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Original Research

Antioxidant activities, anticancer activity and polyphenolics profile, of leaf, fruit and stem extracts of *Pistacia lentiscus* from Tunisia

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Abstract: Plant derived compounds have played an important role in the development of several clinically useful anticancer drugs. The aim of the present study was 1) to evaluate for the first time the anti-proliferative activity of a polyphenol enriched extract obtained from leaf, fruit and stem of Tunisian variety of *Pistacia lentiscus* against two cultured cancer cells, and 2) to carry out a phytochemical analysis of vegetable extracts particularly by determining the chemical composition of phenolics (total polyphenols, flavonoids and condensed tannins content in solvents with varying polarities), 3) to evaluate the antioxidant activity and identify the major compounds by RP-HPLC. Leaf extract using methanol/water (8:2) showed the highest polyphenol content (124.1 mg GAE/g DW). Moreover, total antioxidant capacity, reducing power and antiradical capacities against DPPH were maximal in leaf extracts with IC_{50} significantly lower than that standard (BHT). In MTT assay, methanol (8:2) extract exerted the most potent cytotoxic effect. The leaf extract exhibited an important antiproliferative activity (IC_{50}: 135.67 ± 2.5 and 250.45 ± 1.96 µg/ml in CaCo2 and AGS cells respectively) but the infusion extracts of fruit stems and leaves were inactive. The RP-HPLC analysis revealed the presence of several phenolic compounds in *P. lentiscus* leaf, fruit and stem including tannic acid, gallic acid, digalloyl quinic acid derivative, quercetin and p-coumaric acid as major phenolics. The high phenolic content and the important antioxidant activities of *P. lentiscus* extract could be a useful source of natural products and may be increasingly important for human consumption, prevention of damages caused by oxygen free as well as for the agro-food, cosmetic and pharmaceutical industries.

Key words: Anti-proliferative activity; Antioxidant activity; Phenolic compounds; HPLC analysis; *Pistacia lentiscus.*

Introduction

There is growing interest in the use of plants in the maintenance of human health. Plants have played an important role as a source of effective anti-cancer agents, and it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (1, 2).

Compounds found in vegetables, pharmaceutical plants and fruits that may help to protect against cancer and are attracting a lot of interest in their perceived ability to act as highly effective chemopreventive agents. Nutritional or dietary factors can influence the prognosis after the diagnosis of cancer and the quality of life during cancer treatment. Dietary approaches also considered a reasonable strategy to prevent cancer. In fact, many efforts are being carried out to isolate the bioactive products from pharmaceutical plants to use them in the treatment of cancer (3).

On the other hand, natural antioxidants present in plants have attracted interest because of their abilities to scavenge free radicals, which are reactive molecules with unpaired electrons that are able to damage vast varieties of cellular macromolecules including proteins, DNA and lipid bilayer membranes. Reactive oxygen species (ROS) are linked to some diseases such as cancer. Natural antioxidants presented in plants have attracted interest because of their abilities to scavenge these active species (4). Moreover, considerable attention has been paid to antioxidant properties of plants that may be used for human consumption. Moreover, phenolic compounds of plants are attracting significant interest in the field of food, chemistry and medicine due to their promising antioxidant potential (5), these natural antioxidants have easy and unlimited access to metabolic processes in the body, and produce virtually none of the side effects associated with synthetic antioxidants (6).

In Tunisia, a considerable diversity of plant with multiple interests including therapeutic practices exists, and a number of them have not been subject to chemical investigations. For example *Pistacia lentiscus* a famous herb drug in Tunisia. In fact this species *Pistacia lentiscus* L., which belongs to the Anacardiaceae family, is a dense bush with a strong characteristic aroma and green leaves, which grows in many Mediterranean countries (7). Historically, this plant has been widely used; indeed its aerial part has traditionally been used in the treatment of hypertension and possesses stimulant and

diuretic properties (8). The composition of its berries, essential oil, and mastic-gum resin being more studied than that of the leaves (9, 10). Berries from *P. lentiscus* are rich in anthocyanins, which confer antioxidant properties and induce autophagy, a mechanism which enhances chemoprevention (11).

P. lentiscus leaves contain different types of secondary metabolites, among these, the most abundant compounds are reportedly flavonoids, which display a powerful antioxidant capacity (12) as well as hepatoprotective, anti-inflammatory and anticancer effects (13, 14). In Tunisia, this plant species has not been subjected to detailed studies concerning its quantitative determination of anticancer and antioxidant potentials, as well as their secondary metabolites content. However, in the present article, we reported for the first time on the antiproliferative effects of different extracts from *P. lentiscus* aerial parts on two cultured cancer cells (AGS and CaCo2). The effect of extraction solvents (methanol/water 8:2, ethanol/water 7:3, and boiling water) on phenolic contents (polyphenols, flavonoids and condensed tannins) from leaves, fruits and stem of *P. lentiscus* was also carried out. We also identified the presence of phenolic and flavonoid compounds detected by RP-HPLC and evaluate the antioxidant activity by three different model systems such as phosphomolybdenum assay, reducing power, and DPPH• radical scavenging activity.

