biçer *et al*. in 2017 found the incidence of giving physical punishment was 23.4%, with the rate of parents reporting they had applied violent disciplinary punishments within the last 6 months identified as 44.9-47.8% (Biçer *et al*., 2017). In Turkey only one research investigating the attitudes of medical students about child discipline was encountered (Orhon *et al*., 2006). Orhon *et al*. revealed that 43.3% of pediatric assistant doctors and medical students accepted beating children as discipline. Apart from this research attracting attention to the importance of the attitudes of medical students in Turkey, there is no other research investigating "attitudes to violent disciplinary methods applied to children, social gender roles and children's rights" and the correlation between these attitudes. To fill this gap in the research and to take precautions against violations of children's rights, it is important to determine the attitudes of medical students. As a result, the aim of this research is to investigate attitudes relating to "violent disciplinary methods", "traditional applications harming the body", "social gender roles" and "children's rights to benefit, life and development, free participation in life, protection from abuse and prohibition of torture" and the correlation between these attitudes and to contribute to ending violence against children.

2. MATERIAL AND METHODS

2.1. Research Region and Population

This study is a cross-sectional type research completed on students attending Çanakkale Onsekiz Mart University Faculty of Medicine in years 1-5. At the time of the research, the number of students attending was 611. A total of 304 students responded to the survey.

2.2. Ethics Committee Permission and Scope of the Study

Ethics committee permission was received from Çanakkale Onsekiz Mart University Human Research Ethics Committee (Project Ethics No: 2011-KAEK-27/2015-131). Additionally, to administer the survey, permission was granted by the Faculty of Medicine institute. Generally the research project used a survey form developed by the researcher

containing 80 questions related to the sociodemographic characteristics of the students and their attitudes to children's rights. For definitions and concepts used on the survey form, the United Nations (UN) Convention of the Rights of the Child and other Committee documents and medical literature about child abuse were used. The questions included on the survey form were collected under the following headings: a) sociodemographic characteristics of students, b) corporal punishment applications, c) use of some behavior involving humiliating, embarrassing and demeaning the child as a disciplinary method, d) some traditional applications known to cause transient injury aiming to increase the stamina of the child, e) torture or other cruel and degrading treatment and criminal law, f) social gender roles, g) children's rights, h) child abuse and neglect, i) child labor, j) definition and general principles of early childhood and k) other topics. This article is limited to data collected under the scope of items a-g. Within this scope, the attitudes of medical students participating in the research related to "violent disciplinary methods", "traditional applications harming the body", "social gender roles" and "children's rights to benefit, life and development, free participation in life, protection from abuse and prohibition of torture" and the correlation between these attitudes were investigated.

2.3. Data Collection Tools

The 80-question survey form developed by the researcher was used as data collection tool. The sociodemographic descriptive form comprised 20 questions in the first section of the form. The second section comprised a total of 60 questions coded as A1 to A60. Questions in the second section were created based on the UN Convention of the Rights of the Child and General Comment No. 7 of the UN Committee on the Rights of the Child entitled "Implementing Child Rights in Early Childhood" and supported by the medical literature. Questions in the second section with the aim of investigating attitudes were prepared with 5-point Likert type answers. "Strongly agree", "agree", "undecided/do not know", "disagree" and "strongly disagree" were given as possible options to choose. The questions investigated within the scope of this article are given below.

2.3.1. Questions Related to Violent Disciplinary Methods, Traditional Applications Harming the Body and Social Gender Roles

1) A6. Among manners and disciplinary methods used in raising children; behavior with the aim of embarrassing, humiliating, diminishing and demeaning the child may be included.

2) A7. In some situations children may need to be hit or given physical punishment.

3) A22. While ensuring the upbringing and development of a child, the principle responsibility belongs to the mother (negative formulation from UNCRC Article 18.1)

4) A27. Some traditional applications known to cause temporary injury to the body and with the aim of increasing stamina of the child may be carefully used (negative formulation from UNCRC Article 24.3)

5) A32. While ensuring the upbringing and development of a child, the responsibility lies with the mother and father together (UNCRC Article 18.1)

6) A39. Female children should help with the housework.

7) A40. Female children should help the mother in caring for their siblings.

8) A41. Male children should work to contribute to the family income.

2.3.2. Questions About Children's Rights, and Child Neglect and Abuse

1) A10. Every child has the right to a standard of living that is adequate for the child's physical, mental, spiritual, moral and social development (UNCRC Article 27.1)

2) A12. Each child has the right to rest and leisure, to engage in play and recreational activities appropriate to the age of the child and to participate freely in cultural life and the arts (UNCRC Article 31.1).

3) A17. Children must be protected from all forms of abuse and maltreatment while in the care of any person (UNCRC Article 19.1).

4) A33. In all actions concerning children, the best interests of the child shall be a primary consideration (UNCRC Article 3).

5) A44. Conscious deprivation of the basic needs of the child (such as food, clothing, shelter and medical care) shall be counted as neglect.

6) A50. The presence of events of physical violence (such as injury of the child apart from accidents), mental violence (such as things that may cause psychological injury) and sexual abuse indicate that child abuse has occurred.

7) A53. No child shall be subjected to torture or other cruel, inhuman or degrading treatment and punishment (UNCRC Article 37.a).

2.4 Data Collection

In this research, the observed survey administration technique was used. Classes were entered on different days at different times. Surveys were distributed to medical students who volunteered to participate during lesson hours. Necessary explanations relating to privacy and the aim of the research were made. Participants did not write their names on the surveys. The survey questions were answered within 20-30 minutes. The surveys completed by the students were mixed while collected.

2.5 Data Analysis

The data were analyzed with Statistical Product and Service Solutions (SPSS) (version 20.0; SPSS /IBM Inc., Chicago, IL, USA). The numbers, percentages, mean, and standard deviation were calculated for the presentation of descriptive data. Correlation among the variables was analyzed with Kendall's Tau Correlation. Statistical significance was accepted as $p<0.05$.

3. RESULTS

3.1. Sociodemographic Characteristics of Study Group

The total number of medical students participating in the research was N=304. Of participants 54.1% (n=164) were female and 45.9% (n=139) were male, with mean age of 21.3 ± 2.7 years. The majority of the research group comprised preclinical (2nd and 3rd year) students. Parents of 93.4% of students lived together. Parental educational level was mainly high school and above. Only 10.6% of the research group reported receiving training about children's rights. The sociodemographic characteristics of students are shown in Table 1 (Table 1).

Table 1. Sociodemographic characteristics of medical students participating in the research

Variables	**n***	**%****
Gender		
Female	164	54.1
Male	139	45.9
Studying year		
Preclinical (<4)	203	68.4
Clinical (4-5)	94	31.6
Secondary boarding school		
Yes	70	23.2
No	232	76.8
Mother's education		
Secondary school graduate	122	41.4
High school and above	173	58.6
Father's education		
Secondary school graduate	82	27.8
High school and above	213	72.2
Mother's marriage age		
≤18	58	20.2
>18	229	79.8
Father's marriage age		
≤18	8	2.8
>18	279	97.2
Sibling(s)		
Yes	255	87.3
No	37	12.7
Parental status		
Parents Together	226	93.4
Parents Separated	16	6.6
Any training on children's rights		
Yes	32	10.6
No	271	89.4

*n=number,** %=percentage is distributed for each question

3.2. Attitudes of medical students

Table 2 shows that answers given by medical students to questions about violent disciplinary methods, traditional applications that may harm the body and social gender roles (Table 2).

Table 2. Attitudes of medical students to violent disciplinary methods, traditional applications harming the body and social gender roles

Codes and Variables	SD[1] **n (%)**	D[2] **n (%)**	U[3]/DNK[3] **n (%)**	A[4] **n (%)**	SA[5] **n (%)**
A6. Among manners and disciplinary methods used in raising children; behavior with the aim of embarrassing, humiliating, diminishing and demeaning the child may be included.	226 (74.3)	51 (16.8)	12 (3.9)	8 (2.6)	7 (2.3)
A7. In some situations children may need to be hit or given physical punishment.	190 (63.3)	64 (21.3)	21 (7.0)	17 (5.7)	8 (2.7)
A22. While ensuring the upbringing and development of a child, the principle responsibility belongs to the mother (negative formulation from UNCRC Article 18.1)	47 (15.7)	96 (32.0)	42 (14.0)	80 (26.7)	35 (11.7)
A27. Some traditional applications known to cause temporary injury to the body and with the aim of increasing stamina of the child may be carefully used (negative formulation from UNCRC Article 24.3)	99 (32.8)	77 (25.5)	73 (24.2)	31 (10.3)	22 (7.3)
A32. While ensuring the upbringing and development of a child, the responsibility lies with the mother and father together (UNCRC Article 18.1)	0 (0.0)	3 (1.0)	1 (0.3)	20 (6.7)	274 (91.9)
A39. Female children should help with the housework.	57 (19.5)	66 (22.5)	67 (22.9)	76 (25.9)	27 (9.2)
A40. Female children should help the mother in caring for their siblings.	62 (20.9)	69 (23.2)	71 (23.9)	72 (24.2)	23 (7.7)
A41. Male children should work to contribute to the family income.	119 (39.9)	115 (38.6)	43 (14.4)	11 (3.7)	10 (3.4)

n=number, %=percentage, 1: "Strongly Disagree", 2: "Disagree", 3: "Undecided/Do Not Know", 4: "Agree", 5: "Strongly Agree"

Table 3 shows the distribution of responses to questions about children's rights as defined by the UNCRC. The highest proportion of medical students (93.3%) responded to the question about the right to life and development (A10) with "strongly agree". In second place was the question about prohibition of torture (A53) with 89.3% responding "strongly agree" to this question (Table 3).

Table 3. Knowledge and attitudes of medical students to children's rights and child abuse and neglect

Codes and Variables	SD[1] **n (%)**	D[2] **n (%)**	U[3]/DNK[3] **n (%)**	A[4] **n (%)**	SA[5] **n (%)**
A10. Every child has the right to a standard of living that is adequate for the child's physical, mental, spiritual, moral and social development (UNCRC Article 27.1)	1 (0.3)	3 (1.0)	2 (0.7)	14 (4.7)	280 (93.3)
A12. Each child has the right to rest and leisure, to engage in play and recreational activities appropriate to the age of the child and to participate freely in cultural life and the arts (UNCRC Article 31.1).	1 (0.3)	3 (1.0)	4 (1.3)	29 (9.6)	264 (87.7)
A17. Children must be protected from all forms of abuse and maltreatment while in the care of any person (UNCRC Article 19.1).	3 (1.0)	2 (0.7)	3 (1.0)	31 (10.2)	264 (87.1)
A33. In all actions concerning children, the best interests of the child shall be a primary consideration (UNCRC Article 3).	4 (1.3)	4 (1.3)	30 (10.1)	79 (26.5)	181 (60.7)
A44. Conscious deprivation of the basic needs of the child (such as food, clothing, shelter and medical care) shall be counted as neglect.	13 (4.4)	4 (1.4)	4 (1.4)	49 (16.6)	226 (76.4)
A50. The presence of events of physical violence (such as injury of the child apart from accidents), mental violence (such as things that may cause psychological injury) and sexual abuse indicate that child abuse has occurred.	4 (1.3)	2 (0.7)	5 (1.7)	38 (12.8)	249 (83.6)
A53. No child shall be subjected to torture or other cruel, inhuman or degrading treatment and punishment (UNCRC Article 37.a).	1 (0.3)	3 (1.0)	8 (27)	20 (6.7)	266 (89.3)

n=number, %=percentage, 1: "Strongly Disagree", 2: "Disagree", 3: "Undecided/Do Not Know", 4: "Agree", 5: "Strongly Agree"

3.3. Correlation Findings

The correlation between the attitudes of medical students about the topics of "violent disciplinary methods, traditional applications that harm the body and social gender roles" and "children's right to benefit, life and development, free participation in life, protection from abuse and prohibition of torture" were investigated with Kendall's Tau correlation (Table 4). For attitudes related to violent disciplinary methods, the responses to the statements "A6. Among manners and disciplinary methods used in raising children; behavior with the aim of embarrassing, humiliating, diminishing and demeaning the child may be included" and "A7. In some situations children may need to be hit or given physical punishment" were investigated.

Attitudes about traditional applications harming the body were investigated using the responses to the statement "A27. Some traditional applications known to cause temporary injury to the body and with the aim of increasing stamina of the child may be carefully used".

There was a statistically significant positive correlation between A6, accepting degrading behavior as a disciplinary method for children, with A7 and A27. [(r:0.312, p<0.001), (r:0.156, p:0.002), respectively] (Table 4). There was a statistically significant positive correlation identified between A6 and "traditional social gender roles" (A39. Female children should help with the housework; A40. Female children should help the mother in caring for their siblings; A41. Male children should work to contribute to the family income). There was a statistically significant negative correlation between A6 and "egalitarian social gender roles" (A32. While ensuring the upbringing and development of a child, the responsibility lies with the mother and father together) [(r:0.261, p<0.001), (r:0.235, p<0.001), (r:0.193, p<0.001), (r:-0.207, p<0.001), respectively] (Table 4). There was a statistically significant positive correlation identified between A7 about corporal punishment and "A27: traditional applications harming the body" and "traditional social gender roles (A39, A40, A41)". There was a statistically significant negative correlation identified between A7 and "egalitarian social gender roles (A32)" [(r:0.155, p:0.002), (r:0.342, p<0.001), (r:0.247, p<0.001), (r:0.199, p<0.001), (r:-0.159, p:0.004), respectively] (Table 4). There was a statistically significant positive correlation present between statement "A22: in raising a child the principle responsibility belongs to the mother" with other traditional social gender roles (A39, A40, A41) and "traditional applications harming the body (A27)" [(r:0.171, p<0.001), (r:0.128, p:0.007), (r:0.151, p:0.002), (r:0.119, p:0.012), respectively] (Table 4). There was a statistically significant positive correlation identified between "traditional applications harming the body (A27)" with "violent disciplinary methods (A6, A7)", "traditional social gender roles (A22, A39, A40, A41)" and "child's benefit" accepted as one of the basic principles of child rights [(r:0.156, p:0.002), (r:0.155, p:0.002), (r:0.119, p:0.012), (r:0.169, p<0.001), (r:0.198, p<0.001), (r:0.280, p<0.001), respectively] (Table 4). There was a statistically significant negative correlation between A6 with the children's rights as defined by the UNCRC as "life and development, free participation in life, protection from abuse, benefit of the child, prohibition of torture (A10, A12, A17, A33 and A53)" and "egalitarian social gender roles (A32)". [(r:-0.258, p<0.001), (r:-0.217, p<0.001), (r:-0.117, p:0.034), (r:-0.129, p:0.017), (r:-0.198, p<0.001), (r:-0.207, p<0.001), respectively] (Table 4). There was a statistically significant negative correlation between A7 with the children's rights as defined by the UNCRC as "life and development, free participation in life, protection from abuse, benefit of the child, prohibition of torture (A10, A12, A17, A33 and A53)" and "egalitarian social gender roles (A32)" [(r:-0.193, p<0.001), (r:-0.107, p:0.049), (r:-0.145, p:0.008), (r:-0.116, p:0.030), (r:-0.147, p:0.007), (r:-0.159, p:0.004), respectively] (Table 4). The correlations between other attitude questions are shown in Table 4.

