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Separation and identification of phenolics and flavonoids from wild *Pistacia palaestina* extract and its antioxidant activity

Saleh Abu-Lafi[1], Fuad Al-Rimawi[2]*, Jehad Abbadi[3], Saleh. A. Naser[4] and Khaled Qabaha[5]

[1]Faculty of Pharmacy, Al-Quds University, P. O. Box 20002, Jerusalem, Palestine.
[2]Chemistry Department, Faculty of Science and Technology, Al-Quds University, P.O. Box 20002, Jerusalem, Palestine.
[3]Biology Department, Faculty of Science and Technology, Al-Quds University, P.O. Box 20002, Jerusalem, Palestine.
[4]Division of Molecular Microbiology, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, Florida.
[5]Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Arab American University, Jenin, Palestine.

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An *in-vitro* evaluation of the antioxidant activities of wild Palestinian *Pistacia palaestina* extracts was done. In parallel, the total phenolic content (TPC) and the total flavonoids content (TFC) were measured. The antioxidant activities were determined spectrophotometrically by DPPH, FRAP, CUPRAC and the ABTS methods. The phenolic and flavonoid contents were separated and identified using LC-PDA-MS. The *P. palaestina* extract was found to contain many phenolic and flavonoids that enhance its reducing activity and free radical scavenging ability. Total phenolic content, and total flavonoids contents were found to be 66.5 ± 2.2 mg Gallic acid/g, and 20.3 ± 1.1 mg catechin/g, respectively. Antioxidant activity represented as FRAP, CUPRAC, DPPH and ABTS was found to be 23.5± 1.2 mmol Fe^{+2}/g, 4562 ± 63 µmol Trolox/g, 344 ± 11 µmol/g, 53.1 ± 6.6 µmol/g, respectively. The aim of the study is therefore to employ different antioxidant tests to evaluate the antioxidant activities of crude ethanol leaf extracts of *P. palaestina,* and to determine its phenolic and flavonoids content.

Key words: *Pistacia palaestina,* flavonoids, antioxidants, Liquid chromatography–mass spectrometry (LC-MS).

INTRODUCTION

Humans face different environmental stressors that increase the release of reactive oxygen species (ROS) in the body (Pryor and Stone, 1993; Flora, 2007). These stress conditions include ultraviolet (UV) radiation, microorganisms of different allergens, rise in ozone, cigarette smoke, and polycyclic aromatic hydrocarbons (Pryor and Stone, 1993). Free radicals are non-stable compounds rich with energy and have electrons that do not form pairs in their external atomic orbitals. They are formed by removing or adding an electron from a non-radical atom or molecule (Flora, 2007). Radicals are very reactive because they have reductive or oxidizing effects in the body by giving or taking electrons to or from other molecules. ROS forms most of the free radicals which are known by an oxygen atom with an unshared electron in the external orbit (Valko et al., 2007). Moreover, free

*Corresponding author. E-mail: falrimawi@staff.alquds.edu.

oxygen radicals initiate other free radical-forming chain reactions with a non-radical or oxygen-centered free radical unpaired electron. This includes superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), alkoxyl radical ($RO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$), or ROS; the free oxygen intermediate product carrier metabolite then oxidizes other compounds and initiates free radical-forming chain reactions leading to new free radicals (Pham-Huy et al., 2008).

Biological systems are known to possess antioxidant defense system based on enzymatic and non-enzymatic mechanism (Valko et al., 2007). Exogenous antioxidant sources include vitamin C and E, carotenoids, and of course polyphenols. They are required to achieve homeostasis to reflect on maintaining balance between oxidant/antioxidant ratio. This becomes essential since our endogenous antioxidant system is not sufficient to neutralize ROS action during mitochondrial respiration system and normal cellular metabolism by depending on molecular oxygen such as NADPH oxidase and xanthine oxidase. On the contrary, high doses of antioxidants often have pro-oxidant activity by enhancing oxidative damage (Ullah et al., 2013).