Materials and Methods

Plant Material

The leaf, fruit and stem of *P. lentiscus* were harvested in forestry areas from Bizerte, North of Tunisia. Identification was carried out by Pr. Ouni (Department of Botany Faculty of Sciences. University of Carthage. Tunisia), according to the flora of Tunisia (Cuénod, 1954). A voucher specimen (Pl-11-12) is kept in our laboratory for future reference. The samples were washed and dried under shadow. Dried plant material was then ground to fine powder (diameter < 63 mm) using an electric mill (Moulinex) and stored in deep freeze at -20◦C until use.

Preparation of the plant extracts

Plant materials were ground and macerated for extraction. Three solvents were used for extracting phenolic compounds from leaves, fruit and stems (methanol, ethanol, boiling distilled water). Five gram of each sample was weighed into 100 ml Erlenmeyer flasks, and then 10 ml of solvent with varying polarities: 80% methanol (80% MeOH), 70% ethanol (70% EtOH) and boiling distilled water (infusion in hot water at 100°C for 30 min) were added to the plant samples. Extraction was carried out by shaking at room temperature for 30 min. The extracts were then kept for 24 h at 4°C, and were centrifuged at 5000 g for 10 min, at room temperature, and the supernatants were then filtered using a filter paper (Whatman No. 4). Finally the extracts were evaporated under vacuum to dryness and stored at 4°C until analyzed.

Determination of total polyphenols content

Colorimetric quantification of total polyphenols was determined by the Folin-Ciocalteu method (16). Hundred and twenty five microliter of diluted extract was dissolved in 500 µl of distilled water and 125 µl of the Folin-Ciocalteu reagent was added. The mixture was shaken, before adding 1250 µl Na_2CO_3 (7%) and adjusting with distilled water to a final volume of 3 ml. After incubation for 90 min at 23 °C in the dark, the absorbance was read at 760 nm. A standard curve of gallic acid was used. Total phenolic content of plant was expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid (0-400 µg ml^{-1}). All samples were analyzed in three replications.

Estimation of total flavonoids content

Total flavonoids were measured by a colorimetric assay according to Dewanto *et al.,* (16). An aliquot of methanol, ethanol and water diluted extract or standard solution of (+)-catechin was added to a 75 ml of $NaNO_2$ solution, and mixed for 6 min, before adding 0.15 ml of $AlCl_3$ (10%) solution freshly prepared. After 5 min, 0.5 ml of NaOH was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the same mixture, without the sample, as a blank. Total flavonoid content was expressed as mg catechin g^{-1} DW (mg CE/g DW), through the calibration curve of (+)-catechin. The analysis was repeted three times.

Total condensed tannins

Total tannin content was measured using the modified vanillin assay (17). Three milliliters of 4% methanol vanillin solution and 1.5 ml of HCl (6M) were added to 50 ml of suitably diluted sample. The mixture was kept for 15 min, and the absorbance was measured at 500 nm. The amount of total condensed tannins was expressed as mg (+)-catechin equivalent per gram of dry weight (mg CE/g DW) through the calibration curve with catechin. The calibration curve range was 0-300 µg/ml ($R^2 = 0.995$). Triplicates measurements were taken for all samples.

Identification of phenolic compounds using reversed-phase HPLC (RP- HPLC)

Dried samples from leaves were hydrolysed according to the method of Proestos *et al.,* (18) slightly modified. Forty ml of methanol containing butylated hydroxytoluene: BHT (1g l^{-1}) were added to 0.5 g of a dried *P. lentiscus* sample. The obtained mixture was filtered through a 0.45 mm membrane filter and injected to RP-HPLC. The phenolic compounds analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP–HPLC) coupled with an UV-Vis multiwavelength detector. The separation was carried out on a 250×4.6 mm, 4µm Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of methanol with 0.2% formic acid (solvent B) and water with 0.2% formic acid (solvent C). The flow rate was kept at 1 ml min^{-1}. The gradient program was as follows: 35% B/65% C 0-6 min, 60% B/40% C 6-9 min, 80% B/20% C 9-14 min, 100% B/0% C 14-25 min. The injection volume was 20 µl, and peaks were monitored at 280 nm. Peaks were identified by congruent retention times compared with standards.

Analyses were performed in triplicates. Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard.