Table 4. Correlation between attitudes to violent disciplinary methods, social gender roles and children's rights

A6	A6**													
A7	0,312*	A7**												
A10	-0,258*	-0,193*	A10**											
A12	-0,217*	-0,107*	0,527*	A12**										
A17	-0,117*	-0,145*	0,393*	0,477*	A17**									
A22	0,010	0,058	-0,004	0,029	-0,029	A22**								
A27	0,156*	0,155*	-0,113*	-0,181*	-0,075	0,119*	A27**							
A32	-0,207*	-0,159*	0,331*	0,318*	0,273*	-0,073	-0,056	A32**						
A33	-0,129*	-0,116*	0,217*	0,321*	0,265*	0,101*	-0,061	0,261*	A33**					
A39	0,261*	0,342*	-0,044	-0,126*	-0,034	0,171*	0,169*	-0,018	-0.035	A39**				
A40	0,235*	0,247*	-0,083	-0,131*	-0,061	0,128*	0,198*	-0,018	0,005	0,751*	A40**			
A41	0,193*	0,199*	-0,171*	-0,200*	-0,226*	0,151*	0,280*	-0,094	-0,176	0,358*	0,388*	A41**		
A44	-,0149*	-0,034	0,336*	0,376*	0,292*	0,041	-0,115*	0,211*	0,223*	-0,010	-0,016	-0,157*	A44**	
A50	-0,179*	-0,072	0,321*	0,379*	0,397*	-0,024	-0,209*	0,203*	0,152*	-0,106	-0,100	-0,266*	0,288*	A50**
A53	-0,198*	-0,147*	0,407*	0,326*	0,459*	0,067	-0,099	0,224*	0,337*	-0,040	-0,097	-0,237*	0,385*	0,308*

Kendall correlation analysis, correlation coefficient, *: statistical significance $p<0.05$, **: The codes are clarified in the method section.

4. DISCUSSION

This research is the first study to research the attitudes of medical students to violent disciplinary methods, social gender roles and children's rights and the correlation between these attitudes. One of the most important results of the study is that a statistically significant positive correlation was found between attitudes supporting "corporal punishment may be applied to children in some situations and some behavior with the aim of degrading children may be used as a disciplinary method" and attitudes supporting "traditional social gender roles". Another important result is the statistically significant negative correlation between these attitudes and "children's rights". According to these results, attitudes supporting violent disciplinary methods and traditional social gender roles can be blamed for violations of children's rights.

Research based on the attitudes of parents to corporal punishment reported *corporal punishment partly serves socialization of social gender roles and social control mechanisms* (Wonde and Baru, 2014). In terms of social gender roles male and female roles may be defined as traditional or egalitarian (Esen *et al*., 2017) with stereotypical gender roles the most important element in continuation of traditional attitudes and inequality between females and males (Altınova and Duyan, 2013). This research completed with medical students is the first study to quantitatively show the correlation between attitudes supporting "violent disciplinary methods" with attitudes supporting "traditional social gender roles". There was a statistically significant positive correlation between these attitudes. Though the identified correlations are not causative, they are important in terms of indicating obstacles due to related attitudes preventing children's rights from being upheld. In relation to ending violence toward children, Pinheiro stated *"For lasting change in the belief that adults have unlimited rights in the upbringing of a child, the attitudes that condone or normalize violence towards children, including stereotypical gender roles need to be challenged."*(Pinherio, 2006). The identified correlations in this study appear to support Pinheiro's thoughts. In Turkey several studies researching the correlation between tendency toward violence and social gender roles among university students reported a significant correlation between the students' tendency toward violence and traditional social gender roles (Uçar *et al*., 2017; Uçtu and Karahan, 2016; Çetinkaya, 2013). Additionally in the literature no attitude research focusing on the correlation between social gender roles and violent disciplinary methods from a children's rights perspective was found. Thus, the results of the study fill a significant gap in the research. Also, similar attitude-based studies to be completed in the field of pediatric health may be an important resource.

In all cultures, an inseparable part of raising children is stated to be teaching the child to control themselves and what is acceptable behavior (WHO, 2009). Cultural beliefs and social roles (Orhon et al.,2006; WHO, 2009; Akduman, 2010; Wonde and Baru, 2014; Biçer et al., 2017), personal experience of childhood, attitudes supporting corporal punishment (Gershoff, 2002; Ateah and Durrant 2005; Orhon et al., 2006; Gagné *et al*., 2007; Taylor *et al*., 2011; Fréchette *et al*., 2015), and lack of education regarding appropriate discipline techniques (Orhon et al.,2006; Akduman, 2010; Wonde and Baru, 2014) are reported as the main factors determining the use of corporal punishment by parents. However, having attitudes supporting corporal punishment was shown to be the strongest predictor (Dawes *et al*., 2004; Ateah and Durrant 2005; Vittrup *et al*., 2006; Gagne *et al*., 2007; Fréchette *et al*., 2015), and the strong predictors of attitudes are perceived injunctive and descriptive social norms related to corporal punishment (Taylor *et al*., 2011). When the research findings are examined in terms of violent disciplinary methods, 4.9% of medical students agreed (those responding strongly agree, and agree) with the thought that "among manners and disciplinary methods used in raising children; behavior with the aim of embarrassing, humiliating, diminishing and demeaning the child may

be included" while 3.9% reported they were "undecided/did not know". There were 8.4% who agreed (those responding strongly agree, and agree) with the thought "in some situations children may need to be hit or given physical punishment" while 7.0% stated they were "undecided". Orhon *et al*. revealed that nearly 56% of pediatric doctors and medical students believed that beating children was an acceptable disciplinary route (Orhon *et al*., 2006). Ferreire identified that 37.2% of medical students found physical punishment an acceptable method of training (Ferreira, 2014). Heward *et al*. determined that corporal punishment was accepted as an effective disciplinary method in raising children by 56.0% of students in a Chinese medical faculty and 22.2% of students in an American medical faculty (Heward, 2011). In this research, lower rates were identified for attitudes supporting violent disciplinary methods among medical students. The reason for this may be due to the sociodemographic characteristics of the study population. However, it is thought that this result may have been more affected by the fact that the study did not inquire about different types of physical punishment (e.g., spanking, slapping, pushing, throwing, biting, pinching, squeezing, clipping ears, hair pulling and other). In addition, 17.6% of medical students stated they "agreed" or "strongly agreed" with the thought that "some traditional applications known to cause temporary injury to the body and with the aim of increasing stamina of the child may be carefully used", with the undecided rate 24.2%. According to this result it may be said the consideration that mildly harmful behavior is for the benefit of the child increases the acceptability rates for this behavior. The obtained results support the idea that to prevent violence against children it is necessary not to tolerate any damaging behavior, even if proposed to benefit children (United Nations Committee on the Rights of the Child (UNCRC), *General comment No. 13*; WHO, 2009). A positive result of this study is that 95.9% of medical students participating in the study approved of "prohibiting torture and other cruel, inhuman and degrading treatment and punishment" (those saying strongly agree and agree). High rates of participants, 93.3% to 87.1%, had attitudes supporting children's rights to life and development and rights protecting against abuse. A study by Biçer *et al*. reported 79.6% of medical students stated "children have the right to express their views on all topics related to themselves" (Biçer *et al*., 2016). According to these results, it may be said that in general in Turkey medical students have positive attitudes to children's rights. However, it is noteworthy that in this research medical students had attitudes supporting violent disciplinary methods, stereotypical gender roles and traditional harmful applications. Traditional attitudes linked to these cultural and social norms should be dealt with as a system causing violations of children's rights.

The Convention of the Rights of the Child states that all types of punishment degrading to children, including corporal punishment, violate children's rights to protection from violence and need to be prohibited (UNCRC, *General Comment No.8*, 2006; UNCRC, *General comment No. 13*, 2011; Gershoff *et al*., 2017). To date 51 countries have banned all corporal punishments directed at children (Gershoff *et al*., 2017; Global Initiative to End All Corporal Punishment of Children, 2017). Legal interventions to corporal punishment have increasingly emphasized its role in preventing violence against children (Durrant and Ensom, 2012; Gershoff *et al*., 2017). After legal prohibition, it is reported the frequency of corporal punishment and attitudes supporting corporal punishment reduce (WHO, 2009). Durrant and Ensom reported three forces including "research, declaration of children's rights and legal reform" change the dimensions of corporal punishment (Durrant and Ersom, 2012). As a way of expressing social, behavioral and moral standards (Gershoff *et al*., 2017) and a part of some social-based preventive studies, in Turkey all types of degrading punishment of children, including corporal punishment, should be legally prohibited.

In this research including medical students, 76.4% and 83.6% of students stated they "strongly agree" to knowledge questions about definitions of neglect and child abuse. When

the proportion of those who stated "agree" is added, these rates rise to 93.0% and 96.4%. Additionally some research in recent years has determined that the attitudes of health professionals about corporal punishment is effective on the identification and reporting of cases (Orhon *et al*., 2006; Ferriera, 2014). Gershoff *et al*. attracted attention to the roles of professionals like doctors and psychologists in targeting selective prevention. The importance of this group is due to their roles in advising parents about appropriate disciplinary methods (Gershoff *et al*., 2017) and in the reporting process ensuring identification, treatment and exposing violence during interviews with children (Orhon *et al*., 2006; Ferriera, 2014; Polat, 2014). According to these results, it is necessary to perform further research into attitudes of medical students supporting corporal punishment and other degrading violent disciplinary methods, correlation of these attitudes with traditional social gender roles and interventions to change these attitudes. Reviewing the medical training syllabus from this aspect may be beneficial in ensuring continuous changes in attitudes.

4.1. Strengths and Limitations

The strongest aspect of this study is that it is the first research to focus on the correlation between "attitudes supporting violent disciplinary methods", "attitudes supporting traditional social gender roles" and "children's rights". In this study, there was a statistically significant positive correlation identified between attitudes of medical students supporting "violent disciplinary methods and traditional social gender roles" and a statistically significant negative correlation identified between traditional attitudes and children's rights. In general, medical students were found to have positive attitudes to children's rights to life and development and rights to protection against abuse and prohibition of torture. Additionally though there were low rates of support for violent disciplinary methods, it is noteworthy that there were higher rates of support for harmful traditional applications with the aim of increasing children's stamina. The bifurcation between these attitudes is considered to be due to the fact that corporal punishment types were not asked individually in the research. This result is assessed as a limitation of the research method. The sampling group in this research is a significant limiting factor for generalizing these results to the whole of Turkey. However, the students came from different provinces in Turkey which may have ensured variety in terms of attitudes. Additionally the results of the research are noteworthy for the possible effect of the attitudes of medical students about violent disciplinary methods and stereotypical gender roles in prevention of violations of children's rights.

5. CONCLUSION

The results of this study about the attitudes of "the use of corporal punishment and some behavior with the aim of degrading children as disciplinary methods" and "acceptance of traditional social gender roles", and the correlations identified, are considered to be significant factors in attitudes preventing protection and provision of children's rights. The medical student sample chosen for the study does not provide the opportunity to generalize these results. However, this small sample study shows the importance of determining the attitudes of medical students related to this topic and reveals the need for educational interventions targeting changes in the attitudes of students. In terms of selective prevention studies, educational interventions targeting medical students and health professionals may ensure attitude changes and this may provide significant contributions to "changing norms accepting corporal punishment, reducing the incidence of punishment and thus provision children's rights". In conclusion, in the medical field there is a need for research in different populations to determine violations of children's rights and to allow discussion of the findings in this article on a broader scale. This research may provide helpful data *to change attitudes ignoring or normalizing violence against children, including stereotypical gender roles*.

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RESEARCH ON THE COMPOSITION AND CYTOTOXIC ACTIVITY OF PINUS BRUTIA GUM

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ABSTRACT

Pinus Brutia Ten., traditionaly used as sugar regulator, antioxidant and antitumoral is consumed by chewing in Turkey. The aim of the study is to investigate the components and the effect of the gum. The base of the study depends on the way of comsumption of the resin. For in-vitro analysis, artificial salvia is used. The samples is stayed in artificial salvia at 37° C in ultrasonic bath for different time period and then determined by U-HPLC, PDA detector. Three samples were prepared from each sample and three injections were made. Caffeic acid was investigate in the samples. The optimum waiting period is set at 5 hours, which is considered to be a possible period since the gum adheres to the tooth and is a hardly soluble substance. Considering the anticarcinogenic effect of caffeic acid, cytotoxic test was carried out on specimens which were kept in saliva for 24 hours acid. After incubation of the saliva extract that including 10, 20, 50 µM caffeic acid in liver cancerous cells 1, 3, 6 hours, cell viability was observed.

