

ORIGINAL ARTICLE

In vitro antioxidant and antitumor activities of six selected plants used in the Traditional Arabic Palestinian herbal medicine

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Abstract

Context: Despite several pharmacological applications of the medicinal plants in the Traditional Arabic Palestinian Herbal Medicine in Palestine (TAPHM), studies on their antioxidant properties are still scarce.

Objective: This work evaluates the antioxidant and antitumor activities of the ethanol extracts from different parts of six plants: [*Arum palaestinum* Boiss (Araceae), *Urtica pilulifera* L. (Urticaceae), *Coridothymus capitatus* (L.) Reichb (Lamiaceae), *Majorana syriaca* (L.) Rafin. (Lamiaceae), *Teucrium creticum* L. (Lamiaceae), and *Teucrium capitatum* L. (Lamiaceae)] used in the TAPHM.

Materials and methods: The antioxidant activity was evaluated for the ethanol extracts by DPPH and β -carotene–linoleic acid assays together with total contents of phenols and flavonoids. For the anti-carcinogenic evaluation, the extracts were tested for the ability to inhibit the proliferation of breast cancer cells (MCF-7) using the MTT reduction assay.

Results: Among the extracts, the *U. pilulifera* had the highest amount of total phenolics, possessing the second highest total flavonoids. It also showed a maximum cytotoxic activity ($IC_{50} = 63 \mu\text{g/ml}$), followed by *C. capitatus*, and *A. palaestinum*. Otherwise, the extract of *T. creticum* was demonstrated to be an efficient scavenger of O_2 ($IC_{50} = 83 \mu\text{g/ml}$), followed by *M. syriaca*, *C. capitatus*, *T. capitatum*, *A. palaestinum*, and *U. pilulifera*.

Discussion and conclusion: The results suggest that the investigated plants have shown varied antioxidant capacities which were strongly correlated with their contents of phenolics. Accordingly, this study proposes that the therapeutic benefit of these plants can be, at least in part, attributed to its potential inhibition of oxidative processes.

Keywords

Anti-scavenging activity, Araceae, folk medicine, Lamiaceae, Mediterranean flora, reactive oxygen species (ROS), total flavonoids, total phenols

History

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Introduction

Plant-derived therapeutics are important resources, especially in developing countries to combat human ailments and diseases. About 60–80% of the world's population still relies on traditional medicine for the treatment of common illnesses (WHO, 2003). Traditional remedies have a long-standing history in Palestine and continue to provide useful means for treating ailments (Ali-Shtayeh & Jamous, 2006; Ali-Shtayeh et al., 2000, 2008). Nevertheless, in the literature, little work has been carried out to investigate the plants of Palestine used in the Traditional Arabic Palestinian Herbal Medicine (TAPHM) (Ali-Shtayeh & Jamous, 2008; Ali-Shtayeh et al., 1997, 1998, 2003; Saad et al., 2005).

In the course of our investigations, we found that several plants of the Palestinian ethnomedicine possess promising biological activities, which could be of interest in the field of

folk medicine in the Mediterranean region, besides to other parts of the world (Abu-Lafi et al., 2007).

Over the last two decades, interest in the antioxidant activity of plant extracts has increased considerably (Lin & Huang, 2002; Ranjbar et al., 2006) due to the fact that free radicals, e.g., reactive oxygen species (ROS), can be responsible for various human chronic diseases, e.g. heart diseases, stroke, arteriosclerosis, and cancer, as well as the aging process (De La Fuente & Victor, 2007; Ruiz-Terán et al., 2008). Cancer is a major health problem in the world. Moreover, in many countries, cancer represents the second leading cause of death after heart diseases. The estimated worldwide incidence of different carcinomas is about 10 million, half of which exist in the developed countries (Abu-Dahab & Afifi, 2007; Figueroa-Hernández et al., 2005). In contrast, populations with a high level of natural herbal product use have experienced a reduced incidence of gastric cancer (Deenehy & Tsourounis, 2002; Frantz et al., 2000). In addition to this, the low incidence of colon cancer in Asian countries has been attributed to the high consumption of soybean products (Oh & Sung, 2001; Zhu et al., 2002).

