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Antioxidant, antimicrobial and cytotoxic activities of extracts from some selected mediterranean shrub species (Maquis)

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ABSTRACT

In this study *in vitro* antioxidant, antimicrobial and cytotoxic activities of ethanol extracts of some plants from Urla region in Turkey were investigated. Plant material samples of *Pistacia lentiscus*, *Vitex agnus-castus*, *Cistus creticus* and *Nerium oleander* were collected in October, November and December. The harvesting time significantly affected their antioxidant, antimicrobial and cytotoxic activities of these plant extracts. The highest biological activities in terms of antioxidant, antimicrobial and cytotoxic activities were observed for the leaf extract of *C. creticus*. The applied doses of leaf extracts of *C. creticus*, *P. lentiscus*, and *N. oleander* resulted in higher Bax and GAPDH expressions than those for control cells. These plant extracts may trigger apoptosis and may be a promising natural source for prostate cancer treatment.

Keywords: *Plant extract; antioxidant activity; antimicrobial activity; cytotoxicity; bax expression.*

1. INTRODUCTION

Plants have an almost limitless ability to synthesize phytochemicals, most of which are polyphenols. These substances serve as plant defense mechanism against microorganisms, insects and environmental effects-stress, drought [1]. With this contribution, plant extracts have become important and many researches were performed in order to investigate their notable biological activity, including antioxidant, antimicrobial and cytotoxic properties.

The role of free radicals in oxidative events such as initiation and progression of cardiovascular, tumoral, inflammatory and neurodegenerative disorders is clearly stated and it promotes to the search for new antioxidant molecules. An important source of such molecules is plants with their potent antioxidant activities. Many pharmacological activities such as cyto-protective, anti-tumor promoting, anti-inflammatory, antipyretic and anti-platelet, have been associated with the ability of plant antioxidants to scavenge highly reactive free radicals [2, 3].

Polyphenols, which are one of the most numerous and widespread groups of natural constituents in the plant kingdom, are responsible of high antioxidant activity by inhibiting free radical mediated processes [4]. It was clearly indicated that antioxidant activity increases in parallel by increasing the number of OH groups present in structure [5, 6]. Screening of plant kingdom for active components by pharmacological purposes becomes important. Additionally, antimicrobial properties of natural products are important to discover the new bioactive natural products for health and food industry. Antimicrobial compounds of plant origin are of interest to the pharmaceutical industry for the control of microbial pathogens. Although the introduction of antibiotics dramatically improved the treatment of bacterial infections, the emergence of antibiotic resistant strains of bacteria has led to the continuing search for useful natural antimicrobials. Beside antioxidant and antimicrobial properties, the cytotoxic activities of plant extracts are another interesting subject in the field of natural products research. Increasing number of investigations on polyphenols and their effects on cell cycle progression and cell cycle associated proteins is promising concept on cancer therapy.

The antioxidant, antimicrobial and cytotoxic activity of plant extracts can be affected by numerous factors, including genotype, growing season and location, maturity and post harvest storage conditions [7-10]. Although many factors that affect the polyphenolic content, determination of the optimum harvest time for maximum polyphenolic content is crucial in order to obtain high antioxidant, antimicrobial and cytotoxic activity.

Our region has a great potential by means of biological diversity due to the geographical position with more than 9,000 flowering plant species [11]. Mastic tree-*Pistacia lentiscus*, chaste tree-*Vitex agnus-castus*, Cistus-*Cistus creticus* and Oleander-*Nerium Oleander* are widely found plant species in the region. Several researchers have also studied the antifungal, antimicrobial, cytotoxic and antioxidant activities of these species found in this region [12-15].

The aim of the present work was to evaluate in vitro antioxidant, antimicrobial and cytotoxic activities of the plant extracts of four different plant species harvested in October, November, and December.

2. EXPERIMENTAL SECTION

2.1. General.

All chemicals and solvents used in this study were of analytical grade and supplied from Sigma-Aldrich (Steinheim, Germany).

2.2. Plant materials.

Mastic tree- *Pistacia lentiscus* (leaves and berries), chaste tree- *Vitex agnus-cas*tus (leaves and berries), Cistus-*Cistus creticus* (leaves) and Oleander-*Nerium oleander* (leaves) were collected during the beginning of October, November and December from Urla region (Izmir, Turkey). *Pistacia lentiscus* (Anacardiaceae), *Vitex agnus-castus* (Verbenaceae), Cistus-*Cistus creticus* (Cistaceae) and *Nerium oleander* (Apocynaceae) were identified by Dr. Nihal Özel in Ege Forestry Research Institute (Urla, Izmir).