1. INTRODUCTION

Pinus brutia Ten. resin is known as pine gum because it can be chewed like gum among the people in many regions of Turkey. Among the people the gum is used as antiseptic, sugar regulator, and also in Ottoman time, the compound was used in the composition of the mixtures that used for tumor healing (Arıtuluk *et al*., 2012, Saçlı *et al.,* 2001 and Atıcı 2007). There are not many published scientific studies on resins. Resin-based studies are more like volatile oil analyzes (Ulukanlı *et al*., 2014, Avnı *et al*., 2016).

A small number of active substance analyses are done on the different pine tree shells. Various studies have shown that the pine tree shells have beneficial effects on inflammation, C-deficient scurvy disease and flavonoids in the immune system diseases, glucose metabolism (Ince *et al.,* 2009, Maimoonae *et al,*2011, Kim *et al*., 2004 and Kim *et al.,* 2005). *Pinus* species have economic importance in pharmaceutical and cosmetic sectors. For instance; turpentine has been known to have a long record of remedial utilization primarily as topical counter irritants for the treatment of rheumatic disorders and muscle pain. Pine bark extract is also used in anti-aging cosmetics (Yonei *et al.,* 2004)

In Chinese medicine, pine resin is used for the treatment of skin diseases and burn scald wounds (Yang *et al.,* 2010). There are few reports are available on chemical components of P *Brutia resin* in Mediterinian countries (Satil *et al*., 2011, Iconomou *et al.,* 1964, Schiller *et al.,* 1987). The poisonous effect of the material on organism is determined by cytotoxic assay. Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss *et al*., 2013). There is no cytotoxic study on *Pinus* resin. The resin is used as a gum and while chewing it sticks the teeth. The main aim of the study to analyse the content and the amount of the gum is solved in salvia in different time period.

The other aim of the study is to analyse the effect of pinus gum which is solved in salvia on cell vitality.

2. MATERIAL AND METHODS

2.1. Instumentation

U-HPLC equipped with Thermo Scientific, Accela Model 1250 Pump, Autosampler ve PDA dedector is used for the ingredient analysis.

2.2. Material and Reagents

The resin was bought from markets and the identification was done Ass. Prof. Zeki Haznedaroglu, Katip Çelebi University, Faculty of Pharmacy, Department of Pharmaceutical Botany. The resin was milled and put into plastic tubes to react 5 mL artifical salvia (0.2 g.K_2HPO_4, 0.330g. KSCN,0.260 g. Na_2HPO_4, 1.500 g. $NaHCO_3$,0.700g. NaCl, 1.200g. HCl,1.300g.urea in L, pH=4) (Can *et al*., 2006). Merck branded chemicals was used for the preparation of artificial salvia. The tubes were shaken and left in the ultrasonic bath for 1, 2, 5,10 minutes and 5,10,18, 24 hours at 37^{O}C. Three of each sample were prepared. Full time samples were filtered through the filter paper.

Merck branded chemicals, methanol (HPLC gradient, J.T. Baker 8402), o-phosphoric acid (Redel-de Haen 30417) and bi-distilled water were used for mobile phase. Distilled water was used after filtered with 0.22 µm pore diameter Millipore Express©- PVDF vacuum filter unit. 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium MTT bromide was obtained from Sigma. Standards used in HPLC analysis were obtained from Sigma. 5891 Schwarzband 125 mm filter paper is used also.

2.3. Standard Preparation

Stock solution of dihydroxybenzoic acid, caffeic acid, t-ferrulic acid and m-cumaric acid were preperad in methanol (1mgmL^{-1}). 6, 8, 10 µgmL^{-1}diluted mixtures of the solutions were used for working standards. The mixtures were injected at three times (n: 3). Merck branded standard were used for the analysis.

2.4. Cell Viability: MMT Cytotoxicity Assays

300 mg milled resin gum waited in artificial salvia for 24 hours, at 37^{O}C in ultrasonic bath. This extract that contains 10, 20, 50 µM caffeic acid were incubated for 1, 3, 6 hours in liver cancer cells and cell viability was detected. (Kim *et al.,* 2015). For each hour 3 measurement were done.

Percent cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mossman *et al.,* 1983). MTT (5 mg / ml) was prepared in 5 ml PBS in falcon. It was stored in the dark and at -20 ° C. 96 well culture plate (flat bottom), 2x10 ^{3} cells / well, 150 µl / well were added, to stick to the cells for 24 hours were kept. Used medium: DMEM / F12 + 10% FBS + 100 U / ml penicillin + 100 U / ml streptomycin. A mixture of 10.63 µL (10 µM) or 21.27 µL (20 µM) or 53.15 µL (50 µM) was added to the wells without media removal and incubated for 1, 3 and 6 hours at 37°C in a CO_2 humidified incubator. 20 µl MTT (5 mg / ml in PBS) was added to each incubator and incubated at 37° C in a CO_2 incubator for 3 hours until the formation of purple crystals. Then, 100 µl dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken at room temperature for 1 h and then the absorbance was measured at 570 nm with a reference setting of 650 nm using a microplate reader (Mossman, *et al.,* 1983, Bruggisser, *et al*., 2002).

Calculation: Aresult = A570-A650. The Ablank is removed from the resulting value. % Cell viability is calculated**.**

3. RESULTS AND DISCUSSION

The first step of an analysis is to determine the extraction method. There is no study for Pinus resin extraction. In Turkey, traditional use of *Pinus brutia* Ten resin is chewing, therefore the study depends on the extract of salvia and finding the active ingredients of the gum that pass through the salvia.

There is no information about the contents of the prepared samples, two mixture standards were prepared and measured by U-HPLC. Preliminary studies were carried out by comparing the chromotograms obtained by injection of 24 hour samples with mixture standard chromotograms, to determine possible agents. Based on the recovery time, it was decided to evaluate samples against 6-10 µg / mL 3,4-dihydroxybenzoic acid, caffeic acid, t-ferulic acid, m-coumaric acid standards. The LOD and LOQ Limits of the samples are given in Table 1.

Table 1. Limit of dedection and limit of quantitation of the standards.

Standards	Retantion time (RT), min	Wave lenght, nm	LOD, µg/mL	LOQ, µg/mL
3,4_dihydroxysibenzoicacid	5.73	295	~ 0.5795	~ 1.9317
Caffeic acid	8.02	324	~ 0.3279	~ 1.0931
t-ferrulic acid	10.04	322	~ 0.5076	~ 1.6920
m-cumaric acid	10.50	278	~ 0.1533	~ 0.5112

In chromatogram analysis, the recovery and wave length of the chromatogram of standards and 24 hour salvia extract were compared and caffeic acid was found in gum extract. There were different peaks in the sample chromatogram which can not be determined. Chromatograms of 10 µg / mL standard mixture and 24 hour extracted gum samples were given in Figure 1. In the analysis done by U-HPLC, the presence of caffeic acid was determined. Some studies have shown that caffeic acid is found in pine bark (Yesil Çeliktas *et al.,* 2010). However, if there is no information about the content of the resin, it is necessary to make the comparison with the pine bark.

Possible pharmacological effects of caffeic acid have been shown in several studies. Rahenda Prasad et al. (2011) reported caffeic acid is reduced cell degeneration in cancerous tissue, antioxidant and immunomodulator and it has anti-inflammatory properties (Olthof *et al*., 2001, Oroion *et al*., 2015, İlhami, 2006 and Oktan *et al*., 2010). In addition, the effects of caffeic acid in diabetic mice were investigated (Durmus *et al*., 2008). It is noteworthy that traditional the reasons for the use of resin gum overlap with these effects

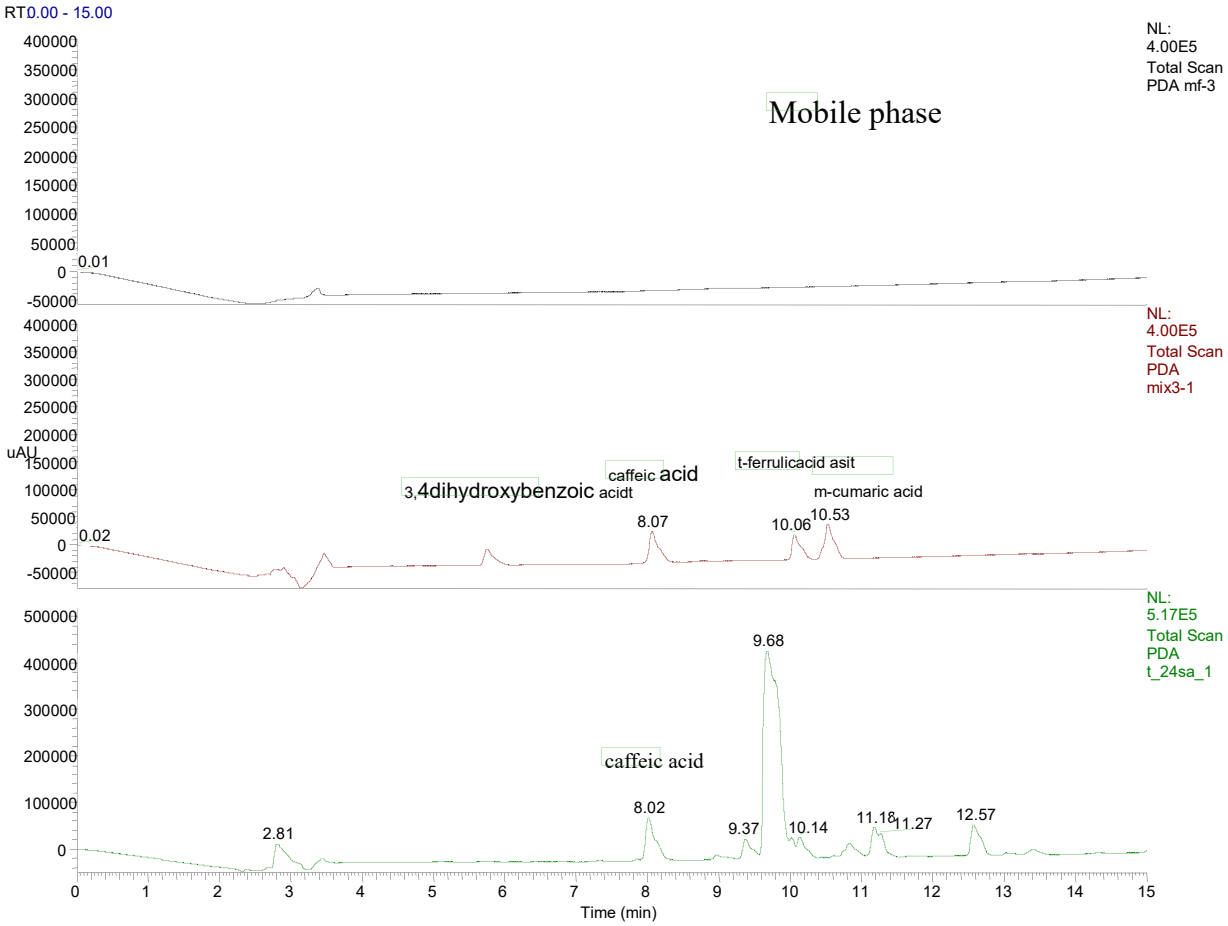


Figure 1: Chromatogram of mobile phase, standards mixture and artificial salvia that gum stayed for 24 hours.

To determine the caffeic acid quantity that passed through the salvia from resin, the same quantity of the resin were stayed 1, 2, 5, 30, 60 minutes and 5, 10, 18, 24 hours at 37 °C in ultrasonic bath in artificial salvia and were analysed by UPLC in triplicate. The calibration equation is found as y= 31954,9 + 216040 and r^2= 0.999. The results is shown in Table 2. The amount of caffeic acid passing through the salvia varies with time is shown in Figure 2.

Table 2: Caffeic acid concentration of the resin extracts

Time	Concentration µg/mg
1 min	0.0939 ± 0.0058
2 min	0.0399 ± 0.0001
5 min	0.0176 ± 0.0007
10 min	0.1177 ± 0.0218
30 min	0.0972 ± 00179
60 min	0.1093 ± 0,0001
5 hour	0.6810 ± 0.1272
10 hour	0.4038 ± 0.1075
18 hour	0.3757 ± 0.0341
24 hour	0.7064 ± 0.1964

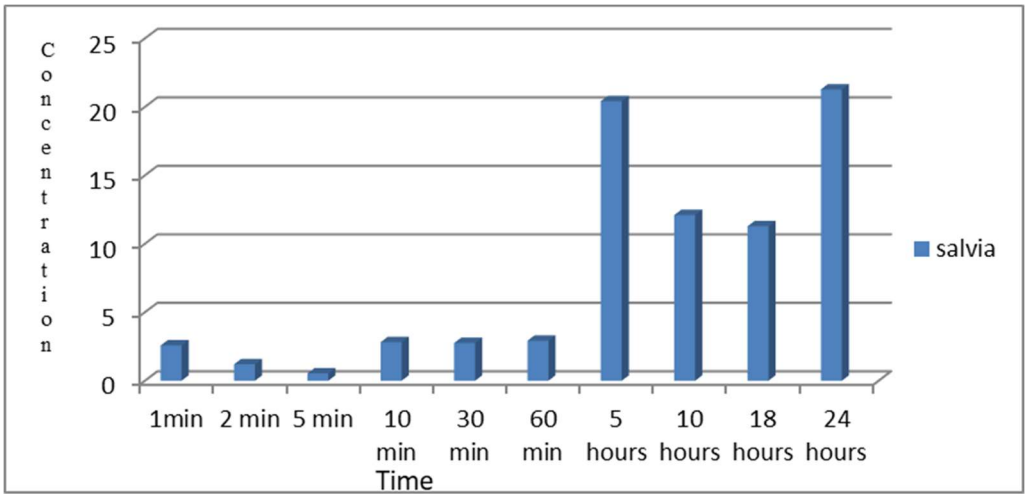


Figure 2: Changes in the amount of caffeic acid concentration (µgmg^{-1}) passing from resin to salvia

The amount of caffeic acid passing through the salvia varies with time. When Figure 2 is examined, it is found that the passage of the active substance from the sample is getting higher after 5 hours. The resin is insoluble in saliva and forms a solid at the bottom. The 1 minute sample was higher than the 2 and 5 minute samples, it was thought that at the beginning the gum was in powder form and in minutes getting harderer and active material release were

getting down. In hours the concentration is getting higher. This can be explained by the breakage of the outer shell of the hard resin. In MMT test results, different concentration of the resins were increased the cell quantity and stopped the cell death in cell culture in 1, 3, and 6st hours. The results are given in Figure 3. The microscopic photographs of the cristals in cells at 3 and 6 hours are given in Picture 1 that is shown the cell viability.