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From its very beginnings, almost half a century ago, cancer chemotherapy has faced remarkable problems. Lack of selectivity of conventional anticancer agents which damage not only malignant but also normal cells in particular, blood cells, has made scientists aware of the need for more specifically selective drugs (Pisha et al., 1995). Another drawback, which arose just after cancer chemotherapy was started, was the appearance of drug-resistant cancer cells (Gottesman, 1993). This in turn justifies the interest in searching for possible anticancer agents from the flora of different countries, which are found in the market under the name of “natural products” (Madhuri & Pandey, 2009). Since there are no efficient synthetic drugs available in the pharmacopeias, there is a great interest in screening plants used in traditional medicine for their beneficial effects. Various medicinal plant extracts that are claimed to be effective as anticancer agents have been used since ancient times (Bauer, 2000). Recently, many studies showed the ability of medicinal plant extracts to control the proliferation of prostate cancer cells (Hiremath et al., 1997; Hryb et al., 1995). Otherwise, the Cernitin pollen-extract (Cernilton, CN), which is a preparation made from eight kinds of pollen and used in Japan and Europe for various prostatic diseases, was found to be highly active in inhibiting the growth of DU145 cells, a prostate cancer cell line. One of the active constituents of this extract was later identified as cyclic hydroxamic acid (Habib et al., 1995). Oenothien B was identified as the active compound of *Epilobium parviflorum* Schreb. (Onagraceae), a plant used for the treatment of prostate disorders (Evans et al., 1995).

In this context, the present work determines the antioxidant, anticancer activities, together with the total phenols and flavonoids, of until now, mostly uninvestigated medicinal plants (Afifi et al., 1999; El-Desouky et al., 2007; Esmaeili & Yazdanparast, 2004; Fontana et al., 1999; Irshaid & Mansi, 2009), collected from different locations in the northern West Bank of Palestine.

In this study, the ethanol extracts prepared from six plants [*Arum palaestinum* Boiss (Araceae), *Urtica pilulifera* L. (Urticaceae), *Coridothymus capitatus* (L.) Reichb (Lamiaceae), *Majorana syriaca* (L.) Rafin. (Lamiaceae), *Teucrium creticum* L. (Lamiaceae), and *Teucrium capitatum* L. (Lamiaceae)] have been evaluated for their antioxidant activity using DPPH-free radical scavenging and β -carotene–linoleic acid assays, and total flavonoid and phenolic compounds. In addition, the current study reports a screening program of anticancer activity against breast cancer MCF-7 human carcinoma.

Materials and methods

Chemicals

β -Carotene, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and α -tocopherol were purchased from Sigma, (Sigma Aldrich GmbH, Steinheim, Germany), while pyrocatechol, quercetin, Tween-40, Folin–Ciocalteu’s phenol reagent (FCR), sodium carbonate, ethanol, chloroform, and other chemicals and reagents were purchased from Merck (Darmstadt, Germany). Trypsin, RPMI 1640 culture medium,

fatal calf serum, glutamine, amphotricine B, Hank’s balanced solution, Trypan blue solution, penicillin and gentamicin, all other reagents were of analytical grade.

Types of cancer cell lines

Breast cancer cell line: MCF-7 human carcinoma obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Plant material

The medicinal plant species screened in this study were collected in April–June 2011 from Nablus region and were identified by Prof. M. S. Ali-Shtayeh from the Biodiversity and Environmental Research Center, BERC, Til Village, Nablus (Table 1). Specimens were air-dried in a shady place at room temperature for 10 d. The dried plant parts were then ground using a Moulinex (Modele Depose type 241, Paris, France) for a minute and the resulting powder was stored in refrigerator at -20°C until use. Voucher specimens are deposited in the Herbarium of BERC.

Extracts preparation

Each ground dried plant sample (50 g) was extracted by continuous stirring with 500 ml ethanol at 24°C for 72 h and filtered through Whatman No. 4 filter paper. The residue was then washed with additional 200 ml ethanol. The combined ethanol extracts were dried using rotary evaporator followed by freeze drying and stored at -20°C for future use.