Evaluation of the total antioxidant capacity

The assay is based on the reduction of molybdate (Mo), Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH [19]. An aliquot of sample extract was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g DW). All samples were analyzed in triplicate.

Reducing power

The method of Oyaizu (20) was used to assess the reducing power of leaf, fruit and stem of *P. lentiscus* extracts. Methanol extracts (1 ml) and standard (ascorbic acid) were mixed with 2.5 ml of a 0.2 M sodium phosphate buffer (pH = 6.6) and 2.5 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$), and incubated in a water bath at 50◦C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid were added to the mixture that was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was then mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride solution.

The intensity of the blue-green color was measured at 700 nm and the reducing power of the extracts was presented as mg AAE /g of DW.

DPPH assay

The electron donation ability of the obtained methanol extracts was measured by bleaching of the purple colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato *et al.,* (21). Extracts (2 ml, 10-1000 µg ml^{-1}) were added to 0.5 ml of 0.2 mM DPPH methanolic solution. After an incubation period of 30 min at room temperature, the absorbance was determined against a blank at 517 nm. Percentage inhibition of free radical DPPH (PI %) was calculated as follow:

PI% = [(Ablank − Asample)/Ablank] ×100, where Ablank is the absorbance of the control reaction and Asample is the absorbance in the presence of plant extract. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the regression equation prepared from the concentration of the extracts and the inhibition percentage. BHT was used as a positive control. Samples were analyzed in triplicate.

Cell culture

AGS human gastric carcinoma cells (ATCC CRL-1739, Rockville, MD, USA) and CaCo2 human colon adenocarcinoma cells (ATCC 169, DSMZ collection) were maintained in Ham's F12K medium (Invitrogen, Cergy-Pontoise, France) and in modified Eagle's medium (Invitrogen), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5 units/ml of penicillin and 5µg/ml of streptomycin (Sigma, Saint-Quentin-Fallavier, France) at 37°C in a 5% CO_2 humidified atmosphere. Cells were maintained as a monolayer in 75 cm^2 culture flasks.

Cell viability by MTT assay

MTT assay was used to determine the inhibition of cancer cell proliferation by leaf, fruit and stem extracts with different solvents of *P. lentiscus*. It is a colorimetric assay based on the fact that mitochondrial oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to its insoluble formazan, which has a purple color. The cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present (22). The CaCo2 and AGS cells were seeded in triplicate for each experimental condition in 96-well microtiter plates (100 µl/well) at a concentration of $2x10^3$ cells/cm^2 and incubated prior to the addition of test compounds. After 48 hours in incubation, the cultivated cells were exposed to various concentrations (25, 50, 100, 250, 500, and 1000 µg/ ml) of extracts for 24h. Cells in the control group received only media containing 0.1% DMSO. Doxorubicin was used as a positive control in this experimentation. After the treatment, normal culture medium was substituted with 100 µl of fresh media and 20 µl of MTT reagent (5 mg/ml in PBS), except for the three cell-free blank control wells. Cells were maintained in 5% CO_2 at 37˚C and total humidity for 4 h. Subsequently, the MTT solution was replaced with 100 µl DMSO and 12 µl of Sorensen buffer (0.1 M NaCl, 0.1 M glycine adjusted to pH: 10.5 with 1M NaOH) and incubated for 15 min at 37˚C. The optical density of the wells was measured at 570 nm using a spectrophotometric plate reader (SPECTROstar Nano). The anti-proliferative activity of the extracts was reported as IC_{50} values (IC_{50} value was defined as the concentration of extract that inhibits cell proliferation by 50%) (23).

Data analysis

All analyses were performed in triplicate and the results are expressed as mean values ± standard deviations (SD).The data were subjected to statistical analysis using statistical program package STATISTICA. The one-way analysis of variance (ANOVA) followed by Tukey's range test was employed and the differences between individual mean values were deemed to be significant at $p < 0.01$ and $p < 0.05$.

Results and discussion

Determination of total polyphenol content

The results of the extracts obtained from different parts of the plant using three solvents (methanol 80%, ethanol 70%, and boiling water) are presented in Table 1.Results revealed that methanol was better than the other solvents in extracting phenolic compounds from plant materials due to their polarities and good solubility (24, 25). It can be noticed that the highest extract yield was obtained by using methanol 80% extraction of all parts of plant, followed by ethanol 70% and water. The total phenolic content of both methanol and ethanol were: 124.1mg and 93.53 mg GAE/g DW for leaf

extract; 45.5 mg and 41.8 mg GAE/g DW for fruit and 12.3 and 8.1 for stem extract. Water efficiency as a solvent was lower than the efficiencies of all other solvents for extracting phenolic compounds. The total phenolic content of the leaves was much higher than that of the fruit and stem with the three extracting solvents.