2.3. Extraction of plant material.

Plant samples were immediately dried at 35 ºC in dark. After grinding of samples, they were extracted with 70% aqueous ethanol solution with a solid-liquid ratio of 1/10 at 37 ºC and 180 rpm in thermo shaker for 2 hours. The samples were then separated from solid plant material by filtration and they were centrifuged at 5,000 rpm for 5 min. Ethanol in extract was completely removed with rotary evaporator below 40 ºC in dark. Finally, dried extracts were obtained by freeze-drying and kept in the dark at +4 ºC until tested.

2.4. HPLC analysis of prepared plant extracts.

The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 mm × 4 mm i.d.) with a particle size of 5 mm thermostated at 30 ºC. The flow rate was 1ml min^{-1} and the absorbance changes were monitored at 260 and 280nm for *P. lentiscus* leaf extract and rest of the plant extracts, respectively.

In the HPLC analysis of *P. lentiscus* leaf extract the method developed by Vaya and Mahmood was used [16]. Briefly, mobile phase used was; (A) water and (B) acetonitrile: water (MeCN-H2O, 1:1, v/v), both containing 2.5% acetic acid (AcOH). The elution gradient was applied as follows: between 0-25 min, B was increased from 40% to 80% (linearly), and between 25-35 min B was maintained at 80% (isocratic); and between 35-37 min B was decreased from 80% to 40% (linearly).

For the rest of the plant extracts the HPLC method was carried out in gradient mode. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) during 20 min; it changed to 50%(A) and (B) in 20 min (40 min, total time); in 10 min it changed to 20% (A) and 80% (B) (50 min, total time), after reequilibration in 10 min (60 min, total time) to initial composition.

2.5. Determination of antioxidant activity.

A Photochemiluminescence (PCL) detection method using a Photochem (Analytik Jena AG, Jena, Germany) system was used to measure lipid-soluble (ACL) and water-soluble (ACW) antioxidant capacities. PCL method involved the photochemical generation of superoxide free radicals combined with chemiluminescence detection. PCL is the most suitable method since it is used to measure the superoxide radical, which is the free radical generated also by human body [17]. For this purpose, extract samples obtained just after the 2 hour extraction were used. After the centrifugation, supernatant with appropriate dilutions was used in the analysis. The antioxidant activity of these samples was measured using "ACW" and "ACL" kits provided by Analytik Jena, and the procedures were followed as described by the manufacturer. The antioxidant capacity (ACW or ACL) of a sample is expressed in equivalent concentrations of the standard compounds ascorbic acid or Trolox, respectively.

2.6. Determination of antimicrobial activity.

Four pathogen microorganisms, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, were obtained from the stock cultures of Faculty of Science and Arts, Izmir Institute of Technology. Microorganisms were maintained at -80 ºC within 20% glycerol-nutrient broth. Overnight cultures were grown in Mueller Hinton (MH) broth at 37ºC in an incubating shaker and colonies grown on the MH agar plates by streaked were used for antimicrobial tests. Antimicrobial tests were carried out by disc-diffusion method. For this purpose, the dried plant extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 mg ml^{-1} and sterilized by filtration through a 0.20 µm membrane filter into sterile falcon tubes. From overnight cultures of test microorganisms, approximately, 5 colonies was picked up by using sterile cotton swabs and dissolved in 2.5 ml phosphate-buffered saline (PBS) solution and turbidity was adjusted to McFarland 0.5. Then, the swab was streaked on the Mueller Hinton agar plates. The discs were impregnated with extract solution and placed on the inoculated agar and incubated at 37ºC for 24 hours. The clear zones around the discs were measured and recorded as inhibition zone that indicate antimicrobial property. DMSO was used as negative control, whereas antibiotic discs of gentamycin, vancomycin and penicillin were used as positive controls.

2.7. Determination of cytotoxic activity.

For the estimation of the *in vitro* cytotoxic activities of the investigated plant extracts, Human Prostate Cancer (PC3) cell line was provided by Professor Kemal S. Korkmaz (Ege University, Engineering Faculty, Department of Bioengineering). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (BIO-IND), 50µg/ml gentamicin sulfate, 250 UG ml^{-1} Fungizone Amphotericin B (GIBCO Invitrogen), incubated at 37 ºC in the dark with 5% CO2 humidified incubator and passaged when they reached 80-85% confluency. Cells used in the experiments were maintained from 10-20th passages.