Determination of antioxidant activity

Scavenging activity of DPPH radical

Free radical scavenging activity of the extracts was determined using the free radical 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH), which is a molecule containing a stable free radical (Sharma & Bhat, 2009). In the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical for free DPPH radical decays and the change in absorbance at 517 nm is followed spectrophotometrically. The test could provide information on the ability of a compound to donate a hydrogen atom, on the number of electrons given molecule can donate, and on the mechanism of antioxidant action. Thus, 4 ml of 0.004% methanol solution of DPPH was mixed with 1 ml of various concentrations of the extracts in ethanol. The mixture was allowed to stand at room temperature in the dark for 30 min, and then the decrease in absorbance at 517 nm was measured against a control (methanol solution) by using UV–Vis spectrophotometer. A mixture consisting of 1 ml methanol and 3 ml of DPPH solution was used as the control. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH ($I\%$), and it was calculated according to the formula:

$$\%I = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \quad (1)$$

where A_{control} (absorbance = 0.427) and A_{sample} are the absorbance values of the control and the test sample, respectively.

Table 1. List of plants screened.

Plant	Family	Part tested	Traditional uses
<i>Arum palaestinum</i> Boiss	Araceae	Leaf	Cure several chronic diseases such as stomach acidity, atherosclerosis, cancer, and diabetes and food toxicity
<i>Coridothymus capitatus</i> (L.) Reichb	Lamiaceae	Arial part	Antimicrobial activities, cosmetics, flavoring and pharmaceutical industries, as refreshing drink or in cooking, against cold, influenza and throat infection, antiseptic and antimicrobial agents
<i>Majorana syriaca</i> (L.) Rafin.	Lamiaceae	Arial part	Flavor and fragrance, strong biological activity
<i>Teucrium creticum</i> L.	Lamiaceae	Arial part	Antidiabetic
<i>Teucrium capitatum</i> L. (<i>T. polium</i> L.)	Lamiaceae	Arial part	Diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive, anorexic, analgesic, antibacterial and antidiabetic effects
<i>Urtica pilulifera</i> L.	Urticaceae	Arial part	Cure for rheumatism, hemorrhage. Diuretic, depurative. Seeds are used for renal stones and inflammation of the bladder, diuretic, and aphrodisiac. Diabetes mellitus and other ailments

The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (100, 200, 300, 400, and 600 $\mu\text{g/ml}$). Antioxidant capacities of the extracts were compared with those of BHA, α -tocopherol, and control. Tests were carried out in triplicate.

Determination of total antioxidant activity:

β -carotene–linoleic acid assay

The antioxidant activity of the ethanol extracts, based on coupled oxidation of β -carotene and linoleic acid emulsion, was evaluated following a modified method of Miller (1971) and Gazzani et al. (1998). Thus, 2 ml of β -carotene solution (0.5 mg/ml in chloroform) were pipetted into a round-bottomed flask containing 20 μl of linoleic acid and 200 μl of Tween 40. Chloroform was completely evaporated using a rotary evaporator under reduced pressure at low temperature ($<30^\circ\text{C}$), and then 200 ml of distilled water saturated with oxygen were added immediately to the flask with vigorous shaking for 30 min. After agitating the mixture, 5 ml aliquot of the resulting emulsion was transferred into a series of tubes each containing 0.1 ml of extract or tocopherol. Each type of the sample was prepared in triplicate. The test systems were placed in a water bath at 50°C for 2 h. The same procedure was repeated with synthetic antioxidant, BHT, BHA and α -tocopherol (2 mg/ml) as a positive control as well as a blank (prepared exactly as before but without adding antioxidants). The absorbance of each sample was read spectrophotometrically at 470 nm, just immediately after the sample preparation and at 15 min intervals until the end of the experiment ($t = 120$ min).

Determination of total phenolic compounds

Total soluble phenols in the plants ethanol extracts were determined with the Folin–Ciocalteu reagent (Slinkard & Singleton, 1997), using pyrocatechol as a standard. Briefly, 1 ml from extract solution (2 mg/ml) was transferred into a volumetric flask of 50 ml, and made up to 46 ml with distilled water. The Folin–Ciocalteu reagent (1 ml) (previously diluted 10-fold with distilled water) was added, and then the contents of flask were mixed thoroughly. After 5 min, 3 ml of 2%

aqueous solution of sodium carbonate (Na_2CO_3) was added; thereafter, the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the ethanol extracts was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol calibration curve as follow:

$$\text{Absorbance} = 0.004757 \mu\text{g pyrocatechol} + 0.0022 (R^2 : 0.9997)$$