Comparing to literature data, studies carried out on the leaves of *P. lentiscus* showed in contrast that more phenolics were extracted using water (infusion) than using the other extraction methods (70% ethanol or 100% ethanol).

The total phenolic content varied also according to species and extraction method, ranging between 1.5 and 7.5% when expressed as tannic acid equivalents, or 2-11.5% when expressed as quebracho equivalents (26).

Determination of total flavonoid content

Results of the total flavonoid contents of leaf fruit and stem extracts obtained using different solvents are presented in Table 1. Different solvents showed different amounts of total flavonoids. Significant differences ($p < 0.05$) in the flavonoid content of methanolic, ethanolic and boiling water extracts from leaves were observed, with values of 12.5, 10.26, and 7.31 mg CE/g DW, respectively. Similarly, the flavonoid contents in fruit were markedly higher in the methanolic extract, with a value of 9.2 mg CE/g DW compared to the ethanolic extract at 7.06 mg and the boiling water extract with a value of 6.24 mg CE/g DW. Methanolic (8:2) extract of stem had a higher flavonoid yield than ethanol extract (7:3) and boiling water but the stem extract was much lower in flavonoid and phenolic content compared to leaf and fruit extracts.

Condensed tannins content

As shown in Table 1, proanthocyanidin content exhibited also significant variations depending on the extraction solvents. In contrast to the total polyphenol and flavonoid, highest yield condensed tannins was observed with the polar solvent (boiling water) with values 10.10, 8.62 and 1.75 mg CE/g DW for the leaves, fruit and stems respectively. However, the lower yields were obtained with ethanol (7: 3) giving 5.95 mg, 4.10 and 0.88 mg CE/g DW for leaves, fruit and stem respectively. These results are in agreement with those of other studies (27) showing that aqueous fraction of *P. lentiscus* leaves was more important than other solvent extract reaching 997.8 mg Eq Tannic acid/g extract. While these results are far from ours, the differences can be explained by the type of soil and climate conditions.

Finally, the plant part, the solvent polarity, and the extraction process largely influence the yield of phenolic compound extracted from plants (28, 29). This is also true for the different parts of *P. lentiscus*, in which the different values obtained between leaves, stems and fruit was important, suggesting that plant part was more important on phenolic content than other endogenous factors notably developmental stage (30), where the trend of total phenolic, flavonoid and tannins compound in the leaves were much higher than in the fruit as well as the stem part of *P. lentiscus*.

Identification of phenolic compounds

Pistacia lentiscus are known to contain a range of secondary metabolites, such as terpenoids and flavonoids. Therefore qualitative analysis of the three investigated methanolic plant extracts made using high performance liquid chromatographic (RP- HPLC) as described in the experimental part is presented in Table 2.

As it is shown in Table 2, *P. lentiscus* was rich in both phenolic acids and flavonoids in the three parts of plant (leaf, fruit and stem). In fact, the two classes were present at 91.92% and 40.68%, respectively for leaf and 48.97% and 16.12% respectively for fruit extract and the phenolic composition of stem extract was dominated by the phenolic acids representing 86.73% of the total compounds. Flavonoids were present at a moderate level, about 10% of the total compounds. Moreover, the leaf extract seemed to be richer in phenolic compounds than fruit and stem extracts (Figure1). In fact 14 phenolic compounds were identified including eight phenolic acids (tannic acid, gallic acid, digalloyl quinic acid derivative, chlorogenic acid, cafeic acid, vanillic acid, p-coumaric acid, ferulic acid, 3,4-dihydroxybenzoic acid and trans-cinnamic acid) and six flavonoids (catechin hydrate, epicatechin, taxifolin, quercetin, rutin hydrate and myricetin). Besides, *P. lentiscus* collected in Israel revealed the presence of gallic acid, cathechin, digalloyl quinic acid, chlorogenic acid and rutin (26). Another study reported that several phenolic compounds, non-

Table1. Phenolic content (total polyphenol content, flavonoid and condensed tannin) of different *P. lentiscus* leaf, fruit and stem extracts.