2.7.1. Treatment of Cultured Cells with Extracts and Cell Viability Assay.

To investigate the cytotoxic activity of the extracts, 95µl of cell suspension was inoculated into 96-well microculture plates at $1x10^4$ cells density per well in culture media containing FBS, fungizone, gentamicin sulfate. Extracts were dissolved in dimethyl

sulfoxide (DMSO) (Sigma Chemical Co.), filter sterilized, diluted at the appropriate concentrations with the culture medium. In all well, 1% DMSO concentration was fixed. Dilutions of extracts were freshly prepared before each experiment. After 24h cultivation for cell attachment, extracts were added at a final concentration of 1, 50, 100, 200, 300, 400, 500 µg ml^{-1}. For extracts of *N. oleander* cells were treated with a final concentration of 0.01, 0.05, 0.1, 0.5, 1, 50, 100 µg ml^{-1}. Cells were treated with the extracts for 24 hours, 48 hours, 72 hours and cytotoxic effects were determined by tetrazolium (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co.) based colorimetric assay. This method depends on the cleavage of tetrazolium salt to purple formazan crystals by mitochondrial enzymes of metabolically active cells [18]. Briefly; 4 hours before the end of each incubation period, medium of the cells was removed and wells were washed by pre-warmed phosphate-buffered saline (PBS) to remove any trace of extracts and to prevent color interference while optical density determination. MTT stock solution (5 $mgml^{-1}$) was diluted at 1:10 ratio into complete culture media, 100µl of MTT dilution was added into each well and incubated. After 3.5 hours plates were centrifuged at 1800 rpm for 10 minute at room temperatures to avoid accidental removal of formazan crystals. Crystals were dissolved with 100µl DMSO. The absorbance was determined at 540nm. Results were represented as percentage viability and calculated by the following formula:

% viability=100-[(ODs-ODB/ODc-ODB) x 100]

Where ODB, ODs, and ODc are the mean optical densities of blank, sample and control, respectively. The assays were performed in triplicate to determine the percent viabilities.

For median inhibition concentration (IC_{50}) determination, dose-response curves were conducted with a series of different concentrations of extracts. IC_{50} was then determined by nonlinear regression analysis of the corresponding dose response curve.

2.8 Quantitative RT-PCR.

The LightCycler® 2.0 Real-Time PCR System (Roche Applied Science) was used for the RT-PCR analysis to determine the mRNA expression of Bax gene in PC3 cell line. For 48 and 72 hours, in a 24 well plate approximately $1x10^6$ cells per well were incubated with the specific extract at different concentration levels listed in Table 1. Extract dilutions were prepared as described in cell viability assay section. At the end of each incubation period, extract treated cells were harvested and total RNA was extracted using High Pure RNA Isolation kit (Roche Applied Science). Isolated RNA was stored -80 ºC until its use. RNA (1µg) was transcripted into cDNA using 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Applied Science) according to manufacturer's instruction. Synthesized cDNA samples diluted 1:10 ratio and 10µl diluted cDNA template was added into pre-cooled capillaries. Fast Start DNA Master SYBR Green 1 kit (Roche Applied Science) was used to perform RT-PCR analysis (ROCHE Applied Science). The reaction conditions as follows: 10 minute at 95 ºC for denaturation period followed 35 cycles of 10 second at 95 ºC for denaturating, 10 second at 68 ºC for annealing, and 16 second at 72 ºC for extention. GAPDH was used as a house-keeping gene. Different concentrations of Bax and GAPDH primers were used to obtain standard curve and mRNA concentrations of the samples were calculated from this curve. GAPDH was used as a positive control and endogenous normalizer. The results of the individual samples were normalized according to the endogenous normalizer.

Table 1. Applied extract concentrations for RT-PCR analyses (48 and 72 h incubation periods).

Plant Extracts	Harvest time[a]	48 hour incubation Concentration(µg/ml)	72 hour incubation
Leafof *P. lentiscus*	Oct.	75	20
Leafof *P. lentiscus*	Nov.	-	20
Leafof *P. lentiscus*	Dec.	200	200
Leafof *C .creticus*	Oct.	50	50
Leafof *C. creticus*	Dec.	100	200
Berries of *P. lentiscus*	Oct.	-	100
Berries of P. lentiscus	Nov.	-	100
Berries of P. lentiscus	Dec.	-	75
Leafof *V. Agnus-castus*	Oct.	1	1
Leafof *V. Agnus-castus*	Nov.	1	-
Leafof *V. Agnus-castus*	Dec.	1	1
Leafof *N. oleander*	Nov.	0.5	0.5
Leafof *N. oleander*	Dec.	0.5	0.5

[a]Months: Oct., October; Nov., November; Dec., December, -:not tested

3. RESULTS SECTION

In this study, six different plant extract samples were obtained from four plant species including Mastic tree-*Pistacia lentiscus*, chaste tree-*Vitex agnus-castus*, Cistus-*Cistus creticus* and Oleander-*Nerium oleander*. The prepared plant extracts were analyzed for their polyphenolic contents. Then these extract samples were evaluated for their antioxidant, antimicrobial and cytotoxic properties.