Determination of total flavonoid compounds

Flavonoid concentration was determined as follows: from each ethanol extract solution (1 ml) was diluted with 4.3 ml of 80% aqueous ethanol. Subsequently, to the test tubes, 0.1 ml of 10% aluminum nitrate $\text{Al}(\text{NO}_3)_3$ and 0.1 ml of 1 M aqueous potassium acetate (CH_3COOK) were added. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as a standard according to the equation:

$$\text{Absorbance} = 0.005358 \mu\text{g quercetin} - 0.0984 (R^2 : 0.9994)$$

Cell culture

The cells were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin, and 50 $\mu\text{g/ml}$ amphotericin B. After checking for the absence of mycoplasmas and bacteria, cells were grown at 35°C as monolayer confluent cells in RPMI 1640 medium supplemented with 10% calf serum. To avoid cell membrane sensitization, no antibiotics were used. For the assay, cells were washed three times with phosphate buffer saline (PBS). PBS was decanted, cells detached with 0.025% trypsin–EDTA, then RPMI 1640 medium was added to make up a volume of 10 ml. The cell suspension was centrifuged at 1000 g for 10 min, and then the pellet was re-suspended in 10 ml medium to make a single

cell suspension. Viability of cells was determined by Trypan blue exclusion and it exceeds 96% as counted in a hemocytometer. Stock cultures were duplicate weekly after inoculation. The cell line was cultured in 6-well tissue culture plates (9.8 cm²) and incubated at 35 °C in a humidified atmosphere containing 5% CO₂. After 24 h, the cells were treated with extracts or pure compounds. Last, 0.1 ml of each extract or pure compound was diluted into serial dilutions (500, 250, 125, and 62.5 µg/ml).

Results and discussion

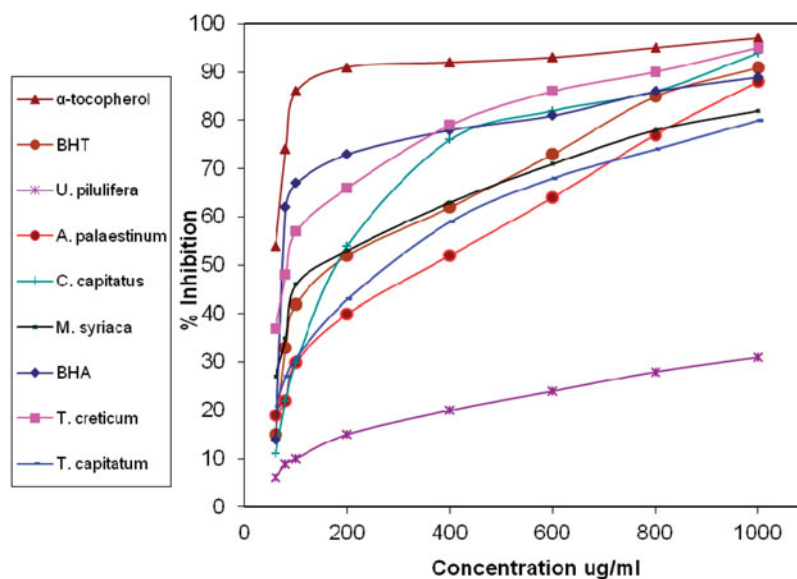
The antioxidant activity of plant extracts largely depend on the composition of the extracts and conditions of the test system. The antioxidant capacities are also influenced by many factors which cannot be fully described with one single method. Therefore, it is necessary to perform more than one type of antioxidant capacity assay to take into account the various mechanisms of antioxidant action (Wong et al., 2006).

This article describes the antioxidant and cytotoxic activities of a number of plants used in the TAPHM for the treatment of several diseases such as stomach acidity, atherosclerosis, cancer, antimicrobial, and many other diseases (Ali-Shatyeh & Jamous, 2008). A total of six plant extracts representing six plant species belonging to four different botanical families, were included in the present screening study.

The antioxidant activity of ethanol extracts of the six plants was studied using the following complementary assays: DPPH free radical scavenging, β-carotene-linoleic acid assay, total phenolics, and flavonoid compound determination.

All plant extracts studied, except *U. pilulifera*, showed remarkable free radical scavenging activity. The inhibition values of both plant ethanol extracts and the standards increase with concentration. At 200 µg/ml for example, *T. creticum* (IC₅₀ = 83 µg/ml) showed the highest free radical scavenging (65% inhibition value compared with 90% inhibition value of α-tocopherol), followed by *M. syriaca*, *C. capitatus*, *T. capitatum*, and *A. palaestinum* (Figure 1). Otherwise, *U. pilulifera* showed the lowest DPPH activity.