Extract	Total phenolic content (mg GAE/g DW)	Total flavonoids content (mg CE/g DW)	Condensed tannins content (mg CE/g DW)
Methanol / water(8 :2)			
Leaf	$124.1 \pm 2.64^{*}$	$12.5 \pm 1.01^{*d}$	$8.88 \pm 2.23^{*g}$
Fruit	$45.53 \pm 1.66^{*a}$	$9.2 \pm 1.19^{*}$	$6.17 \pm 1.46^{*g\,j}$
Stem	$12.3 \pm 2.30^{*b}$	$3.48 \pm 0.33^{*e\,f}$	$1.03 \pm 0.56^{*i\,l}$
Ethanol / water (7 :3)			
Leaf	$93.53 \pm 1.68^{*}$	$10.26 \pm 0.90^{*d}$	$5.95 \pm 1.28^{*h}$
Fruit	$41.8 \pm 1.03^{*a}$	$7.06 \pm 0.46^{*}$	$4.10 \pm 0.92^{h\,i\,j}$
Stem	$8.1 \pm 1.15^{*b\,c}$	$2.11 \pm 0.57^{*e}$	$0.88 \pm 0.70^{*i\,l}$
Boiling water			
Leaf	$82.3 \pm 3.10^{*}$	$7.31 \pm 0.6^{*}$	$10.10 \pm 1.53^{*k}$
Fruit	$31.25 \pm 2.74^{*}$	$6.24 \pm 0.55^{*}$	$8.62 \pm 2.23^{*k}$
Stem	$5.05 \pm 2.72^{*c}$	$1.53 \pm 0.40^{*f}$	$1.75 \pm 0.73^{*l}$

Means (three replicates) followed by at least.*$p < 0.01$ are significantly different. One same letter is not significantly different at $p > 0.05$.

Table 2. Major phenolic compounds (% of total) identified in leaf, fruit and stem methanolic extract of *P. lentiscus* by RP- HPLC.

Compounds	Approximate RT(min)	% of the total		
		Leaf	Fruit	Stem
Phenolic acids		**91.92 ± 1.5**	**48.97 ± 0.5**	**86.73 ± 1.94**
Tannic acid	2.37	13.12 ± 1.34	5.93 ± 0.7	11.98 ± 1.83
Gallic acid	2.56	9.4 ± 0.27	8.88 ± 0.48	8.18 ± 0.83
Digalloyl quinic acid derivative	2.95	45.11 ± 0.71	13.50 ± 0.53	61.38 ± 1.34
Chlorogenic acid	5.11	1.07 ± 0.48	--	--
Cafeic acid	5.96	1.86 ± 0.15	--	--
Vanillic acid	9.66	9.95 ± 0.43	4.31 ± 0.5	--
p-coumaric acid	10.37	12.14 ± 1.28	5.92 ± 0.36	--
Ferulic acid	11.13	6.46 ± 0.84	3.24 ± 0.24	--
3,4-dihydroxybenzoic acid	4.16	--	--	2.86 ± 0.27
Trans-cinnamic acid	14.42	--	--	2.33 ± 0.19
Flavonoids		**40.68 ± 2.05**	**16.12 ± 0.65**	**9.34 ± 0.5**
Catechin hydrate	3.86	9.45 ± 0.85	3.28 ± 0.24	1.63 ± 0.91
Epicatechin	5.18	3.97 ± 0.56	--	--
Taxifolin	10.85	15.10 ± 2.01	4.95 ±0.46	--
Quercetin	10.66	10.48 ± 1.88	5.10 ± 0.19	--
Rutin hydrate	11.98	1.37 ± 0.23	2.05 ± 0.56	--
Myricetin	12.7	0.31 ± 0.01	0.74 ± 0.04	5.56 ± 0.16
Chalcone	18.82	--	--	2.15 ± 0.47

Values (means of three replicates ± SD).