3.1. Composition of prepared plant extracts.

The composition of plant extracts were determined using HPLC analysis although it was not an easy test to determine individual components of each plant extracts the preliminary results of HPLC analysis indicated the presence of polyphenolic compounds in the prepared plant extracts. Also, the HPLC analysis were performed only for qualitative determination of active compounds, not quantitave analysis.

The presence of taxifolin, myricetin, 7,4'-dihydroxyflavone and luteolin were detected in the leaf extract of *P. lentiscus*.(Figure 1.A) The similar observations were also made by Vaya&Mahmood [16]. In the seed extract of *P. lentiscus*, rutin, luteolin and apigenin glucoside were determined (Figure 1.B). Since the detection was carried out at 280 nm using DAD detector HPLC method determines mainly polyphenolic compounds and glycosides. After carefully checking HLPC chromatogram given in Figure 1. the unidentified peaks observed can be attributed to the presence of other types of glycosides in the extracts. In the literature, delphinidin-3-O-glucoside, cyanidin 3-O-glucoside and cyanidin 3-O-arabinoside were determined in the seed extract of *P. lentiscus* [19]. Also in the leaf extract of mastic tree gallic acid and galloyl derivatives of both glucose and quinic acid; flavonol glycosides, i.e. myricetin and quercetin glycosides; and anthocyanins, namely delphinidin3-O-glucoside and cyanidin 3-O-glucoside were reported by Romani et al. [20].

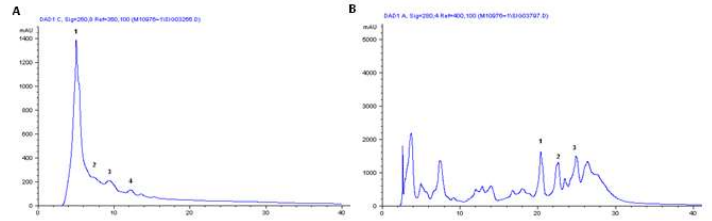

Figure 1. The chromatograms of *P. lentiscus* (A) seed and (B) leaf extracts. In A (1); taxifolin, (2); myricetin, (3); 7,4'-dihydroxyflavone, (4); luteolin and in B (1); rutin, (2); luteolin, (3); apigeninglucoside.

The similar chemical composition were observed seed and leaf extracts of *V. agnus castus* based on the HPLC chromatogram shown in Figure 2. The main peak observed at 15.4 min for both leaf and seed extracts, can be attributed to the presence of casticin. For both seed and leaf extracts the presence of luteolin-7-glycoside and apigenin-7-glucoside were observed in the HPLC chromatograms. Since HPLC was performed at 280 nm it was also possible to observe other types of glycosides appeared as unidentified peaks in our chromatograms. The fruits, flowers and leaves of *V. agnus-castus* were reported to contain flavonoids, tannins, iridoids and diterpenoids[21]. For the seed of *V. agnus castus*, casticin, lypophilic flavonoid, can be determined as a main compound in the fingerprint chromatogram at 270nm [22].

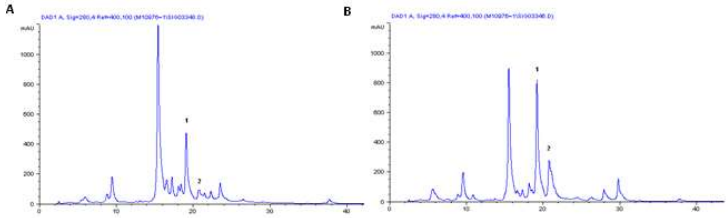

Figure 2. The chromatograms of *V. agnus-castus* (A) seed and (B) leaf extracts. In A and B (1); luteolin-7-glycoside, (2); apigenin-7-glucoside.

The peaks corresponding to catechin, vanillic acid, rutin and luteolin-7-glucoside were observed in the HPLC chromatogram for *C. creticus* leaf extracts (Figure 3.). In the literature presence of seven labdane-type diterpenoids, together with quercetin, myricetin, kaempferol, apigenin, luteolin, aesculin and also the sesquiterpenes and anthocyanins were reported for the leaf extract of *C. creticus* [23, 24].