Natural products derived from plants play a crucial role in the development of several clinically useful anticancer drugs (Yemmen et al., 2017). Exogenous antioxidants have been confirmed to be essential for counteracting oxidative stress (Yemmen et al., 2017). These phyto-antioxidants include phenolic compounds such as flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids (Laguerre et al., 2007).

Polyphenols occur naturally in plants and are of great interest for application in food chemistry, pharmacy and medicine because of their biological effects benefit (Cook et al., 1996). The antioxidant activity of phenolics is mainly due to their redox properties, by acting as reducing agents (or free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelators (Cook et al., 1996). Flavonoids consist of four main classes including namely, flavones, flavanones, catechins, and anthocyanins and they are classified based on their molecular structure (Rice-Evans et al., 1996). They are well known for their active antioxidants capability. Specifically flavones and catechins are the most active flavonoids against ROS (de Groot, 1994; Grace, 1994).

Alternative medicine has been used in many countries to treat many illnesses and alleviate pain. This type of medicine depends mainly on the medicinal plants which are well known sources of bioactive compounds that have many pharmacological and medical benefits (Sandoval et al., 2002).

Pistacia palaestina is an evergreen tree belonging to the *Anacardiaceae* family. It is found mostly in the Mediterranean area and characterized by poor content of nutrient and water and tolerance to long-term exposure of extensive solar radiation and elevated temperatures (Margaris, 1981). Its mastic has been utilized in folk medicine since the time of the ancient Greeks (Ljubuncic et al., 2005). The aerial part of this plant is known to contain diuretic properties and has been used to treat hypertension, coughs, sore throats, eczema, stomach aches, kidney stones and jaundice (Ljubuncic et al., 2005; Bentley and Trimen, 1980; Ljubuncic et al., 2005; Khedir et al., 2017; Trabelsi et al, 2012).

P. palaestina (also named known as mastaki) has antimicrobial, antioxidant, antifungal, and anti-inflammatory activities (Sofowora, 1982; Paraschos et al., 2012; Orhan et al., 2006). It has good anti *Helicobacter pylori* effect which helps in curing many gastrointestinal diseases (Orhan et al., 2006; Dabos et al., 2010). *P. palaestina* methanolic extract was documented for its antimicrobial activity on *Escherichia coli* bacteria; such activity was found to be dose-dependent. Traditionally, *P. palaestina* has been used as an anti-cancer agent against prostatic, colon, breast, liver, stomach, spleen, and uterus cancers. Furthermore, it helps in the protection of the blood vessels as well as the liver by inhibiting the Low-density lipoproteins LDL oxidation (Rahimi et al., 2009). Thyroid cancer cells were found to be sensitive to *P. palaestina* essential oil and showed a remarkable effect as antiproliferative agent while no effect was shown on normal fibroblasts. The oil was extracted from its mastic gum, twigs, leaves, flowers, and berries (Catalani et al., 2017; Mezni et al., 2016). Antiproliferative activity of the *P. palaestina* oil was shown to be through in suppressing the NF-κB signal pathway and NF-κB activity and inhibited the cell cycle at the G1 phase (Giaginis and Theocharis, 2011). Therefore the aim of the present study is to employ different antioxidant tests to evaluate the antioxidant activities of crude ethanol leaf extracts of this plant.

MATERIALS AND METHODS

Plant materials

Plant material and extraction

Wild *P. palaestina leaves* were obtained from Jenin and dried in the shade at room temperature for three weeks then grinded to powder. Fifty grams of the powder was mixed with 500 ml of 96% Ethanol and kept on a shaker for five days at room temperature. The extract was filtered using Whatman blue ribbon No. 41 filter paper. The filtrate was completely dried under vacuum using rotary evaporator (BUCHI Brand) at 50°C. The filtrate was covered to prevent atmospheric contamination. A solution of 3 mg/ml concentration was prepared and filtered through 0.2 μm PVDF membrane filters prior to injection to the LC-PDA-MS

Chemicals and reagents

This study is a follow up to our earlier publication which lists in details all chemicals and reagents including the sources we used

(Al-Rimawi et al., 2017). All the chemicals and reagents were verified to be of analytical grade. Formic acid and acetonitrile and water solvents were of an LC-MS grade from Sigma.