Figure 1. Percent inhibition of selected plants at different concentrations.



The scavenging capacity IC₅₀ of *T. creticum* (IC₅₀ = 83 µg/ml) seems to be fairly comparable to that of the commonly used synthetic antioxidant BHA (IC₅₀ = 85 µg/ml) but higher than that of BHT (IC₅₀ = 180 µg/ml), and lower than that of α-tocopherol (IC₅₀ = 55 µg/ml). However, the scavenging capacity of *T. creticum* was considerably higher than that of the other plants: *M. syriaca* (IC₅₀ = 145 µg/ml), *C. capitatus* (IC₅₀ = 178 µg/ml), *T. capitatum* (IC₅₀ = 280 µg/ml), and finally *A. palaestinum* (IC₅₀ = 372 µg/ml) (Figure 2). The *U. pilulifera* result has been excluded from Figure 2, since it gave a very high IC₅₀ value (>1000).

The results of lipid peroxidation inhibitory activity of the plant extracts, assessed by the β-carotene bleaching activity assay, are shown in Figures 3 and 4. This test is based on the fact that β-carotene loses its color in the absence of antioxidant (Luís et al., 2009). During oxidation, an atom of hydrogen is abstracted from the active methylene group of linoleic acid located on carbon-11 between two double bonds. The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lose their

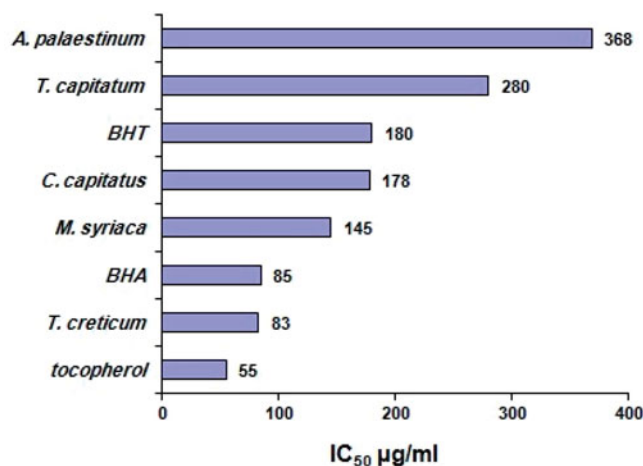


Figure 2. Free radical scavenging capacities IC₅₀ of ethanolic extracts.

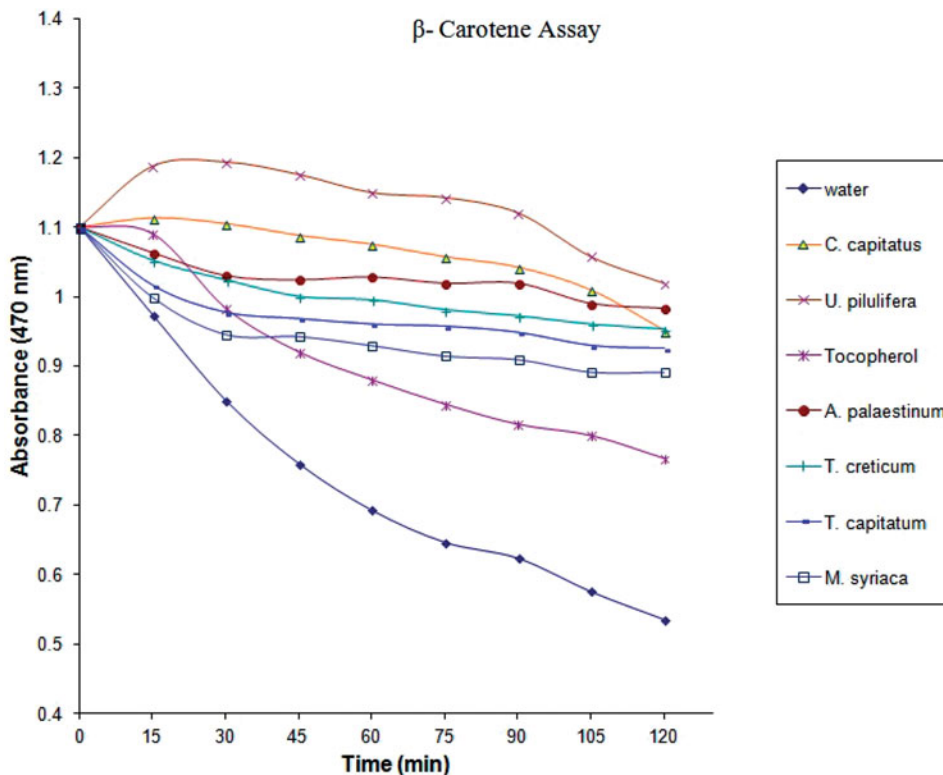
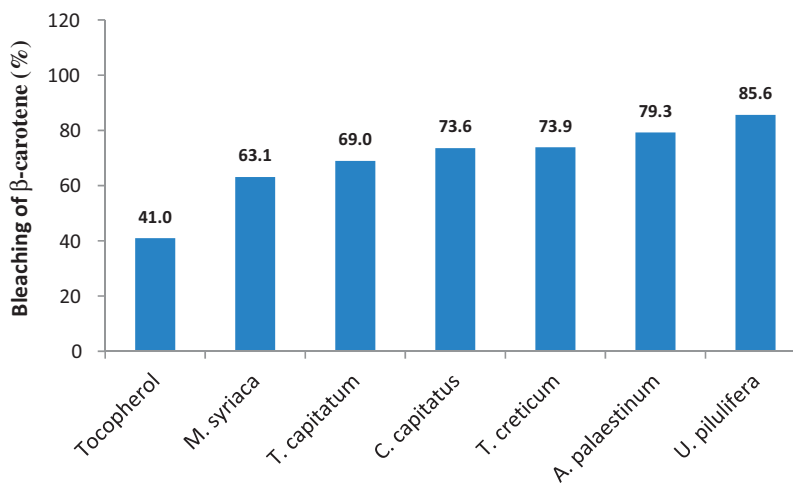