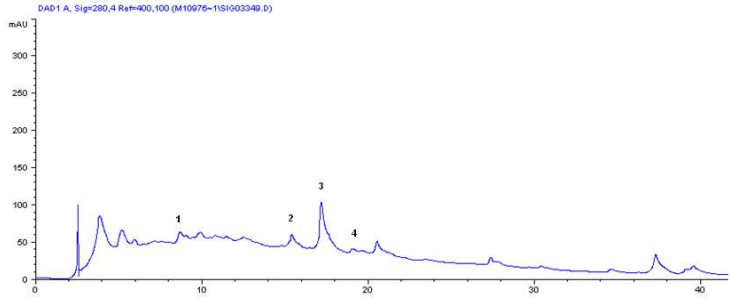

Figure 3. The chromatogram of *C. creticus.* (1); catechin, (2); vanillic acid, (3); rutin (4); luteolin-7-glucoside**.**

The HPLC analysis of *N. oleander* extract revealed the presence of rutin as observed in the chromatogram(Figure 4.). Although the major peak observed at 9.5 min couldn't be identified the information given in the literature indicated that extract of the *N. oleander* contains cardiac glycoside, oleander, as a main component therefore in our chromatogram this peak was attributed to the presence of cardiac glycosides. The presence of kaempferol, rutin and ursolic acid in *N. oleander* leaf was also reported in the literature [25- 26].

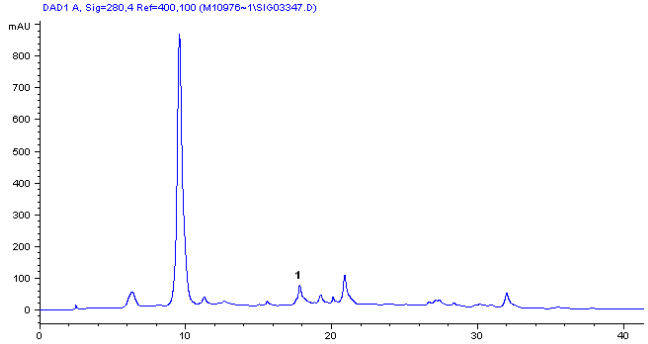

Figure 4. The chromatogram of *N. oleander*. (1); rutin.

These HPLC analyses of prepared plant extracts in this study shown that extracts are rich in polyphenolic contents. Therefore, the possible biological activities which is the main concern of this research comes from these compounds.

3.2. Antioxidant properties.

ACW and ACL antioxidant activities of extracts, which were obtained from plant materials harvested at different period of time, were determined by using PCL assay. ACW antioxidant activity results are given in Figure 5.

The highest ACW antioxidant activity was determined as 487 µg ascorbic acid equivalent per mg of dry plant material for the extract of *Cistus creticus* collected in November. The observed antioxidant activity can be explained by the presence of phenolic compounds such as catechins, rutin, and gallic acid in the cistus extract. In the literature, simultenous determination of catechins, rutin, and gallic acid in lyophilized extracts of Cistus species by HPLC method was also reported by Santagati et al. [27]. Effects of degree of maturity and harvest date on the phenolic and antioxidant contents of plant species are recently investigated issue in the literature [8, 9]. The importance of harvest time on ACW antioxidant activities of extracts can clearly be seen in Figure 5. In order to obtain plant extracts with high ACW activities, the best harvest times were determined as October for leaf of *Pistacia lentiscus*; as November for *Cistus creticus*, and berries of *Pistacia lentiscus*; as December for leaf of *Vitex-agnus castus*. There were no significant differences between ACW

antioxidant activities of extracts obtained from berries of *Vitex-agnus castus* and *Nerium oleander* with changing harvest date. The maturity of berries of *Pistacia lentiscus* could also be observed by monitoring the changes of color from green to dark bright red and purple color throughout the three months, from October to December. As parallel to this maturation, in November berries of *Pistacia lentiscus* with the color of dark bright red were mature enough to obtain extracts with active components for a higher antioxidant activity.

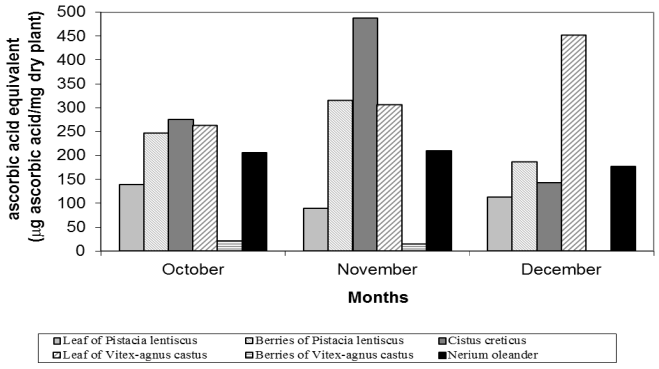

Figure 5. ACW antioxidant capacities of plant extracts obtained from plant species collected at three different harvest times.