Reagents preparation

Ferric reducing ability FRAP reagent was prepared following instructions from Benzie and Strain (1999). Briefly, 2.5 ml of a 10 mM tripydyltriazine (TPTZ) solution was added to 40 mM HCl and 2.5 ml of 20 mM $FeCl_3.6H_2O$. This was followed by the addition of 25 ml of 0.3 M acetate buffer at pH 3.6. Acetate buffer (0.3 M) was prepared following the manufacturer's instructions by dissolving 16.8 g of acetic acid and 0.8g of sodium hydroxide in 1000 ml of distilled water.

Chromatographic instrumentation

According to the manufacturer's description, we used HPLC system which is Waters Alliance (e2695 separations module), and it is equipped with 2998 Photo diode Array (PDA). It has a data acquisition and control compartment which was based on Empower 3 chromatography data software (Waters, Germany). Similarly, the analyses were performed on a Waters Acquity UPLC H-Class system (Waters, Milford, MA, USA) which was equipped with a binary solvent manager, sampler manager, column manager, Acquity QDa detector and PDA that was connected to Waters Empower 3 data station. The Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 µm) was equipped with an Acquity BEH C18 1.7 µm guard column (Vanguard 2.1 × 5 mm). The entire HPLC system was purchased from Waters, Milford, MA, USA.

Measurement of antioxidant activity

Ferric reducing/antioxidant power (FRAP) assay

We measured the antioxidant activity in our extracts samples by following our earlier protocol (Al-Rimawi et al., 2017) which is a modified method of the assay of ferric reducing/antioxidant power (FRAP) published originally by Benzie and Strain (1999). Absorbance readings were recorded against at 593 nm and a reagent blank. We calibrated the reader using 2 to 5 mM Fe^{+2} solution and the results were expressed as mmol Fe^{+2}/g.

Cupric reducing antioxidant power (CUPRAC assay)

The cupric ion reducing antioxidant capacity of the extracts was measured following our earlier published protocol (Al-Rimawi et al, 2017) which is a modification of the method of Apak et al. (2008). In this study we measured the antioxidant activity in 100 ul samples extract and the absorbance readings were rerecorded at 450 nm against the reagent blank. Standard curve was prepared using different concentrations of Trolox. The results were expressed as µmol Trolox/g.

Free radical scavenging activity using DPPH (DPPH assay)

We used DPPH assay in this study based on a protocol with no modification that we published earlier (Al-Rimaw et al, 2017). We measured the change in the absorbance readings at 515nm for up to 30 min or until the steady state was reached. The reagent blank contained Methanol (95%). Trolox at different concentrations was used to establish the Standard curve. The results were expressed as µmol Trolox/g.

Free radical scavenging activity using ABTS (2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid)

ABTS (2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid)) procedure was followed with minor changes as described by Re et al. (1999). The working solution of $ABTS^{+\cdot}$ was obtained by diluting the stock solution in 99.9% ethanol to give absorption of 0.70 ± 0.02 at 734 nm. Sample (200 µl) extract was added to 1800 µl of $ABTS^{+\cdot}$ solution and absorbance readings at 734 nm were taken at 30°C exactly 10 min after initial mixing (A). The radical-scavenging activity of the test samples was expressed as Trolox equivalent antioxidant capacity (TEAC) (in µmol Trolox/g sample) as described earlier (Al-Rimaw et al., 2017).