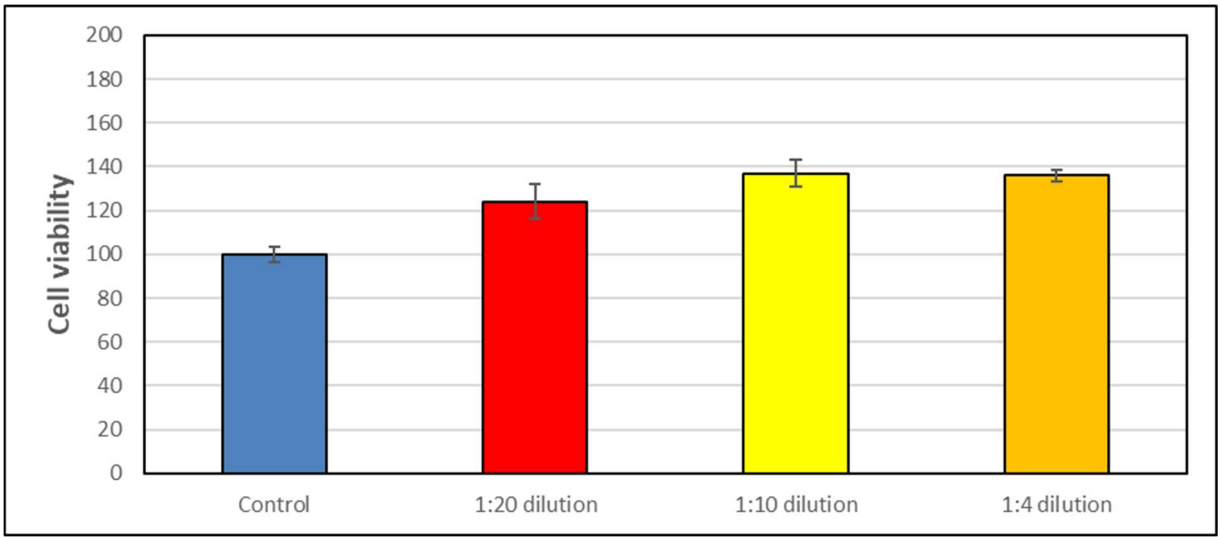
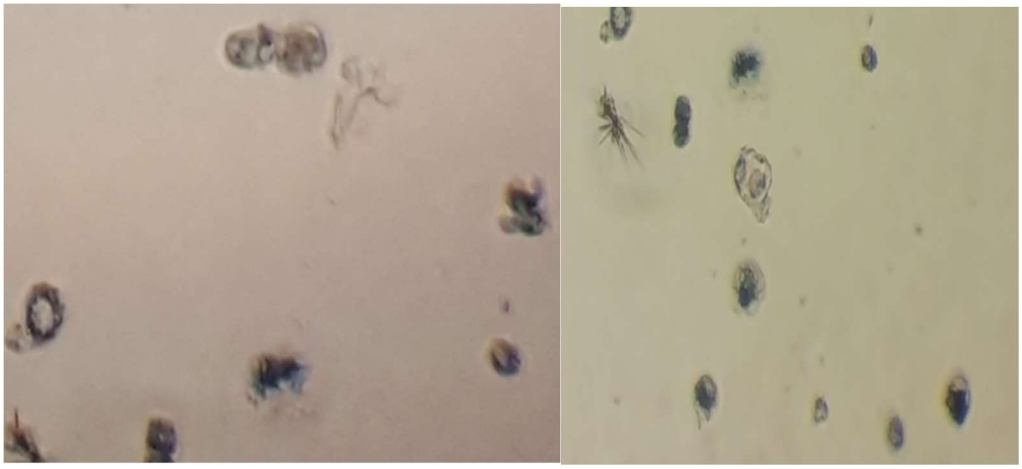


Figure 3: Cell viability results of control group and different concentration of the gum.



Picture 1. 3 hours photo at left and 6 hours photo is at right (The purple inside of the cells are the proof of the cell viability)

4. CONCLUSIONS

It is determined that the pinus resin that used as a chewing gum in public contains various active ingredients. Caffeic acid and the amount of caffeic acid in artificial salvia that passed from the resin is observed. The result of cell culture studies show that chewing gum is hepatoprotective. We can say that these kinds of products are both a protective raw material and a natural product because they are both a pharmaceutical raw material and a preferred product in preventive medicine.

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FORENSIC MEDICAL ASPECT OF RIGHT TO LIFE VIOLATION CASES IN ECHR DECISIONS ABOUT TURKEY FROM 1998 TO 2002: REVISITING THE COMMON ERRORS IN DEATH INVESTIGATIONS

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ABSTRACT

Violation of the right to life is the most severe type of physical assault against humans. In this study decisions by the European Court of Human Rights (ECHR) about violation of the right to life cases in Turkey are investigated, with the aim of determining deficiencies and errors in forensic medical procedures and to discuss the effect of these on the violation decision. Digital court files with decisions made by the ECHR from 1998 to 15.05.2002 published on the internet were retrospectively investigated. Cases with decisions against Turkey for right to life violations were determined. This study assessed data related to death investigations. Data analysis was performed with the Epi-Info 2000 program. From a total of 21 files (22 cases) with decisions of right to life violation and/or torture, there were 12 cases with decision of right to life violation (54.5%). For 11 cases (91.7%) examination of the deceased was performed, with this examination only performed by a forensic medicine specialist for 2 cases (18.2%). It was determined that autopsy was not performed for 8 cases (66.7%). Of the 4 cases with autopsy performed, none (100.0%) had photographs or radiologic imaging taken, skin sampling for microscopic and chemical analysis or sampling of any biological material performed. This research showed that, there were serious deficiencies and errors in forensic medical procedures and those investigations of death are not standardized. To prevent violations of the right to life and to efficiently perform inquiries related to death, doctors with sufficient knowledge and skills about forensic medical procedures should be required to abide by the Minnesota autopsy protocol.

1. INTRODUCTION

The Universal Declaration of Human Rights was proclaimed by the United Nations (UN) General Assembly in 1948 to define and protect human rights was a giant step in the international area (United Nations General Assembly-Declaration, 1948; Gemalmaz, 2001; Gölcüklü and Gözübüyük, 2002). In 1950 the European Convention on Human Rights was signed, and Turkey ratified the Convention in 1954 (Gemalmaz, 2001). Within the scope of the Convention, the European Court of Human Rights (ECHR) is an organ working on a continuous basis since 1998 (Gölcüklü and Gözübüyük, 2002). The second article in the Convention states *"Everyone's right to life shall be protected by law"*. The right to life is a precondition for the existence of all rights and freedoms (Gölcüklü and Gözübüyük, 2002). Violations of this right appear to infringe on the principle of "personal immunity".

In the field of medicine, many regulations have been made related to human rights since the 1970s (Soyer, 1996). These regulations emphasize the principle of using a standard medical approach (Jandoo, 1987; American College of Physicians, 1995; Thomsen and Voight, 1998; Council of Europe, 1997). The basis of this approach comprises the clinical knowledge and skills of doctors and their ethical behavior. The Minnesota autopsy protocol accepted by the UN in 1989 states that it "will guide research into all types of violent, sudden, unexpected and suspicious deaths" (United Nations-Minnesota Protocol, 1991). Investigations into death in Turkey are performed by practitioner doctors and doctors from other branches as well as by forensic medicine specialists and pathologists (Petekkaya, 2012). In the cases associated with the right of life, ECHR decisions against Turkey mention a range of deficiencies and errors related to the application of forensic medicine procedures (Gülsoy, 2002; Kök, 2007; Gültekin, 2011). For this thereby, this study aimed to investigate and reevaluate the files of cases in the ECHR with results against Turkey due to violation of the right to life and to discuss the effect of deficiencies and errors experienced in forensic medicine procedures on the decision-making process.

2. MATERIAL AND METHODS

2.1. Materials

The decisions of the ECHR published in the internet environment were retrospectively investigated (European Court of Human Rights-Decisions, 1998-2002). From 1998 to 15.05.2002, decisions against Turkey involving violations of the right to life and torture laws (Convention articles 2 and 3) were reviewed. Decisions where deficiencies and errors in forensic medicine applications were primarily discussed were determined. Within the scope of this study cases were limited to those with the decision that the right to life was violated. Inclusion criteria for the study were deficiencies and errors in the postmortem forensic medicine procedures shown as justification for the violation decision.

2.2. Data Collection Tools

The data collection tool used was a case analysis form developed by the researcher. This form included a total of 63 questions, with the first 8 questions about sociodemographic data. The other questions were parameters that varied according to the event involved. These parameters included data related to witness statements, crime scene investigation findings, examination after death, autopsy findings, live case examinations, specialist and/or doctor reports and the findings of the court.

2.3. Data collection and statistical analysis

The data analysis form was completed for each case. This broad data set was recorded by obtaining the texts of ECHR decisions. In this study findings of cases resulting in death only were statistically evaluated. Data analysis used the Epi-info 2000 program. For presentation of descriptive data number, percentage and minimum and maximum values were used. Permission for the research was obtained from the Institute of Forensic Medicine Ethics Committee. The results were assessed together with current ECHR statistics and the literature.

3. RESULTS

In the ECHR for the period from 1998 to 15.05.2002 there were a total of 21 court cases resulting in decisions against Turkey with the decision that "right to life and/or torture laws were violated" which abided by the study inclusion criteria. The total number of individuals involved in these court cases was 22. From a total of 22 court cases with decisions of right to life violation and/or torture, there were 12 cases with decision of right to life violation

(54.5%). Of cases 11 were male (84.6%) and 2 were female (15.4%) (Case 8 reported two deaths). The age distribution was minimum 15 and maximum 45 years. In 3 cases (25.0%) both violations, of right to life and torture, were made together. In 3 of the cases (25.0%) death occurred while in custody. The sociodemographic characteristics, province and year of the event, year of ECHR decision and compensation amounts are shown in Table 1 (Table 1).

According to court reports, 11 cases (91.7%) were determined to have examination of death performed. Death examinations of 7 cases (63.6%) were performed by a health organization, while in 4 cases it was performed at the scene (36.4%). Two of the doctors (18.2%) performing the death examination were forensic medicine specialists, while 9 (81.8%) were specialists and practitioner doctors from other branches. Of the 12 cases investigated in the study, 8 cases (66.7%) were determined not to have autopsy performed. The number of cases with autopsy performed was 4 (33.3%). Of these 4 cases, 1 autopsy (25.0%) was performed in a forensic medicine center, while the other 3 (75.0%) were performed in any health organization. For 8 cases (66.7%) it appeared a firearm was used. For 7 of these cases (87.5%) ballistic investigations were performed. For only 1 case (12.5%) were clothes described. None of the 4 cases with autopsy performed (100%) had any photographs or radiological images obtained, had sampling for microscopic or chemical investigations from wounds on the skin or any biological material sampling performed. For 2 of these cases sampling was not performed from tissue or organs for histopathological and toxicological investigations. For all cases, the ECHR stated that the investigations performed were not sufficient or effective. For some cases resulting in death, though medical interpretation of the trauma factor was made, the results reflected in the autopsy reporting were not seen to be persuasive or sufficient by the ECHR. During the court cases, the court obtained expert reports from specialists working in forensic medicine departments outside of Turkey for 2 cases (16.7%).

Table 1. Distribution of cases based on violation type, gender, age, occupation, year of event and year of ECHR decision and compensation amount

Case No	Violation type	Gender	Occupation	Age	Event year	Decision Year	Compensation Amount***
Case1	Violation of right to life and torture	M	Doctor	*	1993	2000	17,500 STR + (15,000 STR -15095 FF)
Case2	Violation of right to life and torture	M	Laborer	23	1993	2002	75,617 Euro - 4100 FF
Case 3	Violation of right to life	M	Doctor	*	1993	1999	15,000 STR + (15,000 STR -13,495 FF)
Case 4	Violation of right to life	M	Farmer	*	1993	1998	27,000 STR
Case 5	Violation of right to life and torture	M	Driver	45	1992	2000	74,320 STR + (21,544 STR -11195 FF)
Case 6	Violation of right to life	M	Guard	*	1990	1999	130,000 FF
Case 7	Violation of right to life	M	*	*	1994	2000	40,000 USD + (2000 USD -3700 FF)
Case 8	Violation of right to life	M and F**	*	*	1993	2002	29,000 Euro + 17,500 STR
Case 9	Violation of right to life	M	Teacher	*	1993	2000	75,000 STR + (13,634 STR - 3600 FF)
Case 10	Violation of right to life	F	Teacher	*	1993	2000	
Case 11	Violation of right to life	M	Student	15	1991	1998	60,000 FF
Case 12	Violation of right to life	M	Manager	*	1993	2000	86,000 STR

*: Data could not be obtained **:Two people reported killed. ***: STR: British sterling, FF:French Franc, USD: American dollars.

