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Phytochemicals Analysis of Different *Teucrium Polium* Leaves Extracts and Evaluating Their Antioxidant and Anticancer Activity against the MCF-7 Breast Cancer Cells

Zineddine Boutefaha[a], Noureddine Belattar[a], Mosaad A. Abdel-Wahhab*

[1]Laboratory of Applied Biochemistry, Faculty of Sciences of Nature and Life, Ferhat Abbes University, Setif 1, Algeria
[b]Food Toxicology and Contaminants Department, National Research Center, Dokki, Cairo, Egypt
*M.A. Abdel-Wahhab (mosaad_abdelwahhab@yahoo.com; ma.abdelwahab@nrc.Sci.eg)

ABSTRACT

This work aimed to determine and identify the total phenolic (TP) and flavonoids (TF) compounds in different *Teucrium polium* leave extracts and evaluation their antioxidant and cytotoxic activity against MCF-7 breast cancer cells. Polyphenols were determined by HPLC; however, the TF and TP were estimated by colorimetric methods using rutin and gallic acid as standards, respectively. The HPLC analysis of the methanolic, ethanolic and aqueous extracts identified a total of 9, 8 and 11 compounds, respectively. Naringenin, quercetin, and ellagic acid were the most abundant, and chlorogenic acid, pyrocatechol. Chlorogenic acid, pyrocatechol and 4`.7-Dihydroxyiso flavone was completely absent in the methanolic and ethanolic extracts; however, catechin was completely absent in the ethanolic and aqueous extracts. The methanolic extract exhibited high antioxidant activity then the ethanolic and aqueous extracts. The three extracts exhibited strong cytotoxic activity against MCF-7 cell line using MTT in a dose-dependent fashion and the methanolic extract was more effective with an IC_{50} of 14.20 ± 1.3 µg/ml. The obtained data indicated that *T polium* could be an ideal alternative to be used for the development of new anticancer agents due to its potential antioxidant and cytotoxic capacities.

Keywords: *Teucrium polium*; Polyphenols; HPLC; antioxidant; anticancer; MCF-7 breast cancer cells

Introduction

Epidemiological studies support that the high consumption of antioxidant-rich vegetables and fruits diminishes the incidence of many cancer types suggesting the ability of these substances to inhibit or prevent cancer and other several diseases [1]. Phenolic compounds are secondary metabolites found in the plant kingdom are considered as the principal antioxidant substances that can scavenge free radicals, reduce cancer risk, and improve immunity [2[. Therefore, phytochemicals present in medicinal plants have received increasing attention in both animal models and humans [3]. The anticancer activities of the natural polyphenolics in the diet may occur via different mechanisms including the activation of antioxidant enzymes, suppression of the anti-inflammatory or anti-cellular growth signaling pathways which lead to cell cycle arrest or apoptosis induction [1].

Recently, cancer has become is the most serious issue affecting humanity worldwide and it ranks the second common causative factor of mortality after heart diseases [4]. Among all cancer types, breast cancer is considered the major common malignancy and the leading cause of cancer death in women all over the world [5]. Treatments such as surgical or radiotherapy have

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several side effects on healthy cells and do not prevent recurrence and metastasis of the tumor. Hence, many studies have focused on finding safe and effective anticancer drugs from plants and natural products [6]. Reactive oxygen species (ROS) are widely known to contribute significantly to tissue injury in several inflammatory, chronic diseases and carcinoma [7]. An imbalance in the cellular redox potential resulted in the alteration in the signaling pathways [8,9]. This phenomenon is the outcome of the lack of endogenous antioxidant capacities or the increase of ROS concentration in biological life. ROS can induce damages to cell micro components including DNA and lead to the peroxidation of lipid and proteins which finally develop several diseases [10]. Despite living organisms and humans have their endogenous antioxidant defense systems against ROS, these systems, under conditions become insufficient and antioxidants supplementation may be required to avoid oxidative damage and maintain the optimal cellular functions [11].

The species of *Teucrium* belongs to Lamiaceae (Labiatae) family, and represented by more than 340 species, and comprises about 12 species in Algeria [12]. Most *Teucrium* species are found extensively in the stony and dry places of the deserts and hills of Mediterranean countries, South-Western Asia, Europe, and North Africa. It is a perennial, pubescent, aromatic plant, up to 40 cm high, possessing white or grey hairs on stems, with green grayish leaves and white flowers [13]. *Teucrium* species have been used as medicinal herbs for more than 2000 years particularly in the Mediterranean countries [14]. Traditionally, the plant is used for its hypoglycemic and antispasmodic activities and it was recommended by herbalists for the treatment of various human diseases, such as gastrointestinal disorders and rheumatic pathologies [15]. It has been reported that *T. polium* contains important amounts of phenolic compounds that showed potent antioxidant activity in its different parts [16]. This study aimed to screen the total phenolic of different solvent extracts of *Teucrium polium* (*T. polium*) leaves by HPLC and evaluation their antioxidant and anticancer activity against MCF-7 breast cancer cells.