Total phenolic content (Folin-Ciocalteau) assay)

Total phenolics were determined using Folin-Ciocalteau reagents as described in our earlier study (Al-Rimawi et al., 2017). *P. palaestina* plant extracts or gallic acid standard of (40 µl) were used in this study as described in our earlier study. Results were expressed as mg gallic acid equivalents (GAE)/ g sample.

Total flavonoid content

Total flavonoids activity was measured following the colorimetric assay of Kim et al. (2003). The absorbance readings were recorded at 510 nm. Catechin at 50 - 100 mg/L was used for calibration and the final results were expressed as mg catechin equivalents (CEQ)/ g sample.

Chromatographic conditions

The entire HPLC system was purchased from Waters, Milford, MA, USA). The HPLC analytical experiments of the crude ethanol extracts were run on Octadecyl column of Waters (XBridge, 4.6 ID × 150 mm, 5 µm) as described by the manufacturer. The PDA wavelengths range was from 210-500 nm. The flow rate was 1 ml/min. Injection volume was 20 µl and the column temperature was set at 25°C. Samples were filtered through 0.45 µm micro porous disposable filter.

The LC-MS column and sample temperature were maintained at 40 and 25°C respectively. Prior to any injection, the mobile phase was equilibrated for 4 min. The injection volume was 1 µl. The detection range of the PDA was from 210-500 nm. The Acquity QDa ionization modes were in the negative and positive ESi, mass range was between 100-1200 Da, cone voltage 15V, capillary temperature 600˚C, capillary voltage 0.8 KV and sampling rate of 8 points/second was used.

RESULTS

Total phenolic content (TPC) and total flavonoid content (TFC)

P. palaestina extract was found to be rich with phenolic and flavonoids with TPC and TFC of 66.5 and 20.3 mg/g, respectively, as shown in Table 1.

Antioxidant activity

Reducing potential of plant extracts

Ferric reducing-antioxidant power (FRAP) assay: This

Table 1. Total phenolic content (TPC as mg gallic acid/g DW*), total flavonoids contents (TFC as mg catechin/g DW), FRAP (mmol Fe^{+2}/g DW), CUPRAC (µmol Trolox/g DW), DPPH (µmol Trolox/g DW), and ABTS (µmol Trolox/g DW) of *P. palaestina* extract.

TPC** (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)	ABTS (µmol/g)
$66.5^{c} \pm 2.2$	$20.3^{c} \pm 1.1$	$23.5^{c} \pm 1.2$	$4562^{c} \pm 63$	$344^{c} \pm 11$	$53.1^{c} \pm 6.6$

*DW: Dry weight.** Results are expressed as an average of three samples. Different small letters within column indicate significant difference ($p < 0.05$, $n = 3$).

method measures the ability of antioxidants to reduce ferric irons. It is based on the reduction of the yellow complex of ferric irons and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the blue ferrous form by electron-donating substances (such as phenolic compounds) at low pH. This reduction is monitored by measuring the change in absorption at 593 nm. The FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe^{2+}. The antioxidant test based on FRAP assay of the extract was found to be 23.5 ±1.2 mmol Fe^{+2}/g of dry material (Table 1).

Cupric reducing antioxidant power (CUPRAC): The antioxidant test based on CUPRAC assay of the extract was found to be 4562 ±63µmole Trolox /g of dry material (Table 1).

Free radical scavenging ability of plant extracts

Free radical scavenging activity using DPPH

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (31). It is a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of the extracts. As antioxidants donate protons to this radical, the absorption decreases. The antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The color changed from purple to yellow and the absorbance at wavelength 517 nm decreased. The DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The bleaching of DPPH solution increases linearly with increasing amount of extract in a given volume. The DPPH antioxidant activity of the extract was found to be 344±11 µmole Trolox/g (Table 1).