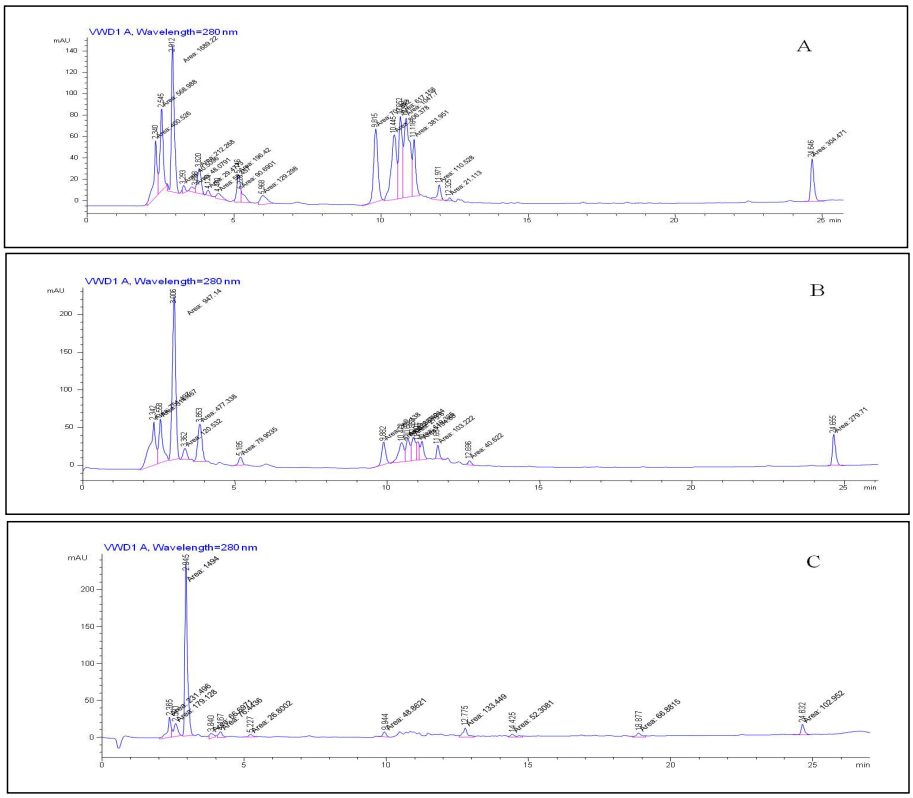

Figure1. HPLC Chromatographic profiles of phenolic acids and flavonoids in the methanolic extract of *P. lentiscus* leaf (A), fruit (B) and stem (C) monitored at 280 nm.

identified in our samples, such as benzoic acid, luteolin, kaempferol, orientin, isorhamnetin, vitexin, luteolin-7-O-glucoside and Quercetin 7-O-glucoside were presented (31).

The HPLC chromatograms of methanolic extract presented in Figure 1, shows that *P. lentiscus* extract contained 17 compounds including: tannic acid, gallic acid, digalloyl quinic acid derivative, catechin hydrate and myricetin wich are present in the leaves, fruit and stems with different percentages. Indeed, digalloyl quinic acid derivative was detected to be the major phenolic component in the three plant parts (leaf, fruit and

stem), contributing to about 60% of the total amount and showing the percentage of 61.38%, 13.5% and 45.11% in stem, fruit and leaf, respectively. It is reported that *P. lentiscus* leaf is a rich source of polyphenol compounds (7/5% of leaf dry weight) especially galloyl derivatives like mono, di, and tri-*O*-galloyl quinic acid and monogalloyl glucose (32). In addition the galloyl derivatives were highly efficient antioxidants using the biologically relevant model of chemically induced LDL oxidation and showed a close similarity with the ability to scavenge hydroxyl radicals (33).

In this article, we report for the first time, the identification of polyphenols in the stems of *P. lentiscus* identified using the RP-HPLC technique. Our results show the presence of several compounds with known antioxidant properties and that 3, 4-dihydroxybenzoic acid, Trans-cinnamic acid and chalcone are only detected only in stems. On the other hand, digalloyl quinic acid derivative and myricetin are predominant in stems with percentages 61.38% and 5.56 % respectively, in comparison with the extract of the leaves and fruit.

According to the research previously done on the phytochemicals of *P. lentiscus* the flavonoids, phenolic acids and their derivatives were the most abundant compounds in leaves, those with the highest concentrations being myricetin glycoside (6216.13 mg/kg of plant), catechin (3354.78 mg/kg of plant), β-glucogallin (2214.461 mg/kg of plant), and quercitrin gallate (1160 mg/kg of plant) (34).

Antioxidant ability assays

Total antioxidant capacity

Phosphomolybdenum assay is based on the reduction of Phosphate-Mo (VI) to Phosphate Mo (V) by the sample and subsequent formation of a bluish green colored phosphate/Mo (V) complex at acid pH. In phosphomolybdenum assay, all the extracts exhibited different degrees of activity as shown in Table 3.

Results indicated that leaf extract has higher antioxidant capacity (113.23 ± 1.08 mg gallic acid equivalent/g dw) than other extracts which showed antioxidant capacity of 39.81 (± 0.91 mg GAE/g dw) in fruit extract and 13.03 (± 0.77 mg GAE/g dw) in stem extract.

Phosphomolybdenum assay measures the reduction degree of Mo (VI) to Mo (V). It is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It involves in thermally generating auto-oxidation during prolonged incubation period at higher temperature. It gives a direct estimation of reducing the capacity of antioxidant (35).

Reducing power assay

In reducing power assay, the presence of reductants (antioxidants) in samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron which serves as a significant reflection of antioxidant activity (36).

All the extracts showed some degree of electron donating capacity and reduced Fe^{3+} to Fe^{2+}. The reducing ability of the extracts was 35.42 ± 1.61, 15.32 ± 0.87 and 6.03 ± 0.68 AAE mg/g dw in leaf, fruit and stem respectively. The ferric reducing antioxidant power of the leaf extract showed higher activity compared to that obtained with the other extract. This could be due to the presence of higher total phenolics and flavonoids, which plays a major role in reducing power activity. This suggests that leaf extract has a significant ability to react with free radicals to alter them into more stable non-reactive species and to terminate radical chain reaction. The ferric reducing power of bioactive compounds was associated with antioxidant activity (37). It was reported that the plants with the highest levels of total phenolics and flavonoids exhibited greater reducing power activity (38).