4. DISCUSSION

In this study investigating court cases with decisions against Turkey in the ECHR from a medicolegal perspective, there were 12 cases found with the right to life violated and in 3 of these cases (25.0%) death was determined to have occurred while in custody. In nearly all of the investigated court cases, 11 cases (91.7%), the death examination reports were discussed in terms of deficient and erroneous aspects. Additionally the ECHR stated that in the majority of cases (66.7%) autopsy was not performed and this situation was a significant deficiency preventing effective investigations. These results show that a standard postmortem forensic medicine procedure was not applied for cases with violations of the right to life.

fAccording to statistical data about the number of applications against Turkey and rights violations, at the beginning of 2001 the country with most court case applications to the ECHR was Turkey (European Court of Human Rights, 1998-2002; Tezcan *et al*., 2002; Tanrıkulu, 2002; Doğru, 2001; Karakuş, 2001). In the period from 1987 to 15.04.2002 there were 148 applications with the claim of right to life violations and it was reported that 23 court cases resulted in violation decisions (Tezcan *et al*., 2002). In the period from 2003 to 2014, there were 100 decisions about right to life violation and 147 active investigations not completed decisions about Turkey (Republic of Turkey Ministry of Justice Department of Human Rights, 2014). According to ECHR 2017 statistics, there were a total of 62800 applications in front of the court, with the distribution of application numbers according to country reported as Hungary 10100 (16.1%), Romania 9850 (15.7%), Turkey 7650 (12.2%) and Russia 7650 (12.2%) (European Court of Human Rights, 2017). When the current statistics for the ECHR are investigated according to the distribution of Convention articles in all decisions given about the party states from 1959 to 2016, Turkey is first place in the general total for violation decisions (European Court of Human Rights, 1959-2016). From 1959 to 2016, there were a total of 133 right to life violations and 204 effective investigation not completed decisions given against Turkey (European Court of Human Rights, 1959-2016). According to this current data, Turkey is in second place after Russia when examined in terms of right to life violations (European Court of Human Rights, 1959-2016). Due to the 2nd article of the European Convention on Human Rights, the positive obligations of states are “to take precautions to protect the right to life and to perform effective investigations into events of death”, with negative obligations of “not to unlawfully end the lives of those who are under their sovereignty” (Kocabaş, 2009; Cengiz, 2011; Stan, 2012; Bilge, 2014). Similarly the court states that in cases of death occurring while in custody, states are obligated to present a reasonable explanation of any kind of worsening of the individual’s health state (Cengiz, 2011; Bilge, 2014). In this study it was identified that in 3 cases (25.0%) deaths were identified to occur while the individual was still held in custody. For these cases the effective research indicated by the court requires a full and accurate autopsy to show whether the death occurred as a result of torture of the individual and objective analysis of results (Stan, 2012; Bilge, 2014). In the literature, research related to right to life violations after 2002, there are court cases cited that ended after 2002 similar to the court case topics cited in this investigation (Kocabaş, 2009; Cengiz, 2011; Bilge, 2014). When these results and the statistics about the ECHR and Turkey from past to present are noted, “right to life violations and lack of effective investigation into deaths” still continues to be a current problem (Republic of Turkey Ministry of Justice Department of Human Rights, 2014; European Court of Human Rights, 2017; European Court of Human Rights, 1959-2016). In medicolegal terms, these results show that the Minnesota autopsy protocol is not routinely applied in researching suspected illegal deaths in Turkey.

The Minnesota protocol is an important guide used in death investigations in events linked to torture and similar illegal deaths including analysis of autopsy and skeletal remains (United Nations-Minnesota Protocol, 1991; Vieira *et al.,* 2012). A significant difference in this

protocol is the approach that "crime scene investigation is an inseparable part of death investigations" (Polat, 2002; İnanıcı *et al.*, 2004).

The cases investigated in this study had the common characteristic of having deficiencies and errors mentioned in ECHR decision; for 11 cases (91.7%) the court debated the death examination reports from a variety of angles. The case without discussion was a death linked to firearm injury. In this case, the court identified deficiencies related to the crime scene and ballistic investigations and that no autopsy was performed, and focused on the failure to protect the right to life of the individual. For the other 11 cases (91.7%), it was identified that deficiencies in the death examination, autopsy, crime scene investigation and ballistic investigation were debated with priority. The court particularly mentioned the critical importance of the autopsy procedure to reveal conditions of death. Additionally, forensic medicine research limited to death examination for cases with accusations of human rights violations was emphasized as being insufficient. When dealing with deficiencies of the autopsies in these cases, the Minnesota autopsy protocol was cited. The main framework of autopsies were criticized in light of the recommendations in this protocol. It was stated that lesions observed on external examination were not described in detail, clinical analysis about the trauma factor was not completed, differentiation of antemortem and postmortem findings was unsuccessful, normal postmortem findings were mistakenly assessed, lesions were not photographed, sampling of wounds on skin for wound age was not completed, clothing was not described, firearm wounds were not numbered and findings related to localization and firing distance identification were not appropriately defined. Crime scene investigations frequently had deficiencies mentioned for topics such as "photography, sketch plan, description of the placement of the body, determination of localization of bullets if a firearm was used and numbering and separate collection of these". The deficiencies and errors identified in death investigations were found to form the basic justification for the violation decisions given by the ECHR. These identifications used as ECHR justification overlap with research findings debating the deficiencies in forensic medicine training and applications in Turkey (Gürpınar *et al.*, 1997; Salaçin *et al.*, 1997; Çolak *et al*., 2001; Gülsoy, 2002; Çolak *et al*., 2004; Kök, 2007; Adli Tıp Uzmanları Derneği, 2007; Demirer, 2007; Gültekin, 2011; Petekkaya, 2012; Kumral and Özdeş, 2014).

The other common characteristics of the court decisions for the investigated cases was the scrutiny of the area of speciality of doctors performing the investigations. Topics of debate were whether doctors performing medical investigations were specialists on the procedures performed or what their previous experience was. For only 2 cases (16.7%) were death examinations performed by forensic medicine specialists. The forensic medicine specialists decided these two cases required autopsies. This result may be interpreted as a finding showing that forensic medicine specialists have a different approach to death investigations. The Minnesota protocol explaining crime scene investigation in systematic form defines this team work between forensic medicine specialists as an important obligation (United Nations-Minnesota Protocol, 1991). To resolve the problems identified by the court with "crime scene investigation, collection and assessment of evidence", it may be beneficial for experienced forensic medicine specialists to contribute to these investigations (Polat, 2002; İnanıcı *et al.*, 2004, Gültekin, 2011).

5. CONCLUSION

In this research, cases with violation of right to life show the necessity of standardizing death investigations. However, the standards emphasized in different autopsy protocols can

only be assured by using audit mechanisms in autopsies (Pakiş and Yaycı, 2006). The ECHR decisions investigated in this study identified serious deficiencies and errors in the postmortem forensic medicine procedures. As a result, in general the "efficient organization of investigations into deaths and protection of the right to life" were found to be unsuccessful. One of the main reasons for this is the lack of a standard and/or official protocol for death investigations such as the Minnesota protocol and lack of auditing of the quality and reporting processes for the performed investigations. In postmortem forensic medicine procedures, experienced doctors play an important role in revealing the truth and as a result protecting the right to life. To fulfill this role death investigations should be performed according to a sufficient protocol. To prevent right to life violations and effectively organize inquiries into deaths, doctors with sufficient knowledge and skills relating to forensic medicine applications should be required to abide by the Minnesota protocol.

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INVESTIGATION OF MUTAGENIC POTENTIAL OF WATER AND SEDIMENT FROM KARAMENDERES RIVER (ÇANAKKALE, TURKEY) USING THE AMES TEST

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ABSTRACT

In this study, the mutagenic effects of water and sediment samples taken from 5 stations between Kumkale and Karaköy locations on the Karamenderes River were investigated with the short time mutagenicity test system of the Ames test. Different extracts (hexane, chloroform and dichloromethane) and five different concentrations (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of water and sediment samples were prepared for testing in the study. Experiments were performed in the presence and absence of S9, which contains the TA98 and TA100 mutant strains of Salmonella typhimurium bacteria and microsomal enzymes. As a result of the mutagenicity studies, it was determined that the water samples had mutagenic effect causing base pair changes at all the stations for the chloroform extract. Mutagenicity was determined at the first station for the hexane extract. It was determined that there are weak mutagenic effects in the dichloromethane extract and in sediment samples from different stations.

1. INTRODUCTION

Today, environmental pollution has become an important problem because of rapid population growth, unplanned industrialization and ignorant agricultural practices. Domestic and industrial wastes and pesticides are discharged into water sources without proper treatment and this causes pollution of already limited water resources. Some of these substances cannot be broken down, sedimented, adsorbed or destroyed in any way. Therefore, these chemicals accumulate in water resources and threaten the health of living organisms in the environment (Ayan, 2005).

Many of the chemicals involved in aqueous ecosystems have mutagenic and carcinogenic effects, causing changes in living DNA (Anonim, 1991). These chemicals can be effective even at very low concentrations. It is not possible to analytically determine the chemical structures of these substances, which accumulate in tissues, with existing chemical methods. For this reason, biological methods and indicators based on carcinogenic and mutagenic substance scanning in tissues have gained importance. Since carcinogens and

mutagenic substances can be found in aquatic organisms with an important place in human nutrition, monitoring of the genotoxic effects of these substances has great importance for human health (Kotelevtsev and Stepanova, 1995).

The Ames test was specifically designed to detect the mutagenesis of chemical substances (Ames *et al.*, 1975). With this testing system, mutagenesis of more than 5000 chemicals and bioactive components isolated from plants and plants, soil, water and air can be identified in a short time; as well as the antimutagenic effect of phytochemicals such as plants, artificial or natural chemicals and vitamins, carotenoids, flavonoids and terpenoids (Maron and Ames, 1983; Hong and Lyu, 2011; Vu *et al*., 2012). The Ames test is one of the most commonly used test systems for bacterial mutagenicity because of its easy of application and sensitivity, with details well known and characterized (Gatehouse *et al*., 1998).

Certain chemical substances become active after being metabolized in vivo. The cytochrome P450 metabolic oxidation system in the liver of humans and lower-level creatures has the ability to metabolize these chemicals to DNA-reactive, electrophilic forms. Since there is no such enzyme system in bacteria, microsomal enzymes are added to the test system. This enzyme mixture (cytochrome P-450 enzyme system and various metabolic enzymes) is referred to in brief as S9 and is usually obtained from the rat liver (Maron and Ames, 1983, Mortelmans and Zeiger, 2000; Sakura *et al*., 2004).

The Karamenderes River, which is our study area, is contaminated with pesticides used in agricultural activities in the vicinity. In addition, many leather and olive oil plants operating in the districts of Bayramic and Ezine, along with the effluent from dairies and slaughterhouses, are sources of pollution due to inadequate refinement (Anonim, 2007).

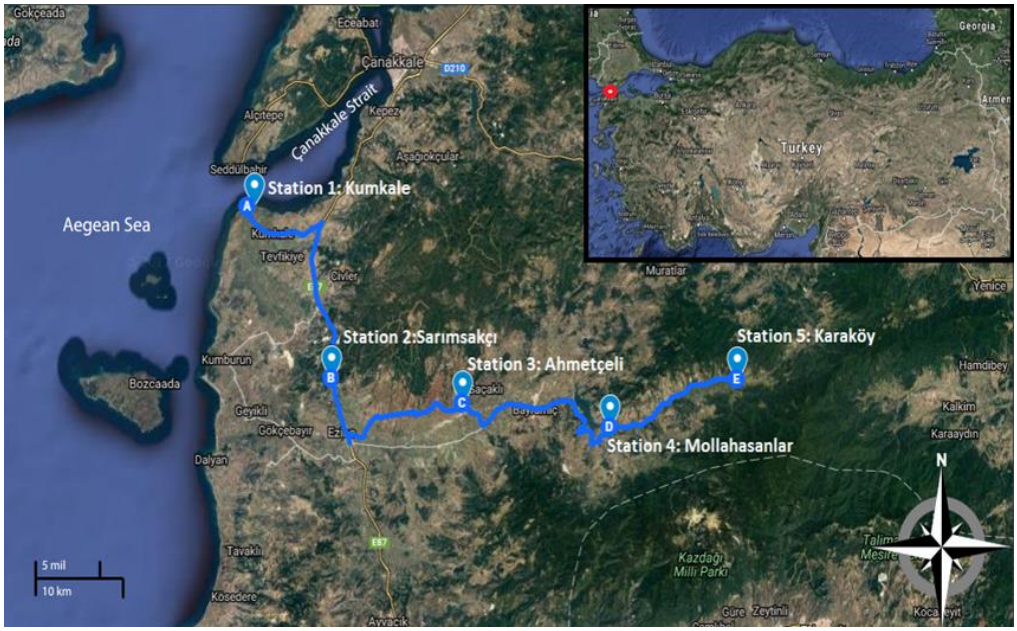
As a result of a literature review, it was determined that there were no previous mutagenic studies about Karamenderes River. In this study, the aim was to investigate the potential mutagenic effects of water and sediment samples taken from Karamenderes River.

2. MATERIAL AND METHODS

2.1. Study Area and Sampling Stations

The Karamenderes River rises in Kazdağı and passes near the Bayramic and Ezine districts of Çanakkale and flows into the Dardanelles near Kumkale. Karamenderes River is the only source of water that constantly flows into the region (Kayacan, 2008). Water and sediment samples were taken from 5 stations (1. Kumkale, 2. Sarımsaklı, 3. Ahmetçeli, 4. Mollahasan, 5. Karaköy) along Karamenderes River in November 2012 (Figure 1). The water samples were taken from the area where the current is not very high in amounts of 5 lt and sediment samples were obtained from the surface of the river bed in amounts of 250 g. The water samples were placed in polyethylene bottles (500 mL) and immediately transported to the laboratory.

Figure 1 . The stations samples are taken at Karamenderes River



2.2. Chemicals and Test Strains

4-Nitro-o-phenylenediamine (NPD), glucose, L-histidine, D-biotin, glucose-6-phosphate and rat liver S9 fraction were purchased from Sigma-Aldrich; sodium azide (SA, NaN_3),2-aminofluorene (2AF), magnesium sulfate ($MgSO_4.7H_2O$), citric acid monohydrate ($C_6H_8O_7.H_2O$), potassium phosphate (K_2HPO_4), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2.6H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) and dimethyl sulfoxide (DMSO) from Merck; bacto agar and nutrient broth were purchased from Oxoid.

The mutagenicity assay was performed with the standard plate incorporation method with and without metabolic activation (S9 mixture) as described by Maron and Ames (1983). *Salmonella typhimurium* TA98 strains were used for frame shifts and TA100 strains were used to identify changes that caused base pair mutations. *S. typhimurium* strains were kindly provided by Prof. Dr. Hülya Sivas (Anadolu University, Faculty of Science, Department of Biology) and maintained as described by Maron and Ames (1983).