Free radical scavenging activity using ABTS

A modified procedure using ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) was used. The $ABTS^{+\cdot}$ stock solution (7 mM) was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The working solution of $ABTS^{+\cdot}$ was obtained by diluting the stock solution in ethanol to give an absorption of 0.70 ± 0.02 at λ = 734 nm. Sample extract (100 µl) was added to 900 µl of $ABTS^{+\cdot}$ solution and absorbance readings at 734 nm were taken at 30°C exactly 10 min after initial mixing. The ABTS assay measures the relative antioxidant ability of extracts to scavenge the radical-cation $ABTS^{+}$ produced by the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate. ABTS antioxidant activity of *the* extract was found to be 53.1 ±6.6 µmol Trolox/g (Table 1).

HPLC-PDA-MS profiles of P. palaestina extract

The crude extracted ethanolic material of pistacia was analyzed using LC-PDA-MS instrument and the UV absorption was high at 270 nm (Figure 1). Almost all the eluted peaks had two wavelength maxima between 270-274 nm and close to 338-360 nm (Figure 1). Figure 2 shows the chromatograms when overlaid at two wavelengths of 270 and 350 nm. When the chromatogram is monitored at 270 nm, more peaks were seen.

Table 2 shows some of the deprotonated compounds derived from *Pistacia palaestina* total ion chromatograms (Table 2). Besides showing their typical $[M-1]^{-}$ peaks, dimer adducts of $[2M-1]^{-}$ were apparently seen because of the high concentration of the injected sample (Figure 3). Figure 3 shows selected MS of flavonoids eluted at 0.8, 1.2, 1.3, 4, 4.5 and 5.2 min. respectively (3a-f).

DISCUSSION

Medicinal plants are good sources of secondary metabolites and antioxidants such as phenolic compounds (like cinnamic acids, flavonoids, stilbenes, coumarins, lignans, and lignins), ascorbic acid and carotenoids (Ning et al., 2012). Antioxidants quench free radicals and hence prevent cellular damage (Fraige et al., 2018).

Traditionally, *P. palaestina* medicinal plant is used for treating many diseases (Sofowora, 1982; Orhan et al., 2006). It is used as a part of different herbal formulas to treat some types of bacterial infections as well as some types of cancers (Rahimi et al., 2009; Catalani et al., 2017; Mezni et al., 2016). Due to absences of adverse

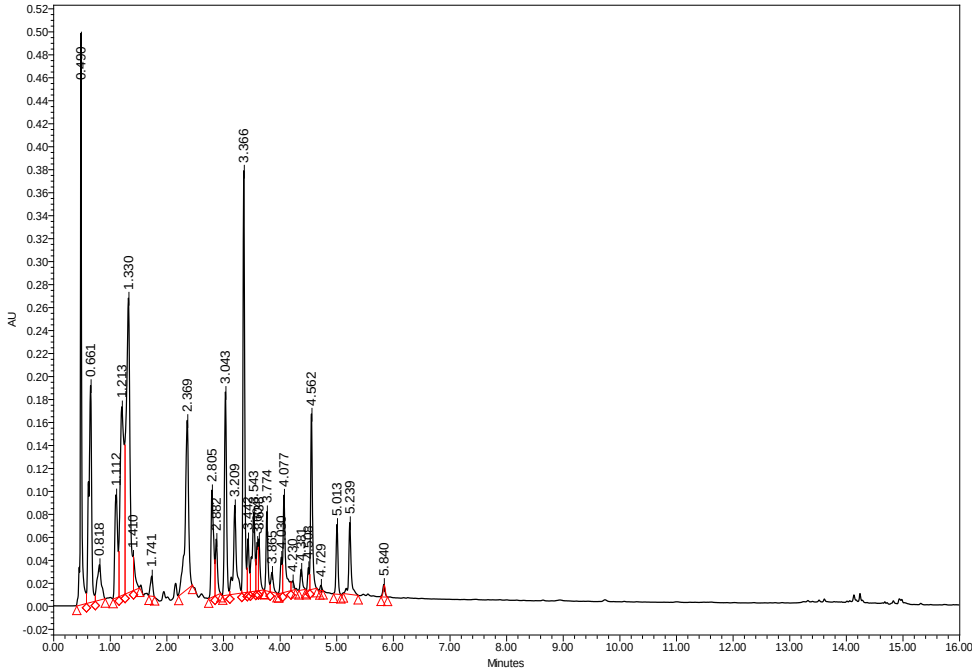

Figure 1. HPLC-PDA chromatograms of crude *P. palaestina* ethanol extract at 270 nm.