DPPH· radical scavenging activity

In order to evaluate the *in vitro* anti-oxidant activities of *P. lentiscus* extract, the DPPH radical scavenging activity of the leaf, fruit and stem extracts were measured and compared to that of BHT (IC_{50} = 8.5 µg/ml) The results were summarized in Table 3.

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow.

As can be seen, the leaf extract was able to scavenge the free radicals. They exhibited antioxidant potential comparable to BHT with IC_{50} = 2.14 ± 0.19 µg/ml. This value is significantly lower than that standard (IC_{50} = 8.5 µg/ml). Compared with positive control, the fruit and stem extracts showed the best scavenging activities with IC_{50} = 23.01 ± 2.54 and 108.24 ± 2.19 µg/ml respectively. In fact, this result suggests that leaves of *P. lentiscus* possess phenolic compounds that have a high potential to neutralize free radicals.

On the other hand, it was observed that the radical scavenging activity is increasing with the increase of phenolic content (figure 2). The methanol extract of leaves, at 10 µg/ml, showed the highest scavenging activity (96.17%), but the value was much higher than that of BHT at 10 µg/ml (58.73%). In the case of DPPH scavenging ability assay, it was found that methanolic extract of *P. lentiscus* leaves reached 76.4% inhibition at 200 µg/ml compared with α-tocopherol (39).

Table 3. Antioxidant activities of different extracts of *P. lentiscus*.

Extract	Total antioxidant capacity[a]	Ferric reducing antioxidant power[b]	DPPH IC_{50} (µg/ml)
Leaf	113.23 ± 1.08*	35.42 ± 1.61*	2.14 ± 0.19*
Fruit	39.81 ± 0.91*	15.32 ± 0.87*	23.01 ± 2.54*
Stem	13.03 ± 0.77*	6.03 ± 0.68*	108.24 ± 2.19*
BHT	n.d	n.d	8.5 ± 0.8*

a: gallic acid equivalent mg/g dw; b: ascorbic acid equivalent mg/g dw plant material. n.d: not determined. Data are expressed as means S.D of triplicate measurements. Differences at *p <0.01 was considered statistically significant.

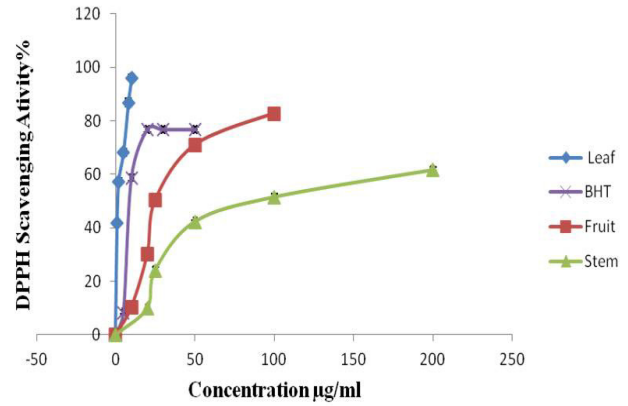

Figure 2. DPPH radical scavenging activities of the various extracts of *Pistacia lentiscus* and BHT. Result represents means of triplicates of different concentrations analyzed.

Our RP-HPLC analysis showed that the phenolic composition of the stem extract was characterized by the presence of a high level of digalloyl derivative quinic acid (61%), however, this extract had a low antioxidant activity. At 200 mg/ml the inhibition reached 60%, despite galloylquinic derivatives is known for its strong antioxidant activity but the stem extract has a low antioxidant activity (33, 40). Nevertheless, strong leaf extract may be explained by the presence of phenolic compounds having an important antioxidant capacity like gallic acid, digalloyl quinic acid, catechin and quercetin (41), this trapping activity may be also suggested by the synergistic effect of polyphenols. Furthermore, compared to the fruit and stem extracts, it is apparent that leaf extracts are more potent, with a higher level proportion of inhibition.

However, a positive correlation between phenolic composition and antioxidant activity was proved (42). In this work and in related references it was observed that the leaf extract reported also a high concentration between DPPH radical scavenging potential and total polyphenol content. Antioxidant activity of methanol extract was probably due to the presence of flavonoids and phenolic compounds which are present in the extract (43). Based on the data obtained from this study, leaf extract of *P. lentiscus* is a powerful free radical inhibitor which may limit free radical damage occurring in the human body.