Similarly, the maturity of the berries of *Vitex-agnus castus* could be investigated by monitoring the color changes from green to dark green. The oily nature of the extract of *Vitex-agnus castus* revealed that it might be used as a possible oil source with an antioxidant activity.

The changes of ACL antioxidant activities of extracts with harvest time are presented in Figure 6. The highest ACL activity was observed for the berries of *Pistacia lentiscus* as 488 mg Trolox equivalent per mg of dry plant material in October. It was followed by leaf extracts of *Cistus creticus*, Pistacia lentiscus, *Vitex-agnus castus* and *Nerium oleander* as 470, 447, 417 and 352 mg Trolox equivalents respectively. It should be noted that the harvest time did not affect ACL antioxidant activity as much as ACW antioxidant activity. Almost all extracts have constant ACL activities with a slight deviations from month to month.

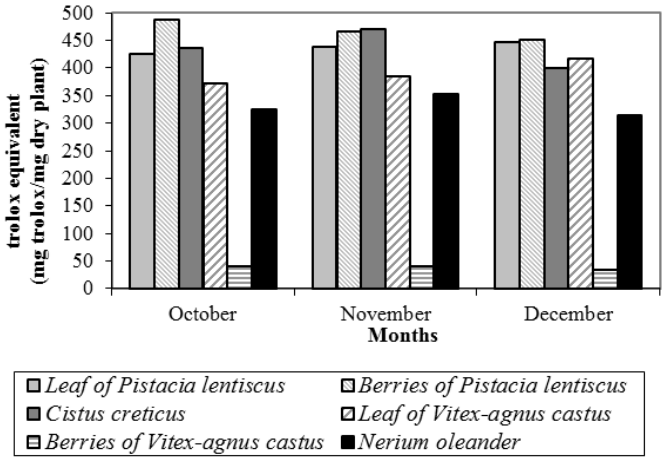

Figure 6. ACL antioxidant capacities of plant extracts obtained from plant species collected at three different harvest times.

As it is expected, because of the oily nature of the extract of *Vitex-agnus castus* berries, ACL activity was higher when compared with the ACW activity of it but relatively low compared with the other ACL activities of other plant extracts.

ACL and ACW activities of extracts can be compared because Kranl et al. reported that ACW activity of ascorbic acid is close to the ACL activity of Trolox [28]. Nearly all extracts had higher ACL activities relative to their ACW activities. This result indicates that the components having an antioxidative effect are more active in lipid, so it may be especially beneficial for preventing oxidative damage of lipids such as lipid oxidation of cell membrane. The berries extract of *Pistacia lentiscus* and *Cistus creticus* leaf extract in November are good source of both water-soluble and lipid-soluble antioxidant components. There was no significant effect of harvest time on both ACL and ACW activities of *Nerium oleander* extract, but ACL activity *Nerium oleander* extract was relatively high compared to its ACW activity.

3.3. Antimicrobial properties.

In the present work antimicrobial potential of ethanolic extracts of four different plant species have been determined (Table 2). These results showed that plant extracts exhibited inhibition effect due to their antimicrobial contents. Main chemical constitutes that are responsible for antimicrobial property have been reported earlier [1, 29].

Table 2. Effect of harvest time on antimicrobial activities of plant extracts.

Plant extracts	Microorganisms/Inhibition zones (mm)											
	Escherichia coli			*Klebsiella pneumoniae*			*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
	Oct.[a]	Nov.	Dec.	Oct.	Nov.	Dec.	Oct.	Nov.	Dec.	Oct.	Nov.	Dec.
Berries of *V. agnus-castus*	-	-	-	-	-	-	7.5	10	8	-	-	9
Leaf of *V. agnus-castus*	-	-	-	-	-	-	7.5	8	7.5	-	-	-
Berries of *P. lentiscus*	-	-	-	-	-	-	14	14.5	14	-	-	15
Leaf of *P. lentiscus*	-	-	-	-	-	-	13	13	13	-	-	9
Leaf of *C. creticus*	-	-	-	8.5	11.5	11	17	15	14	-	8	15
Leaf of *N. oleander*	-	-	-	-	-	-	-	-	-	-	-	-
	Antibiotic Controls											
Gentamycin	-			-			20			-		
Penicillin	-			-			44			-		
Vancomycin	-			-			14			-		

[a]Months: Oct., October; Nov., November; Dec., December

The researches in the literature focused mainly on the antibacterial activities of mastic, resinous gum produced by *P. lentiscus* tree[30]. In this study leaf and berries extracts of *P. lentiscus* showed inhibition against *S. aureus*.