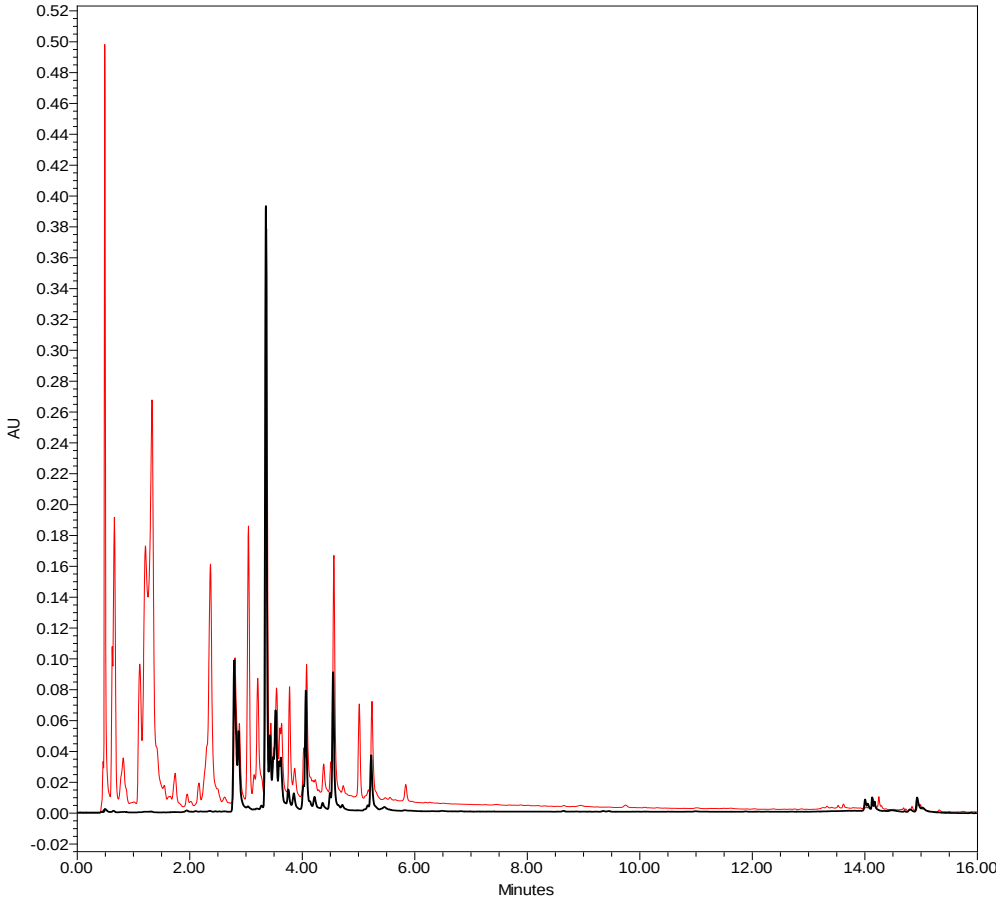

Figure 2. Overlaid HPLC-PDA chromatograms of crude *P. palaestina* ethanol extract at 270 (red) and 350 nm.

Table 2. Some of the identified compounds from ethanol extract of *Pistacia* leaves using UPLC-MS in the negative mode.