Evaluation of cytotoxicity of *P. lentiscus* extracts

All extracts from the leaves, fruit and stem were subjected to cytotoxic screening against CaCo2 and AGS cancer cells and the IC_{50} values obtained are shown in Table 4. Among the three solvent extracts, methanol (8:2) extract exerted the most potent cytotoxicity. Methanol (8:2) extracts of the leaf showed high cytotoxic activity towards CaCo2 and AGS cell lines with IC_{50} values less than 250 µg/ ml. while ethanol and water extracts displayed low cytotoxicity with IC_{50} values higher than 400 µg/ml. Interestingly, the highest toxicity was observed for methanol (8:2) leaf extract in both cancer cell lines indicating the synergistic effect of compounds present in the whole extract. After 24 hours of treatment, the IC_{50} values for methanol (8:2) leaf extract were found to be 135.67 ± 2.5 and 250.45 ± 1.96 µg/ml in CaCo2 and AGS cells respectively. In

Table 4. Cytotoxity activity of *P. lentiscus* extracts, expressed as IC_{50} values (µg/ml).

Extracts	CaCo2	AGS
Methanol 8:2		
Leaf	135,67 ± 2,66^{a,e}	250,45 ± 1,96^{a,e}
Fruit	403,84 ± 3,35^{a}	545,57 ± 0,9^{a}
Stem	886,95 ± 3,5^{b}	>1000
Ethanol 7:3		
Leaf	341,33 ± 0,98^{a,c}	456,48 ± 1,65^{a,c}
Fruit	665,25 ± 2,8^{c}	715,32 ± 3,73^{c}
Stem	>1000	>1000
Infusion		
Leaf	722,33 ± 1.63^{a,b}	887,87 ± 2.2^{a,b}
Fruit	902,01 ± 3,6^{d,c}	989,58 ± 2,3^{d,c}
Stem	>1000	>1000
Doxorubicin (µM)	15.08 ± 2.22^{e}	22.36 ± 1.5^{e}

Doxorubicin was used as positive control. All the values are expressed as mean ± SEM (n = 3).$^{a-e}$ Column wise values with different superscripts of this type indicate significant difference ($P < 0.05$).

contrast, methanol (8:2) and ethanol (7:3) extracts of the fruit displayed a moderate activities towards these cell lines. Since the methanol extract showed an IC_{50} value > 400 µg/ml. The cytotoxicity leaf extract against AGS and CaCo2 cells was significantly higher ($p < 0.05$) than the other extracts. In addition, infusion of the fruit and leaves displayed very low activity with IC_{50} values more than 800 µg/ml. Similarly, all extracts of the stem were not active with strong IC_{50} values being more than 1000 µg/ml.

An important implication of these findings is that, a 24-hours exposure to the leaf extract reduces effectively tumor cell viability with important IC_{50} values. In contrast, the fruit and stem extracts were not cytotoxic against the human gastric carcinoma and human colon adenocarcinoma cells lines.

In another study the MTT assay suggested that both CMG and taxol inhibited the proliferation of YD-10B cells in a time and dose dependent manner. Moreover, 10µg/ml of CMG and 50µg/ml of taxol caused fragmentation of the genomic DNA at 24 hours (44).

In a recent paper it has been demonstrated that *P. lentiscus* leaf extract exerted dose and time dependent inhibitions towards two human neuroblastoma cell lines (SK-N-BE(2)C and SH-SY5Y) (45).In fact from 24 h exposure time, the extract showed activity on the SK-N-BE(2)C cell line with an IC_{50} value of 100.4 ± 1.6 µg/ml and IC_{50} value of 56.4 ± 1.1 µg/ml with SH-SY5Y cell line. However, the IC_{50} value will appear increasingly low after 48 and 72 hours.

From the research that has been carried out it is possible to conclude that among the different solvents used, methanol/water (8:2) extract possess significant amounts of polyphenolic contents, flavonoids and condensed tannin. Additionally ten phenolic acids and seven flavonoids were identified, and digalloyl quinic acid derivative was the dominant phenolic compound in leaves, fruit and stem. Indeed leaves showed significant antioxidant and radical scavenging activity as compared to fruit and stem, which may be accounted for the high polyphenolic content. The results obtained indicate that these plant extracts have a very appreciable antiprolif-

erative activity. It has been demonstrates that *P. lentiscus* leaf extracts potentially inhibited the proliferation of human gastric carcinoma and human colon adenocarcinoma cells. However the identified polyphenols, the important anticancer activity and the high antioxidant activity of *P. lentiscus* proved in this work help to develop and enhance scale of the biological properties of this plant. This, therefore, suggested that *P. lentiscus* can be used as an easily accessible and effective source of natural antioxidants and chemopreventive agents.

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Conflict of interest
The authors declare that there is no conflict of interest.

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