The leaf extract of *Cistus creticus* has shown the highest antimicrobial activity against the tested microorganisms. This extract was effective for inhibiting the growth of *K. pneumonia* and *P. aeruginosa* whereas antibiotics were not effective. When it was compared the sensitivity of microorganisms against plant extracts, the most sensitive bacteria was found to be *S. aureus*. Additionally, *E. coli* was found to be the least sensitive microorganism. This may be attributed to cell wall structure because the cell wall in Gram positive bacteria consists of a single layer, whereas, the cell wall in Gram negative bacteria consists of a multi-layered structure bounded by an outer cell membrane [28]. Control discs with DMSO alone showed no inhibitory effect against the organisms tested. Antibiotics used as positive control were found to be effective only for *S. aureus*. Penicillin provided

the largest inhibition zones. Although studying antimicrobial property at three different harvesting times, it was not possible to draw a significant conclusion from our antimicrobial screening results.

3.4. Cell Viability.

The median inhibition concentration (IC_{50}) values of plant extracts for PC3 cell line are given in Table 3. For 48h and 72h incubation periods, cell viability was reduced in dose dependent manner for the extracts obtained from plants collected at all harvest times. Cell proliferation was observed when leaf extracts of *C. creticus* harvested in October and November were used at a concentration of 1 μg ml^{-1} (data not shown). When these extracts were used at a concentration of 50 μg ml^{-1} the cell viability suddenly decreased below 50 %. For 48 h and 72 h incubation periods, IC_{50} values for the leaf extract of *C. creticus* harvested in December were determined as 97 and 170 μg ml^{-1}, respectively. Significant cytotoxic effects for both berries and leaf extracts of *P. lentiscus* harvested in October were also observed. Leaf extract of *V. agnus-castus* showed nearly same cytotoxicity pattern for all harvest times. Extremely high cytotoxic activities were observed even at very low concentrations of *N. oleander* leaf extracts. It should be noted that at the early stages of apoptosis, cells have high mitochondrial activity. MTT test utilizes degradation of tetrazolium salt to purple formazan crystals by mitochondrial enzymes. That is why high mitochondrial activity can be sign of either apoptosis or proliferation. In addition all cell death does not have to be apoptosis. Discrimination can only be seen with further molecular analysis.

3.5. Quantitative RT-PCR.

There are different pathways of the cells to undergo to apoptosis. One of them is mitochondrial pathway which is regulated by pro- and anti-apoptotic members of Bcl-2 family proteins [31]. There is increasing evidence that members of the Bcl-2 family are important for the regulation of prostate cell apoptosis. Bax is a Bcl-2-related homolog protein that promotes apoptosis [32]. When proapoptotic proteins such as Bax released from the inner mitochondrial membrane into the cytosol, caspase dependent death pathways are triggered. In the present study, after the plant extracts were applied to PC3 cell lines at different concentrations (Table 1) apoptosis related gene expression levels were determined on mRNA level using real time PCR to investigate the presence of caspase dependent cell death pathways. After PC3 cell cultures were incubated with extracts of *C. creticus* leaf harvested in October for 48 hour, *P. lentiscus* leaf harvested in December for 72 hour and *N. oleander* leaf collected in December for 72 hour at a concentration of 50, 20 and 0.5 μg ml^{-1}, respectively the applied doses of these plant extracts resulted in higher Bax and GAPDH expressions than those for both control cells and positive control of primer sets. As seen in Figure 7. the relative values of Bax expressions of cells treated with leaf extracts of *C. creticus*, *P. lentiscus* and *N. oleander* were found to be 1.83, 1.37 and 1.44 times greater than the Bax control, respectively. Relative values of GAPDH in the same conditions were found to be 2.45 times, 1.64 times and 2.18 times greater than those of GAPDH control respectively. Relatively high expressions of these apoptosis related genes revealed that these plant extracts may trigger apoptosis and may be promising natural sources for cancer treatment. No significant differences were observed between the relative values of both Bax and GAPDH expressions for cell cultures treated with the rest of plant extracts listed in Table 1 compared to those for control cells.

Table 3. *In vitro* cytotoxic activities (IC_{50} values as μg ml^{-1} ± Standard deviation) of crude plant extracts tested against PC3 cell line for exposure of 48 and 72 hours.