Peak #	RT (mins)	$[M-H]^-$	MWt	Preliminary identification
1	0.465	191	192	Quinic acid
2	0.629	343	344	3-Galloylquinic acid
3	0.666	169	170	Gallic acid
4	0.827	325	326	5-Galloylshikimic acid
5	1.19	153	154	Gentisic acid
6	1.329	495	496	digalloyl quinic acid
7	2.172	371	372	(17:2)-anacardic acid
8	2.372	323	324	Unknown
9	3.052	197	198	Unknown
10	3.216	523	524	quinic acid derivative 1
12	3.375	463	464	quercetin 3-O-galactoside
13	4.085	447	448	kaempferol hexoside
14	4.395	349	350	Unknown
15	4.579	615	616	Quercetin 3-(2-galloylglucoside)
16	4.896	949	950	Unknown
17	5.023	349	350	Unknown
18	5.249	599	600	quercetin-O-(O-galloyl)-hexoside
19	5.848	501	502	Unknown
20	6.985	327	328	Unknown
21	7.584	329	330	Unknown
22	12.042	265	266	Unknown

effects and good biological activities such as anti-inflammatory, antimicrobial, and antioxidant activities, its application has increased. The results showed that the *P. palaestina* extracts investigated in this study are rich in phenolic compounds and flavonoids. These findings make this herbal plant an excellent source for pharmacological use. The antioxidant activity of *P. palaestina* extract might be attributed to its polyphenols content. These natural substances have been proved to possess different biological activities (Fraige et al., 2015).

Until now, there is no antioxidant activity and polyphenolic content analysis of *P. palaestina* from Palestine and this motivated us to give more insight into the antioxidant and polyphenolic content of this plant. Ethanol was used in this work to extract plant materials proved to be an effective extraction solvent.

P. palaestina has strong antioxidant activity represented by both acting as reducing agent and as free radical scavengers. These results were observed using two types of typical assays used to evaluate antioxidant activity of plant extracts. The first category measured the potential of plant extracts to reduce ions or oxidants (by acting as reducing agents) like ferric ion, cupric ion. The main two assays of this category are FRAP (which measures the reduction potential of ferric to ferrous ion), and CUPRAC (which measures the reduction of cupric to cuprous ions). Although FRAP antioxidant assay has been very popular among researchers, CUPRAC assay is a relatively new assay (Apak et al., 2008). It utilizes the copper (II)–neocuproine [Cu(II)–Nc] reagent as the chromogenic oxidizing agent and is based on the cupric reducing ability of reducing compounds to cuprous.

The second category of AA measures the ability of the plant extracts to scavenge free radicals. The DPPH and ABTS assays (where DPPH and ABTS are stable free radicals) are the two main examples of this category. These assays are quick and simple, and their reactions are reproducible and linearly related to the concentration of the antioxidant(s) present. DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Sakanaka et al., 2005). It is a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The color changed from purple to yellow and the absorbance at wavelength 517 nm decreased. DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The bleaching of DPPH solution increases linearly with increasing amount of extract in a given volume.

The UPLC-MS in the negative mode of *Pistacia palaestina* showed various phytochemicals as phenolics,