2.3. Extraction of The Water and Sediment Samples

Extraction of the water samples was carried out with the liquid-liquid extraction method. A 5-liter sample of water from each station had 500 mL used each time, and 25 mL of solvent was added to quickly flush the balloon in the flask and then left for a while for the phase to form. The solvent was then removed by separation of the solvent and placed in separate glass bottles and this process was repeated twice with fresh extraction solvent. n-Hexane ($CH_3(CH_2)_4CH_3$), chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2) solvents were used respectively for extraction. The extracts were then evaporated at 45 ºC using an evaporator (Spectral, Heidolph, Laborator 4001) and water bath, and dimethyl sulfoxide (DMSO) was added before the test to dissolve the remaining precipitate (Singh *et al*., 1987).

Sediment samples taken from the designated stations were dried at 60 °C in a petri dish. The dried sediments were poured into a porcelain mortar and powdered, weighed to the amount

of 0.1 g and then placed in sterile tubes. They were vortexed by adding 1 mL of hexane, chloroform, and dichloromethane (1:1:1,v:v:v). The supernatant was then sterilized by centrifugation in a refrigerated centrifuge (Hettich, Universal 320R) at +4 °C and 5600 g for 10 minutes and the same procedure was repeated three times. Solvents of the obtained extracts were evaporated with a rotary evaporator in a water bath at 40 °C. Extracts were stored in the refrigerator at +4 °C. When the test was performed, the remaining precipitate was dissolved by adding DMSO (Keijzer et al., 2000).

2.4 Methods

Amounts of 0.1 mL of an overnight bacterial culture and 0.5 mL of sodium phosphate buffer (0.2 M, pH 7.4 for assay) were added to 2 mL aliquots of top agar containing different concentrations of each extract. The resulting complete mixture was poured onto minimal agar plates prepared as described by Maron and Ames (1983). The plates were incubated at 37 ºC for 48 h and the revertant bacterial colonies on each plate were counted.

For the positive control, 4-Nitro-o-phenylenediamine (NPD) for *S. typhimurium* TA98 strain and sodium azide (SA, NaN_3) for TA100 strain were used in the absence of S9. In the presence of S9, 2-aminofluorene (2AF) was used for both strains. In the negative control, dimethyl sulfoxide (DMSO) was used for both strains. All samples were tested on triplicate plates for each concentration and the studies were applied in two independent experiments. An extract was considered mutagenic if the number of revertants per plate was at least doubled compared to the solvent controls (Maron and Ames, 1983). Data were collected as the mean ± standard deviation of three experiments.

3. RESULT and DISCUSSION

In the absence of S9, with the concentration of 10^{-2} *S. typhimurium* TA100 strain was found to be mutagenic in hexane extracts obtained from water samples, while at higher concentrations the number of revertant colonies decreased in parallel to the toxic effect. At station 4, both strains were found to have poor mutagenic activity at all concentrations except 10^{-4} concentration. In the S9 experiment, there was no significant increase compared to the negative control (Table 1).

In chloroform extracts, *S. typhimurium* TA98 strain in the absence of S9 had weak mutagenicity at 10^{-1} concentration, and cytotoxic effect at 10^0 concentration from station 4. In TA100 strains, except for station 4, the highest concentration (10^0) of mutagenic activity was detected at each station. Although there were higher numbers of colonies at the 4th station in the 10^{-1} dose than in the other stations, there was no increase in the number of colonies due to cytotoxic effect at 10^0 doses. In study with S9, the TA98 strain had the highest concentration of mutagenic response at the 4th station. In the TA100 strains, mutagenicity at the 3rd station was determined at the highest concentration, and mutagenicity at the 4th and 5th stations was high (Table 2).

In dichloromethane extracts, in the absence of S9, the highest concentration of *S. typhimurium* TA100 strains showed weak mutagenicity at stations 2 and 3, while at stations 1, 4 and 5, concentration-dependent revertant colony counts were increased. In the TA98 strain, mutagenic results were not found at any station. In the presence of S9 it was determined that the number of colonies due to concentration at the 3rd station increased in the TA100 strain. There was no significant increase in colony number in TA98 strains (Table 3).

Sediment samples were found to have poor mutagenic activity at the 2nd, 3rd and 5th stations at the highest concentration in *S. typhimurium* TA100 strains in the absence of S9. No mutagenic activity was observed in the TA98 strain in the experiment without S9 and in the S9 experiment for both strains (Table 4)

According to the results of the research, it was determined that there is mutagenic activity in the water samples taken from Karamenderes River. In the absence of S9 in *S. typhimurium* TA100 strain, mutagenicity was found at all the stations in the chloroform extract obtained from the water samples. The highest mutagenic response was found in the chloroform extract at stations 4 and 5 in the presence of S9. This result shows that at these two stations, metabolic activation results in metabolites in the living body may cause mutagenic effects. It was also found that weak mutagenic activity was observed in hexane extract at station 4 (Table 1). It is possible that such a result would occur due to the residues of the pesticides used in the apple orchards in this region. The presence of mutagenicity in hexane and chloroform extracts from water samples suggests that pesticide residues may be present in the samples. Organochlorine and organophosphate pesticides in water can be trapped by these two solvents (Aleem and Malik, 2005).

As a result of the studies done, many pesticides have been shown to cause mutagenic effect (Aleem and Malik, 2005; Zhao *et al*., 2010). In a study in India, mutagenic potential of water samples from three stations on the Ganges River were investigated. Mutagenicity was detected even at low concentrations in strains TA98 and TA100. Some pesticides such as dichlorodiphenyltrichloroethane (DDT), β-hexachlorocyclohexane (BHC), aldrin, and dieldrin have been identified in water samples with High Performance Liquid Chromatography (HPLC) analyses (Rehana *et al*., 1995).

In a study carried out on the Karamenderes River and near the soil, the results of the analyses revealed that many pesticides, especially α-endosulfan and hexachlorohexane, can cause pollution (Yıldırım and Özcan, 2007).

At the third station, mutagenicity in the absence of S9 in the *S. typhimurium* TA100 strain in the chloroform extract and weak mutagenicity in the presence and absence of S9 in the dichloromethane extract were determined. It is possible that the observed mutagenic effect is due to the pollution of the wastes from settlements in the surrounding area and the consequent drainage of inadequately treated leather and olive oil production wastes from Bayramiç town. The mutagenic activity, especially in the dichloromethane extract, demonstrates the presence of complex mixtures in this region. This is because in the Ames test, it has been reported that dichloromethane is a suitable solvent for basic-neutral compounds for the extraction of water samples from complex environmental mixtures (Nielsen, 1992; Lippincott *et al*., 1990).

At the second station, the mutagenic response was determined in the chloroform extract. In the dichloromethane extract, the mutagenic effect was weak. The station with the lowest mutagenic activity was determined to be the second station. Although there is agricultural activity in this area, the mutagenic potential is diminished due to the dilution of the pollution load from the third station.

At the first station, mutagenicity was also observed in hexane and chloroform extracts. In the dichloromethane extract, a weak mutagenic effect was found. In this region where the river enters the sea, the accumulation of pollutants can result in mutagenic activity.

In a study in Slovenia, samples from the Sora River contaminated with water from wastewater treatment plants, domestic waste and landfills were examined with the Ames test system and 11 samples were found to have mutagenic activity ((Filipic, 1995).

Strong mutagenic activity was not observed in any sediment samples collected from the Karamenderes River. It was determined that there are poor mutagenic effects at stations 2, 3 and 5. This shows that the amount of mutagenic substances in the river is low in water and insoluble in sediment (Table 4).

Maccubbin *et al*. (1991) tested sediment samples from the Detroit River in Michigan and Lake Michigan using Ames in the United States of America, where industrial, domestic and agricultural wastes are heavily involved. High mutagenic activity was detected at 16 sites resulting in metabolic activation of chemicals containing microsomal enzymes (S9). Strong mutagenic activity was not detected in any of the samples when S9 was not applied.

It is not accurate to combine the results of this study and the mutagenicity obtained in previous studies to a single cause. This is because some chemicals do not have genotoxic effects on their own, but they are known to create this effect when they are combined. For this reason, the analysis of the chemicals in water and sediment samples is important for the correct interpretation of mutagenic results.

Table 1. Mutagenicity results of *S. typhimurium* TA98 and TA100 strains of hexane extracts obtained from water samples

Treatment		Concentration	Number of his^+ revertant colony/Plate			
			TA98		TA100	
			S9 (-)	S9 (+)	S9 (-)	S9 (+)
			mean±SD	mean±SD	mean±SD	mean±SD
Positive control	NPD	10^{-2}	811±94,55			
	SA	10^{-3}			1189±147,33	
	2AF	$5x10^{-3}$		957±107,29		1295±156,70
Station 1		10^{0}	25±4,36	26±1,53	180±38,42	137±15,39
		10^{-1}	19±2,08	26±4,73	179±24,06	135±10,39
		10^{-2}	24±6,24	35±8,18	306±7,09	141±9,85
		10^{-3}	15±4,93	29±3,06	248±32,19	160±26,23
		10^{-4}	13±4,16	31±7,23	179±3,61	192±28,00
Station 2		10^{0}	16±5,03	32±1,53	128±9,24	154±12,77
		10^{-1}	22±4,58	34±1,53	115±9,29	194±17,78
		10^{-2}	19±4,58	38±6,56	167±31,01	160±9,29
		10^{-3}	20±3,79	29±3,46	160±2,08	162±15,31
		10^{-4}	15±4,04	29±6,00	132±4,16	139±17,95
Station 3		10^{0}	33±4,58	38±5,13	168±4,73	130±14,57
		10^{-1}	23±1,53	23±5,13	150±10,60	159±10,97
		10^{-2}	25±2,65	26±6,56	210±15,95	180±15,10
		10^{-3}	26±4,58	30±7,64	148±7,00	139±5,29
		10^{-4}	26±1,73	27±3,21	169±26,54	190±15,57
Station 4		10^{0}	38±2,08	29±5,51	213±34,78	163±7,02
		10^{-1}	41±1,53	38±1,73	285±22,19	149±7,81
		10^{-2}	44±7,21	27±6,81	214±31,09	183±14,05
		10^{-3}	37±6,81	31±7,21	266±11,93	137±19,43
		10^{-4}	26±5,51	34±1,53	162±22,68	135±13,50
Station 5		10^{0}	17±3,79	42±6,56	179±27,23	162±21,08
		10^{-1}	24±4,04	28±4,36	134±13,61	125±13,05
		10^{-2}	26±5,29	30±2,65	143±27,39	145±6,56
		10^{-3}	18±3,06	34±3,21	156±17,39	142±5,29
		10^{-4}	34±5,00	31±4,51	143±29,50	165±10,26
Negative control	DMSO		24±3,50	28±5,98	135±21,06	144±34,76
Spontaneous control			24±6,94	25±6,59	157±27,52	152±18,64

*NPD: 4-Nitro-*o*-phenylenediamine, SA: Sodyum azide, 2AF: 2-aminoanthracene, DMSO: Dimethyl sulphoxide

Table 2. Mutagenicity results of *S. typhimurium* TA98 and TA100 strains of chloroform extracts obtained from water samples

Treatment		Concentration	Number of revertant colony/Plate			
			TA98		TA100	
			S9 (-)	S9 (+)	S9 (-)	S9 (+)
			mean±SD	mean±SD	mean±SD	mean±SD
Positive control	NPD	10^{-2}	811±94,55			
	SA	10^{-3}			1189±147,33	
	2AF	$5x10^{-3}$		957±107,29		1295±156,70
Station 1		10^{0}	36±4,51	36±7,55	359±47,15	222±13,89
		10^{-1}	28±4,36	30±8,50	144±12,66	197±5,51
		10^{-2}	20±4,16	21±1,73	127±5,51	115±15,82
		10^{-3}	28±6,08	22±3,51	116±11,55	138±9,00
		10^{-4}	25±6,42	24±2,65	114±7,77	142±24,38
Station 2		10^{0}	20±3,51	36±4,58	277±23,86	199±17,21
		10^{-1}	20±3,21	26±1,53	120±2,08	121±13,65
		10^{-2}	18±3,51	23±6,24	125±8,39	143±14,74
		10^{-3}	15±2,08	28±2,65	147±24,33	129±5,51
		10^{-4}	19±5,13	28±5,29	139±21,07	126±23,52
Station 3		10^{0}	35±6,51	40±2,52	375±26,21	324±20,42
		10^{-1}	23±5,13	32±6,81	153±11,36	163±32,08
		10^{-2}	18±3,21	30±5,69	151±12,42	118±20,98
		10^{-3}	17±2,08	28±7,09	128±4,58	119±25,03
		10^{-4}	19±4,04	28±3,06	124±27,07	151±18,15
Station 4		10^{0}	17±5,51	64±10,69	219±10,58	498±29,82
		10^{-1}	40±5,69	35±4,04	216±14,53	203±22,85
		10^{-2}	26±5,03	35±1,53	135±16,50	184±29,46
		10^{-3}	22±3,79	32±2,31	94±7,09	125±7,09
		10^{-4}	19±3,21	26±3,06	115±10,82	165±9,29
Station 5		10^{0}	21±1,53	38±6,51	302±7,09	514±13,53
		10^{-1}	22±3,79	19±4,58	216±11,00	202±19,14
		10^{-2}	14±1,53	28±0,58	151±16,44	152±25,16
		10^{-3}	18±4,51	28±4,93	152±15,50	129±5,13
		10^{-4}	19±4,93	19±2,08	171±7,55	132±3,79
Negative control	DMSO		24±3,50	28±5,98	135±21,06	144±34,76
Spontaneous control			24±6,94	25±6,59	157±27,52	152±18,64

*NPD: 4-Nitro-*o*-phenylenediamine, SA: Sodyum azide, 2AF: 2-aminoanthracene, DMSO: Dimethyl sulphoxide