Plant extracts	Harvest time[a]	48h Concentration(μgml^{-1})[b]	72h Concentration(μgml^{-1})[b]
Leaf of *P. lentiscus*	Oct.	25,93	10,11
	Nov.	150,34	34,26
	Dec.	105,16	50>
Leaf of *C. creticus*	Oct.	50>	50>
	Nov.	50>	50>
	Dec.	96,99	169,83
Berries of *P. lentiscus*	Oct.	-	30,92
	Nov.	51,70	51,61
	Dec.	69,88	52,06
Leaf of *V. Agnus-castus*	Oct.	17,57	9,88
	Nov.	18,85	1>
	Dec.	11,00	13,39
Leaf of *N. oleander*	Oct.	-	-
	Nov.	0,64	0,1383
	Dec.	0,0176	0,0241

[a]Months: Oct., October; Nov., November; Dec., December
[b] Data represent the mean±S.D. of three separate experiments

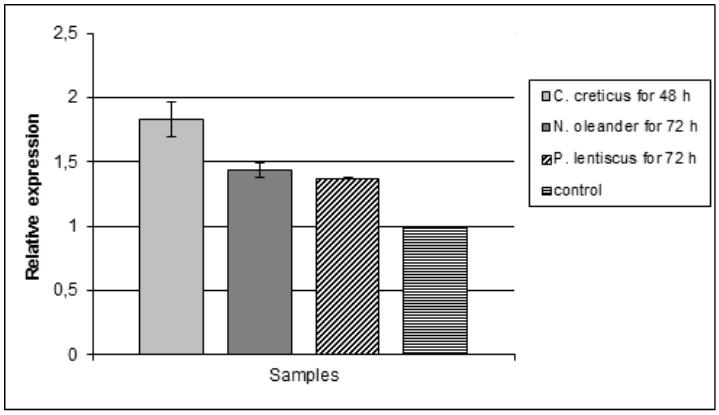

Figure 7. Effects of plant extracts on the relative values of Bax gene expression.

In the literature, extracts of *C.creticus* exhibited IC_{50} value below 20 μg ml^{-1} and induced apoptosis in human leukemic cell lines [23, 33, 34]. The active compounds (Sclareol and ent-3β-hydroxy-13-epi-manoyl oxide belong to the labdane type diterpenes) of *C.creticus* were isolated from the leaves and the fruits of *Cistus creticus* subsp. creticus. These compounds were found to be active against different human cancer cell lines [23, 34]. Investigation on effects of crude aqueous extracts different species of Cistus, *Cistus incanus* and *Cistus monspeliensis*, shown that they have also free-radical scavenging capacity and protective effects againts DNA cleavage [35].

The cytotoxic activity of *P. lentiscus* resin on peripheral blood mononuclear cell has previously been reported in the literature [36]. Ethanolic extract of chios mastic gum, a product derived from *P. lentiscus*, inhibited proliferation and induced death of HCT116 human colon cancer cell line *in vitro* [37, 38]. Recently, Sakami et al. 2009 investigated effects of *P. lentiscus* leaf on thirteen human cell types and found that promyelocytic

leukemia HL-60 was very sensitive to the cytotoxicity of mastic [30]. Further investigation on the mechanisms involved in regulatory systems in prostate cancer cell lines revealed that the gum mastic was effective by suppressing the NF-KB activity and NF-KB signal pathway [39]. Other studies regarding the effect of gum mastic on prostate cancer cells revealed that mastic can regulate expression of a tumor suppressor gene, maspin, by upregulating its mRNA and protein levels [40, 41].

The antitumoral activity of compound called oleandrin present in the extract of *N.oleander* has been investigated on PC-3 cell lines [42]. It was also reported that oleandrin induced apoptosis in androgen-independent human prostate cancer cell lines in vitro[43].

Several recent reports have appeared concerning the apoptotic effects of antioxidants, like some plant flavonoids that induce apoptosis in some tumor cell lines but not in normal cells [44]. Understanding the modes of action of these compounds should provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy. Therefore, further studies are needed to identify the bioactive compounds of each extract.

4. CONCLUSIONS

Six different plant extract samples from four plant species including mastic tree-*Pistacia lentiscus*, chaste tree-*Vitex agnus-castus*, Cistus-*Cistus creticus* and oleander-*Nerium oleander* possess antioxidative, antimicrobial and cytotoxic properties. These biological activities of extracts could be attributed to the their rich polyphenolic composition. In this study, it was found that also, harvest time significantly affects their antioxidant, antimicrobial and cytotoxic activities of these plant extracts. The differences can be explained by variations in the amount of plant active components with harvesting time. The highest biological activities in terms of antioxidant, antimicrobial and cytotoxic activities were observed for the leaf extract of *C. creticus*. Leaf extract of *C. creticus* has been shown to induce apoptotic cell death in PC-3 cell lines with a relatively high expression of Bax. This plant extract may trigger apoptosis and may be a promising natural source for prostate cancer treatment.

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