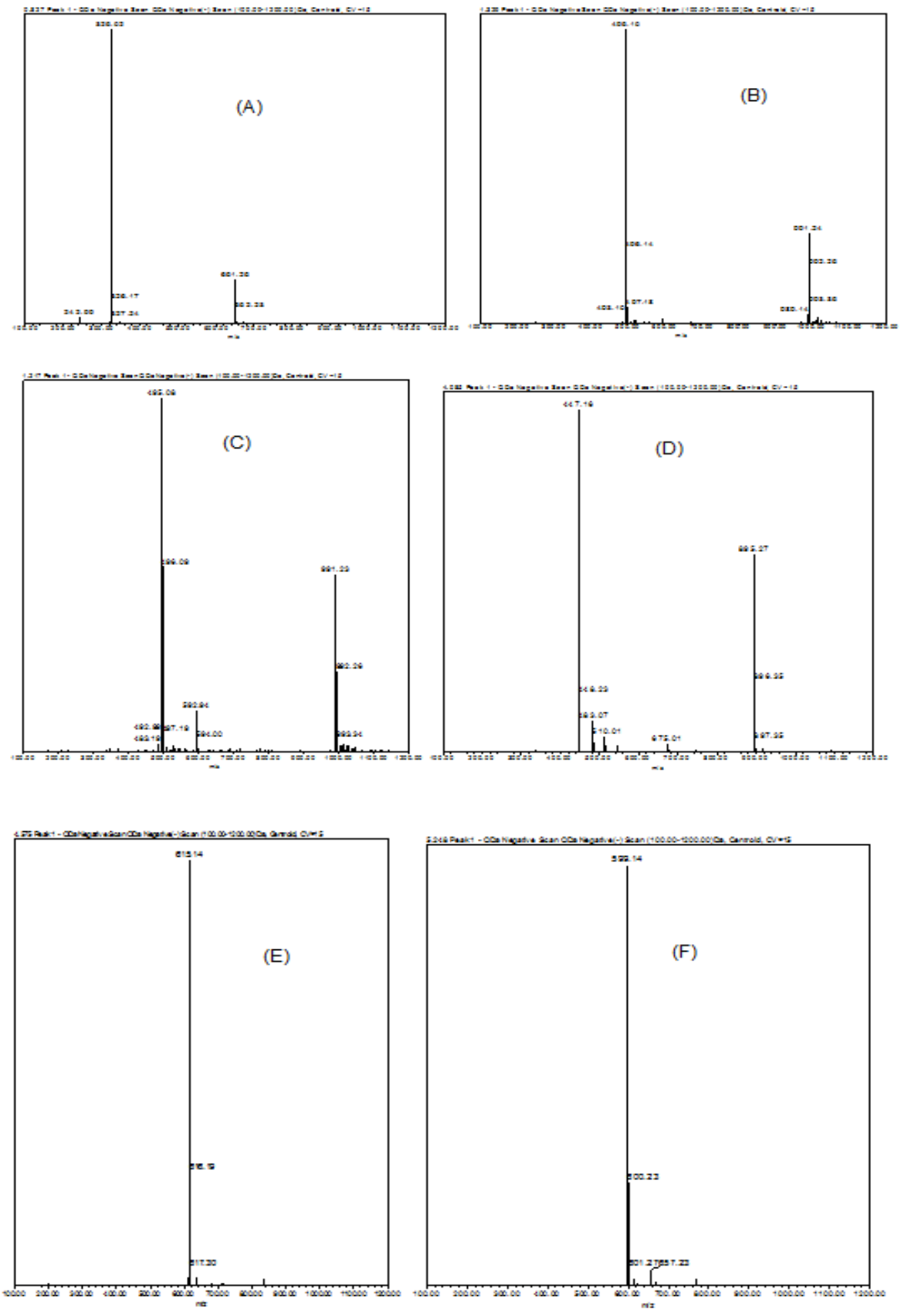

Figure 3 (a-f). -ESi-MS spectra of flavonoids eluted at 0.8, 1.2, 1.3, 4, 4.5 and 5.2 min respectively.

flavonoids and flavonoid glycosides (Al-Rimawi et al., 2017). This is in agreement with the study of Jouki and Khazaei (2010) where different phenolic compounds were detected e.g. gallic, chlorogenic, ellagic, sinapic and protocatechuic acid, (+)-catechin and juglone (Jouki and Khazaei, 2010). The PDA was used to monitor wavelengths from 220-360 nm. All the main peaks shared maximum wavelengths near 270 nm and close to 338-360 nm showing glucosides flavonoids.

Conclusions

P. palaestina extract is rich in phenolic and flavonoids contents and presented an excellent DPPH and ABTS radical scavenging activities. It also showed excellent reducing abilities as represented by FRAP and CUPRAC assays. This activity may be attributed to its polyphenolic compounds and flavonoids. The question now is: 'What should be done with the plant or its extracts that are ich in PP and flavonoids?'

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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