Table 3. Mutagenicity results *of S. typhimurium* TA98 and TA100 strains of dichloromethane extracts obtained from water samples

Treatment		Concentration	Number of revertant colony/Plate			
			TA98		TA100	
			S9 (-)	S9 (+)	S9 (-)	S9 (+)
			mean±SD	mean±SD	mean±SD	mean±SD
Positive control	NPD	10^{-2}	811±94,55			
	SA	10^{-3}			1189±147,33	
	2AF	$5x10^{-3}$		957±107,29		1295±156,70
Station 1		10^{0}	23±2,65	19±2,65	191±17,58	150±14,57
		10^{-1}	28±3,00	24±6,11	166±27,43	171±3,61
		10^{-2}	28±6,24	26±5,69	155±6,00	146±14,74
		10^{-3}	21±4,16	21±4,35	141±4,58	139±15,50
		10^{-4}	19±3,51	22±3,79	120±15,72	132±10,82
Station 2		10^{0}	24±1,53	32±4,93	254±17,06	184±13,05
		10^{-1}	21±3,46	24±4,93	139±9,45	150±15,10
		10^{-2}	22±2,08	27±1,73	108±22,37	182±11,50
		10^{-3}	29±2,65	19±2,52	164±7,64	169±28,57
		10^{-4}	16±3,79	19±3,79	121±5,03	132±3,79
Station 3		10^{0}	29±2,31	17±4,16	227±25,16	206±11,14
		10^{-1}	30±4,04	17±1,15	140±20,03	164±15,52
		10^{-2}	16±2,00	23±4,00	162±30,83	129±6,03
		10^{-3}	30±2,52	20±3,46	143±16,52	132±14,04
		10^{-4}	27±4,73	17±5,86	150±26,91	118±7,55
Station 4		10^{0}	26±2,89	21±3,05	206±26,69	203±9,45
		10^{-1}	18±4,58	22±4,16	157±12,74	123±14,73
		10^{-2}	19±5,51	24±3,21	144±21,45	200±25,94
		10^{-3}	18±3,06	23±4,93	155±4,04	163±11,37
		10^{-4}	21±3,79	23±6,08	140±15,10	153±24,38
Station 5		10^{0}	36±6,11	28±1,53	186±3,79	205±30,02
		10^{-1}	39±3,46	26±3,46	143±12,22	143±7,09
		10^{-2}	25±3,79	21±3,06	137±11,59	161±20,30
		10^{-3}	16±4,93	26±6,08	130±13,58	167±6,66
		10^{-4}	18±3,51	22±3,79	120±12,34	156±16,44
Negative control	DMSO		24±3,50	28±5,98	135±21,06	144±34,76
Spontaneous control			24±6,94	25±6,59	157±27,52	152±18,64

*NPD: 4-Nitro-*o*-phenylenediamine, SA: Sodyum azide, 2AF: 2-aminoanthracene, DMSO: Dimethyl sulphoxide

Table 4. Mutagenicity results of *S. typhimurium* TA98 and TA100 strains of sediment samples

Treatment		Concentration	Number of revertant colony/Plate			
			TA98		TA100	
			S9 (-)	S9 (+)	S9 (-)	S9 (+)
			mean±SD	mean±SD	mean±SD	mean±SD
Positive control	NPD	10^{-2}	811±94,55			
	SA	10^{-3}			1189±147,33	
	2AF	$5x10^{-3}$		957±107,29		1295±156,70
Station 1		10^{0}	32±2,08	17±2,65	167±14,01	155±9,07
		10^{-1}	25±5,03	17±2,52	174±6,03	146±5,51
		10^{-2}	24±4,16	19±2,00	165±5,03	139±9,07
		10^{-3}	28±4,51	19±1,53	198±26,50	154±14,53
		10^{-4}	22±4,04	18±4,51	157±7,09	141±6,56
Station 2		10^{0}	18±2,65	21±2,00	227±37,17	126±12,06
		10^{-1}	17±2,52	17±2,08	165±21,73	121±10,82
		10^{-2}	22±3,51	21±1,53	146±17,50	120±9,17
		10^{-3}	18±3,51	22±1,73	187±20,52	121±6,56
		10^{-4}	22±6,03	20±2,00	201±13,75	111±16,09
Station 3		10^{0}	24±5,51	23±3,21	228±8,19	138±8,50
		10^{-1}	18±4,04	16±3,00	169±16,52	115±14,98
		10^{-2}	23±1,53	16±2,65	159±19,76	113±9,07
		10^{-3}	27±3,51	18±1,15	151±17,52	117±6,51
		10^{-4}	24±4,58	22±3,21	156±25,03	133±8,19
Station 4		10^{0}	32±4,04	32±8,14	174±31,94	128±9,07
		10^{-1}	17±2,08	24±3,51	148±12,70	139±10,54
		10^{-2}	24±5,51	17±4,16	113±10,02	114±9,54
		10^{-3}	27±6,56	23±4,16	165±24,03	134±8,50
		10^{-4}	27±3,61	20±3,06	114±19,97	114±7,77
Station 5		10^{0}	30±5,69	16±3,51	234±31,01	137±8,33
		10^{-1}	28±1,15	29±5,86	163±30,27	142±9,07
		10^{-2}	26±1,53	24±3,00	186±24,50	128±8,00
		10^{-3}	25±5,50	19±3,61	140±22,11	121±9,17
		10^{-4}	26±1,53	17±4,16	168±22,72	141±9,61
Negative control	DMSO		24±3,50	28±5,98	135±21,06	144±34,76
Spontaneous control			24±6,94	25±6,59	157±27,52	152±18,64

*NPD: 4-Nitro-*o*-phenylenediamine, SA: Sodyum azide, 2AF: 2-aminoanthracene, DMSO: Dimethyl sulphoxide

4. CONCLUSION

Today, pollution of water resources as a result of anthropogenic activities presents a great danger. With industrialization and urbanization, many wetlands are polluted, and this pollution directly or indirectly threatens the health of people and other living organisms. As a result of the studies and the different test methods used, it is clear that water and sediment are contaminated with many genotoxic compounds and that these compounds cause mutagenic effects on different organisms.

In this study, water samples and sediment samples taken from the Karamenderes River in Çanakkale were found to have mutagenic activity at all stations in the absence of S9 in the chloroform extract and at the highest concentration at the two stations in the presence of S9. The water samples were found to have a mutagenic response at one station in the absence of S9 in the hexane extract. It was also shown that there are weak mutagenic effects at different stations in dichloromethane extracts and sediment samples. With these results, it is necessary for farmers to be aware of the use of pesticides in agricultural activities carried out around the Karamenderes River. In addition, it is very important to purify and treat the waste from settlements and industry in the surrounding area in order to prevent pollution of the river.

There are more and more unknown mutagenic and carcinogenic compounds found in water sources. Conducting genotoxicity studies in conjunction with chemical analyses is also important in determining the harmfulness of these chemicals by accurately identifying mutagens in the water. Apart from this, other in vitro and in vivo tests should be performed and the results obtained should be supported so that the results can be confirmed.

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BRYOPHYTES AS HIDDEN TREASURE

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ABSTRACT

Bryophytes are the second largest heterogeneous group of terrestrial plants. The bryophytes placed taxonomically between Algae and Pteridophytes, are further divided into three classes; Hornworts (class Anthocerotae), Liverworts (class Hepaticae) and Mosses (class Musci). They are the most captivating exotic species on earth with distinguishing characteristics.

Bryophytes are rich in a variety of secondary biological active compounds. Bryophytes contain potentially useful natural products, including polysaccharides terpenoids, lipids, amino acids and phenylpropanoids. Bryophyte isolated compounds and extracts have cytotoxic, antimicrobial, insecticidal, antiviral, nematocidal effects on smooth and non-striated muscles, weight loss, plant growth regulation and allelopathic activities.

In the present review, the therapeutic uses of bryophytes were focused in detail. This will highlight bryophytes as potential source for phytotherapeutic remedies and chemical products used in different fields of chemistry, pharmacology, bioiogy and different branches of life sciences.

1. INTRODUCTION

The bryophytes comprises the liverworts (Marchantiophyta, 6.000 species), the hornworts (Anthocerotophyta, 300 species) and the mosses (Bryophyta, 15.000 species) were thought to be the second largest group of land pivotal plants after flowering plants in the early land plant evolution (Shaw and Renzalia, 2004). Bryophytes are characterized by dominant perennial gametophytic stages, with small and unbranched sporophyte remain attached to the maternal gametophyte (Cox *et al*., 2010). They may be that are important components in forest ecosystems and constitute a major part of the biodiversity in moist environments and mountain ecosystems (Hallingback and Hodgetts, 2000).

Bryophytes are found in habitats of every ecosystem and play a significant role in each ecosystems for example nutrient cycling, water economy or providing shelter for other organismal groups.

The size of bryophyte species varies from few millimeters to few metres. In liverwort *Monocarpus* to 0.7 m in the self supporting *Dawsonia superba* Turner and *Fontinalis antipyretica* Hedw. to 2 m as observed in the water habitat (Sabovljevic *et al*., 2016).

Some have a cuticle, some absorb water directly through leaf surfaces. They do not have true roots. They instead have multicelled, root like appendages called "rhizoids," which anchor the plants and take in water and minerals.

Bryophytes have pigments, chlorophyll a and b, xanthophyll and carotene. They store starch as energy saver molecule in plastids. Flavonoids are common in this group which is in accordance with their ability to cope with UV radiation (Sabovljevic *et al.,* 2016).

Bryophytes are important environmental indicators and have been used as predictors of climate change to validate climate models and potential indicators of global warming (Rao, 2009).

Bryophytes also can be important contributors to the total stream metabolism, nutrient cycling, food web interactions in streams and as direct food source for some vertebrates (Andrea *et al*., 2011). More importantly, some species are of great source for herbal medicine. Bryophyte are used as indicator species for erosion control, bioindicators of heavy metals in air pollution, aquatic bioindicators, radioactivity indicators, as material for seed beds, fuel, medicines and food sources, pesticides, nitrogen fixation, moss gardening, treatment of waste, construction, clothing, furnishing, packing, genetic engineering, for soil conditioning and culturing (Chandra *et al*., 2016).

Bryophytes are small biomass in various ecosystems and seldom part of ethnomedicine that rarely subject to medicinal and chemical analyses. Hundreds of natural products have been isolated from bryophytes. Bryophytes have potentially useful natural products, like polysaccharides, terpenoids, amino acids, lipids, quinones, phenylpropanoids and other specialized metabolites (Sabovljevic *et al*., 2016).

This slow growing group of plants is stockroom of naturally occurring materials and have been investigated for the antimicrobial, antioxidant, anti-inflammatory, anti-venomous and anti-leukemic activity (Mishra *et al*., 2014).

Bryophyte extracts and isolated compounds may be shown antimicrobial, antiviral, cytotoxic, nematocidal, insecticidal, effects on smooth and non-striated muscles, weight loss, plant growth regulators and allelopathic activities (Sabovljevic *et al*., 2016). In the recent years bryophytes has emerged as a potential biopharming tool for production of complex biopharmaceutiticals. Bryophytes considered as 'remarkable reservoir' of natural products and secondary metabolites, which show interesting biological activity could be used in medicine.

Bryophytes especially moss and liverworts are the source of many biologically active novel compounds pertaining to pharmaceutical uses (Singh *et al*., 2007). About 3.2 % of mosses and 8.8 % of liverworts have been chemically investigated. Species like *Bryum, Marchantia, Sphagnum, Octeblepharum*, *Riccia*, *Barbula*, and *Fontinalis* are used to treat different diseases such as cardiovascular diseases, fever, inflammation, lung diseases, infections, skin diseases and wounds (Glime, 2007).

Bryophytes are known to produce secondary metabolites to combat a number of stress condiotions such as microbial decomposition predation, extreme temperature and UV-radition. They are the large variety source of secondary metabolites, thus provide a great potential for biotechnological and biopharmaceutical applications for bryophytes (Xie *et al*., 2009).

Although bryophytes are important source of various plant derivatives but only few studies have been conducted to get an in depth knowledge regarding role of various metabolites of bryophytes. Present review focused on the therapeutic uses of bryophytes and the various phyotochemical and pharmaceutical constituents obtained from the bryophytes.

2. MATERIAL AND METHODS

In the present review, information about bryophytes, their medicinal properties and biochemical properties was gathered searching scientific databases including Elsevier, Google Scholar, PubMed, Springer, related books and manuscripts online or offline.

3. AIM OF THE PRESENT REVIEW

In the present review, scientific databases and pharmacological properties of bryophyte species were given.

4. MEDICINAL PROPERTIES

4.1. Ethnomedicinal Properties

In general, bryophytes never play a direct role in human life because the uses of bryophytes by ethnic people (for their healthcare or other needs) have been not exactly understanded. It is clear that these little plants do not have ethnobotanical importance in different cultures (Alam *et al.*, 2015). Because bryophytes produce little biomass per locality and are not often used as medicinal plants. However, the small size of these plants as well as usually not huge biomass in the nature, made these plants neglected for wide use.

Miller and Miller (1979), stated that the ancient method of determining the medicinal properties of a plant is 'doctrine signature'deals with resemblence of plant parts to structure and shape of organs in animal or human body for which it is remedial. As an example, some liverworts (e.g. *Marchantia polymorpha* L.) were believed to treat liver aliments because of its shape like liver. Similarly, *Polytrichum commune* Hedw. called hair cup moss, was used for hair treatment (Miller and Miller, 1979).

The first medicinal mosses are mentioned already in Renaissance herbals (by Fuchs, 1543 and Lobelius, 1581). From the 18th century, physicians were interested in using bryophytes as medicinal alternatives (Drobnik and Stebel, 2014).

Flowers (1957), indicated that the majority use of bryophytes as ethnomedicine reported from Chinese, Indian and Native American medicines. Bryophytes are highly used in horticulture in Far East, and Chinese and Indian people use them widely in ethno therapeutics (Kumar *et al*., 2000; Ando and Matsuo, 1984).