Figure 3. Antioxidant activities of ethanolic extracts of the plants (*A. palaestinum*, *U. pilulifera*, *C. capitatus*, *M. syriaca*, *T. creticum*, and *T. capitatum*), and α -tocopherol, as assessed by β -carotene-linoleic acid assay over 120 min.

Figure 4. β -Carotene bleaching percentages of plant extracts and tocopherol determined by β -carotene bleaching test.



conjugation, the carotenoids lose their characteristic orange color. This process can be monitored spectrophotometrically and bleaching percent is calculated according to the following equation:

$$\text{Bleaching \%} = [1 - (A_0 - A_t)/(A_0^0 - A_t^0)] \times 100\% \quad (2)$$

where A_0 and A_0^0 are the absorbance values measured at initial time of the incubation for sample and control, respectively, and A_t and A_t^0 are the absorbance values of sample and control, respectively, at t min.

The results obtained from this test are comparable with those obtained from the DPPH assay. The percentages of bleaching inhibition were 41, 63.1, 69, 73.6, 73.9, 79.3, and

85.6% for tocopherol, *M. syriaca*, *T. capitatum*, *C. capitatus*, *T. creticum*, *A. palaestinum*, and *U. pilulifera*, respectively (Figure 4).

The total phenolic compound amounts ranged from 146.4 to 270 $\mu\text{g}/\text{mg}$ pyrocatechol equivalent in the test plants, with *A. palaestinum* giving the highest yield of phenolics 270 $\mu\text{g}/\text{mg}$ followed by *U. pilulifera* 213 $\mu\text{g}/\text{mg}$ and *T. capitatum* 202.5 $\mu\text{g}/\text{mg}$ (Table 2). However, the remaining plants gave amounts less than 200 $\mu\text{g}/\text{mg}$.

Plant extracts display various degrees of free radical scavenging activity depending on what types of phenolic compounds exist in the extract, since phenolic compounds show different ranges of antioxidant activity depending on the

Table 2. Total phenolic and flavonoid content and antioxidant activity of ethanolic extracts of selected plants.

Plant	TP* μg (PE) mg^{-1}	TF ^S μg (QE) mg^{-1}	TP + TF	DPPH assay at 200 $\mu\text{g}/\text{ml}$	β -Carotene assay at 200 $\mu\text{g}/\text{ml}$
<i>A. palaestinum</i>	3.9	270	273.9	40	11
<i>C. capitatus</i>	103.5	160.2	263.7	54	15
<i>M. syriaca</i>	37.3	146.4	183.7	53	19
<i>T. creticum</i>	35.2	154.2	189.4	66	13
<i>T. capitatum</i>	30.8	203.5	234.3	43	16
<i>U. pilulifera</i>	120.6	213	333.6	15	–8

*Total phenols.