Chemical constituents of these plants have been used as biologically active agents. Many bryophyte compounds have shown biological activity with particular properties to their application in medicine and agriculture (Pant and Tewari, 1998). For example *Polytrichum commune* which is used as antipyretic and anti-inflammatory agent or boiled as a tea for treating the cold. *Rhodobryum giganteum* Schwägr is another species used to treat cardiovascular diseases or angina (Ando and Matsuo, 1984). In different parts of the world, different ethnic groups used plants to cure various diseases. Gaddi tribes people in India, used *Plagiochasma appendiculatum* Lehm. et Lind. for treating skin diseases (Kumar *et al*., 2000). Irular tribe used also *Targionia hypophylla* L. for skin diseases in Kerala state. In South India, people used hair-like thallus *Frullania ericoides* (Nees) Mont for hair-related afflictions (Remesh and Manju, 2009).

Gasuite Indians (Utah, USA) used species such as *Philonotis*, *Bryum*, *Mnium* and some hypnaceous forms to alleviate burn pains (Sabovljevic *et al*., 2001). Ding (1982) indicated that 40 species have been used in Chinese traditional medicine.

The liverworts *Conocephalum conicum* (L.) Dumort and *Marchantia polymorpha* (Hedw.) mixed with vegetable oils, are used as ointments for burns, eczema, cuts and bittes (Sabovljevic *et al*., 2016). For eye diseases, Chinese used Peat-moss *Spaghnum teres* (Schimp.) Ångstr. ex Hartm and for tonsillitis, bronchitis, cystitis and timpanitis. *Haplocladium microphyllum* (Hedw.) Broth. and *Polytrichum commune* Hedw. is widely used as a medicinal cure to antipyretic, diuretic and hemostatic properties (Chandra *et al*., 2016).

136 species bryophytes have been reported that used in ethnobotany for a variety of purposes (Harris, 2006). Nearly half of these species used for their pharmaceutical constituents (Table 1).

Asakawa (2001) indicated that, 500 bryophytes have been studied with respect to their chemistry, pharmacology and application as cosmetics and medicinal drugs in Asia.

Today, ethnobotany has become a crucial area of research and development in resource management of biodiversity. As tribal communities has their own health care systems. Their ancient knowledge referred to as ethno-therapeutics, has provided a more useful and effective strategy for the discovery of active drugs.

4.2. Therapeutical Properties

Bryophytes are natural reservoir products of secondary metabolites. These metabolites have shown biological activity used in pharmacology. Bryophytes especially moss and liverworts are the source of biological active constituents pertaining to pharmaceutical uses (Nath and Singh, 2007).

In past few years, more than 400 chemical compounds were isolated from bryophytes (Asakawa, 2007). Biologically active compounds obtained from mosses includes biflavonoids, terpenes, terpenoid and flavonoids whereas liverworts to contain a large variety of lipophilic mono-, di- and sesquiterpenoids aromatic compounds like bibenzyls, benzoates, cinnamates and naphtalenes (Asakawa, 2007).

Secondary metabolites of plants that are the potential therapeutic introduction of novel drugs has increased in recent years. Investigations on secondary metabolites of bryophytes have revealed the few original compounds, some of which are not isolated from higher plants.

Antibiotic resistant bacteria have motivated researchers to look forward for new plant based natural active compounds. Botanist and microbiologist indicated precious antibiotic substances in bryophytes. They have compounds such as alkaloids, polyphenolic acids and flavonoids.

The antibiosis of bryophytes has been studied in recent years. Some of the species of bryophytes like *Polytrichum* sp. and *M. polymorphya* are used against pulmonary tuberculosis and to treat gingivitis.

Antibiotic polyphenols were identified in *Atrichum, Dicranum, Mnium, Polytrichum* and *Sphagnum* sp. (McCleary and Walkington, 1966). Apigenin, luteolin, kaempferol and orobol glycosides and their dimers are also found in mosses (Zinsmeister *et al*., 1991; Basile *et al.*, 1999). Extracts of various medicinal plants containing flavonoids have been reported to show antimicrobial activity (Waage and Hedin, 1995).

Table 1. Ethanomedicinal uses of bryophytes

Species	Medicinal uses	References
Liverworts		
Riccardia sp.	anti-leukemic activity	Alam, 2012
Plagiochasma appendiculatum	skin diseases	Shirsat, 2008
Reboulia hemisphaerica	blotches, hemostasis, external wounds, and bruises	Asakawa, 2007
Conocephalum conicum	antimicrobial, antifungal, antipyretic, antidotal activity	Ding, 1982
Herbertus sp.	antiseptics, antidiarrheal agents, expectorants *and* astringents	Azuelo *et al.*, 2011
Frullania tamarisci	antiseptic activity	Asakawa, 2007
Frullania ericoides	to get rid from head lice and nourishment of hair	Remesh, 2009
Marchantia polymorpha	inflammation, used as diuretics, for liver ailments, insect bites, used to cure cuts, fractures, poisonous snake bites,	Hu, 1987
Marchantia convoluta	treatment of hepatitis, fever and gastric intolerance	Rao, 2009
Marchantia palmata	acute inflammation caused by the touch of fire and hot	Tag *et al*., 2007
Marchantia paleacea	skin tumefaction, hepatitis and as antipyretic	Sabovljevic *et al*., 2011
Dumortiera hirsuta	source for antibiotics	Azuelo *et al*., 2011
Pallavicinia sp.	antimicrobial agent	Azuelo *et al*, 2011
Plagiochila sp.	anti-leukemic activity/anti-microbial activity and used as perfumes or as perfume components	
Plagiochila beddomei	wound healing	Alam, 2012
Riccia sp.	mixed with jiggery and given to the children affected by the ringworms.	Lubaina *et al*., 2014

Targionia hypophylla	mixed with two tablespoons of coconut oil for scabies itches and other skin diseases	Remesh and Manju, 2009
Hornworts		
Ceratophyllum demersum	purgative, astringent, constipating and antipyretic	Pullaiah, 2006
Mosses		
Cratoneuron filicinum	heart disease	Pant and Tewari, 1998
Leptodictyum riparium	antipyretic in uropathy	Pant and Tewari, 1998
Philonotis fontana	to relieve pain of burn and heal burns, adenopharyngitis, antipyretic	Flowers, 1957
Philonotis sp.	heal burns, for adenopharyngitis, as antipyretic and antidote	Asakawa, 2007
Plagiopus oederi	sedative, epilepsy	Pant and Tewari, 1998
Bryum argenteum	antidote, antipyretic, antifungal	Asakawa, 2007
Rhodobryum giganteum	to treat cardiovascular problem and nervous prostration, to cure angina, anti-hypoxia, diuretic, antipyretic, and antihypertensive	Pant and Tewari, 1998
Rhodobryum roseum	to treat nervous prostration and cardiovascular diseases sedative	Wu, 1977
Leucobryum bowringii	body pain, paste of leaf tips mixed with *Phoenix sylvestris*	Lubaina *et al*., 2014
Oreas martiana	anodyne (pain), hemostasis, external wounds, epilepsy, menorrhagia and neurasthenia (nervosism, nervous exhaustion)	Asakawa, 2007
Ditrichum pallidum	for convulsions, particularly in infants	Pant and Tewari, 1998

Entodon flavescens	used during earache, leaf juice is used as ear drops, during cold, leaf juice is administered daily twice	Lubaina *et al.*, 2014
Fissidens nobilis	for growth of hairs and diuretic activity	Azuelo, 2011
Funaria hygrometrica	hemostasis, pulmonary tuberculosis, bruises, skin infection	Pant and Tewari, 1998
Fontinalis antipyretica	used in chest fever	Drobnik and Stebel, 2014
Taxiphyllum taxirameum	for external wounds, hemostasis	Asakawa, 2007
Aerobryum lanosum	used during burns, decoction of whole plant boiled in goat urine is applied externally	Lubaina *et al.*, 2014
Mnium cuspidatum	for hemostasis, nose bleeding	Pant and Tewari, 1998
Mnium sp.	to reduce pain of burns, bruises and wounds	Azuelo *et al.*, 2011
Plagiomnium sp.	for infections and swellings	Azuelo *et al.*, 2011
Octoblepharum albidum	used as febrifuge and anodyne	Singh, 2011
Dawsonia superba	used as diuretics, hair growth	Azuelo *et al.*, 2011
Polytrichum commune	used for hemostasis, wound healer, antipyretic, antidotal activity, dissolve kidney and gall bladder stones, to speed up labor process during child birth	Turner *et al.*, 1983
Polytrichum juniperinum	to treat prostate, uninary difficulties and skin ailments	Gulabani, 1974
Pogonatum macrophyllum	to reduce inflammation and fever, also used as detergent diuretic, laxative and hemostatic agent	Alam, 2012

Barbula unguiculata	to treat fever and body aches	Azuelo *et al.*, 2011
Barbula indica	used during menstrual pain and intermittent fever	Lubaina *et al.*, 2014
Hyophila attenuata	used during cold, cough and neck pain, leaf decoction is administered with a pinch of pepper powder daily	Lubaina *et al.*, 2014
Weisia viridula	to treat cold and fever	Asakawa, 2007
Sphagnum sericeum	used for dressing wounds, with anti-microbial properties for skin ailments (insects bites, scabies, acne), haemorrhoids and to treat eye diseases	
Sphagnum teres	to treat eye diseases	Ding, 1982
Haplocladium microphyllum	to treat cystisis, bronchitis, tonsillitis pneumonia and fever	Ding, 1982

Studies on *Platyphylla* and *D. scoparium* showed antimicrobial effects on the gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Sarcinalutea*, but no activity against gram-negative *Escherichia coli* (Pavletic and Stilinovic, 1963).

Bryophytes have shown antibacterial activities against gram negative and gram positive bacteria (Basile *et al*., 1999).

Phenolic compounds isolated from *Dicranum, Atrichum, Polytrichum, Mnium,* and *Sphagnum* sp. showed antimicrobial properties (Mishra *et al*., 2014). Also lipids and fatty acids were analyzed in the general of families, *e.g.* Dicranacae, Ditrichaceae and Entodontaceae (Ichikawa *et al.*, 1983; Dembitsky *et al.*, 1993; Wasley *et al.*, 2006).

Nikolajeva (2012), indicated that the antimicrobial activity for two liverwort *Frullania dilatata* and *Lophocolea heterophylla,* and three moss species *Eurhynchium angustirete, Rhytidiadelphus squarrosus* and *Rhodobryum roseum* has been reported.

Decker *et al*., (2003) reported that aqueous extract of few bryophytes have some inhibitory effect on the growth of *E. coli*.

All bryophyte extracts showed a specific antifungal property against the plant pathogenic fungi depending on the concentration. Different crops like tomatoes, wheat and green pepper were infected with *Botrytis cinerea, Phytophora infestans* and *Erysiphe gramini.* After they were treated with alcoholic extracts of different bryophytes species. These alcoholic extracts of different bryophytes species showed antifungal activity for these crops (Frahm, 2014).

Neckera crispa and *Porella obtusata* extracts had showed fungicidal and antifeedant effects several times, and thus commercial product was developed as natural pesticide for Portuguese slug *Aarion lusitanicus* from *Neckera crispa* and *Porella obtusata* extracts (Frahm and Kirchoff, 2002).

Some of the moss and liverworts possess antioxidative activities which helps them to survive in the extreme climate and stress condition (Mishra *et al*., 2014). Heavy metal, desiccation and UV radiation have been found to cause an array of some different enzymes in bryophytes (Dey and De, 2012).

Bryophytes have been found to accumulate some metals and few others were able to insulates the toxic metals.

Antioxidant and free radical scavenging activities are in the focus of pharmacists and nutrition scientists. Free radicals are playing a role in the pathogenesis of many diseases (Castro and Freeman, 2001). Oxidation processes may also decrease the stability of drugs and foods. Bhattarai *et al*., (2009) indicated the potential of Antarctic mosses *Sanionia uncinata* and *Polytrichastrum alpinum* to be used as antioxidants for medicinal and cosmetic purpose.

Antioxidant property, scavenging activities and phenolic content of the aqueous extract of *Brachythecium rutabulum, Calliergonella cuspidata* and *Hypnum mammillatum* have investigated. *B. rutabulum* showed the higher phenolic property than other species (Chobot *et a*l., 2008).

Methanolic and ethylacetate extracts of *M. polymorpha* have also shown antioxidant property. Bryophyte could be the source of many antioxidants which could be used for novel drug discovery (Mishra *et al*., 2014).

Anti-leukemic activity has also been demonstrated in several compounds from leafy liverworts. A new enteudesmanolide called diplophyllin, was isolated from *Diplophyllin albicans* and *D. taxifolium.* Diplophyllin has an alpha-methylene lactone against human epidermoid carcinoma (KB cell culture). Marchantin A from *M. palacea, M. polymorpha,* and *M. tosana,* riccardin from *Riccardia multifida* and perrottetin E from *Radula perrottetii* show cytotoxicity against the leukemic KB cells (Chandra *et al*., 2016). Also compounds from *Plagiochila fasciculata* seemed to inhibit leukaemia (P388 cells) (Saxena and Harinder, 2004).

Apart from ethno-medicinal uses some bryophytes possesses antitumor activities against different cancer cells and thus bryophytes needs to be more focused on the next years.

5. CONCLUSION

Natural products derived from the plants can be used an alternative recipe for development of drug resistance in pathogens. Herbal compounds have been discovered with therapeutic potential. Bioactive compounds used as drugs are a new production system for major problems in medicine.

Bryophytes, a small group of plants, are an important source of biological active compounds. Many of the bryophytes are the source of medicinal recipes with antibacterial, antimicrobial, antifungal and anti-leukemic agents (Bhattarai *et al.*, 2009). Bryophytes being rich source of secondary metabolites could be a source of the bioactive compounds with immense therapeutic potential.

The current researches are going on the medicinal active constituents of bryophytes are used in curing diseases such as skin diseases, cardiovascular diseases, hepatic disorders and many more other ailments.

This evaluation and validation of traditional practices with medicinal active constituents of bryophytes provides significant opportunities for newer drug discoveries for human health care.

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