^STotal flavonoids.

functional groups present and how they are arranged on the compound (Sroka, 2005).

In one hand, the total flavonoid compounds concentrations varied considerably among plants ranging from 3.9–120.6 $\mu\text{g}/\text{mg}$ quercetin equivalents. In our study, *U. pilulifera* and *C. capitatus* gave the highest flavonoid contents. On the other hand, *A. palaestinum* gave the lowest flavonoid content. Furthermore, other tested plants possessed comparatively moderate levels of flavonoids (31–37 $\mu\text{g}/\text{mg}$ as quercetin equivalents).

A weak correlation can be observed between antioxidant activities of plants (in terms of free radical scavenging capacities or total antioxidant activity) and total flavonoids or total phenolics (Table 2). However, free radical scavenging activity and total antioxidant activity were strongly correlated with total phenolics and flavonoid content: $r^2=0.8$, and 0.7, respectively.

This may indicate that the concentration of phenolics and flavonoids is not the only factor related to the antioxidant activity, leading to the fact that the possible synergism of these compounds with other components present in the extracts may be responsible for this observation.

Indeed, the efficacy of those plants as antioxidants has been attributed to the presence of many different chemical compounds such as phenolics and flavonoids (Saeed et al., 2012). In this study, the total phenolics were determined using pyrocatechol equivalent and total flavonoids were determined using quercetin equivalent. The presence of phenolic antioxidant can hinder the extent of β -carotene degradation by “neutralizing” the linoleate free radical and any other free radicals formed within the system. Hence, this forms the basis by which plant extracts can be screened for their antioxidant potential. In conclusion, the present study indicates that the use of these plants in Palestine either in traditional medicine or as edible plants is justified since they may be considered promising sources of natural antioxidants with bioactive properties.

Laboratory experiments show the percent inhibition of the ethanol extracts on breast cancer cells (MCF-7) of each plant at different concentrations (Figure 5). *U. pilulifera* showed the highest cytotoxicity against breast cancer, 85% of the cells were dead at the concentration of 500 $\mu\text{g}/\text{ml}$ then *C. capitatus* showed 80% at the same concentration. In contrast, IC_{50} for *U. pilulifera* was 63 $\mu\text{g}/\text{ml}$ and *C. capitatus* was 100 $\mu\text{g}/\text{ml}$. *A. palaestinum* and *M. syriaca* showed IC_{50} in the range between 500 and 600 $\mu\text{g}/\text{ml}$, while *T. capitatum* did not exhibit any cytotoxicity against breast cancer. These results may be attributed to the presence of phenolics, since phenolic compounds are known to inhibit mutagenesis in humans when

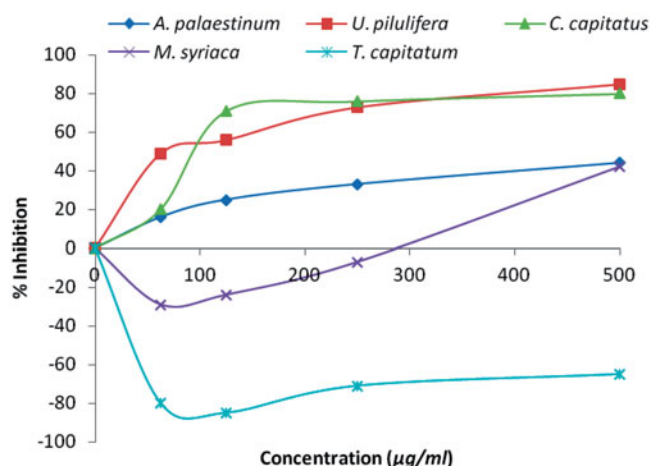


Figure 5. Cytotoxic effects of the extracts in the MTT assay on breast cancer cells (MCF-7).

ingested up to 1.0 g from diet rich in fruit or vegetables (Tanaka et al., 1998).

In conclusion, the results obtained suggest that the six plants tested have demonstrated different antioxidant capacities which were strongly correlated with their contents of phenolics and flavonoids. Accordingly, this study suggests that the therapeutic benefit of these plants can be, at least in part, attributed to its potential inhibition of oxidative processes, in addition to its efficient scavenging of O_2 .

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Declaration of interest

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