Materials and methods

Plant material and preparation of the extracts

Aerial parts of *Teucrium polium (T. polium)* were collected from natural populations in the region of Amoucha in North Algeria during the flowering period in June 2018. The plant was identified by a botanist from the Division of Ecology; University of Setif 1, Setif, Algeria. The leaves of the plant were cleaned and air-dried in darkness at ambient temperature. The dried

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leaves were powdered and sieved and then the extraction was done using water and hydro-alcoholic solvents. The leaf extracts were prepared according to Contini et al. [17] with slight modification. In brief, 50 g of the leaves powder were defatted twice with 400 ml hexane for 2 hours followed by filtration. The defatted residue was then extracted with water, methanol, or ethanol in the aqueous mixture (80%) with constant stirring overnight at room temperature. All water and hydro-alcoholic extracts were then filtered on a sintered glass after centrifugation for 10 min at 3000 rpm. The recovered residues underwent a second maceration in the same conditions for 4 h and the filtrate was obtained after centrifugation and filtration. The two filtrates were mixed and the solvent was evaporated in the oven at 40 ºC until a dry extract was obtained.

Determination of total phenolic

Total phenolics were estimated using the Folin-Ciocalteu reagent method [18]. Briefly, 200 µl of the extract or standard (gallic acid) solutions and 1 ml of Folin-Ciocalteu's reagent dissolved in water (10%) were mixed, after 4 min 0.8 ml of $NaHCO_3$ (7.5%) solution was added to the reaction medium. The samples were shaken and then incubated for 2 h in the dark at room temperature then the absorbance was measured spectrophotometrically at 765 nm. All tests were repeated three times to ensure reproducibility of the results. A standard curve was plotted using different concentrations (0-200 µg/ml) of gallic acid and the results were expressed in µg equivalent of gallic acid per mg of extract (µg EAG/mg).

Determination of total flavonoids

Total flavonoids were determined by the $AlCl_3$ method as described by Djeridane et al. [19]. The reaction mixture contained the extract (1 ml) or standard solutions (2-50µg/ml) and 2% $AlCl_3$ solution (1 ml) in methanol. After incubation for 10 min at room temperature, the absorbance of mixtures (prepared in triplicate) was measured at 430 nm using a UV-Vis spectrophotometer. A calibration curve was done using rutin as standard and then total flavonoids were determined and expressed as µg equivalent of rutin per mg of extract (µg ER/mg).

HPLC analysis of polyphenols of T. polium extracts

Polyphenols composition of different *T. polium* extracts were determined using Agilent 1260 series HPLC. The analysis was performed using C18 column (4.6 mm i.d. × 250 mm, 5 µm,) by injection a volume of 10 µl and then a gradient elution was performed by varying the proportion of solvent A (water) and solvent B (0.02% trifloro-acetic acid in acetonitrile), at a flow rate of 1 ml/min. The solvent gradient was as follows: 0-5 min 80% A; 8-12 min 50% A;

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12-16 min 80% A and the elution fractions were monitored by UV detector at 280 nm. All separations were performed at 35 °C.

Evaluation of the antioxidant activity

DPPH radical scavenging activity

The DPPH scavenging activity was measured as described by Ahmad et al. [20]**.** Firstly, extract solutions at various concentrations were prepared in methanol (5-200 mg/ml), and then, 50 µl of each extract was added to 1950 µl of 0.2 mM DPPH methanolic solution. The mixture was vortexed and incubated for 30 min in dark at room temperature and the absorbance of the solutions was read at 517 nm. Methanol was used instead of the sample to obtain the absorbance of the control. BHA and BHT as standard antioxidant were used for comparison. The scavenging activity of each extract was determined by the following formula:

DPPH scavenging activity (%) = (A0 – A1) /A0 ×100

Where A0 is the absorbance of the control and A1 is the absorbance of the sample**,** and the scavenging effect was estimated by the inhibition concentration at 50% (IC_{50}) that was deduced from the inhibition curve.

Evaluation of reducing power

The reducing power of *T. polium* extracts was performed according to Gül and Pehlivan [21]. For that, 0.5 ml of solution extract at various concentrations was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of potassium ferricyanide (1%). The mixture was then incubated for 30 min at 50 ˚C and 5 ml of trichloroacetic acid (1%) were added to terminate the reaction, and the mixture was centrifuged for 10 min at 3000 rpm. A volume of 1.25 ml of the solution supernatant was mixed with distilled water (1.25 ml) and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was read at 700 nm. A higher absorbance indicates increased reducing power. The reducing power was determined at 50% of EC by plotting the absorbance against extract contents.

Determination of cytotoxicity against MCF-7 breast cancer cells

The cytotoxicity of different extracts was determined by the conventional method based on MTT reduction assay [22]. MCF-7 breast cancer cells (obtained from ATCC via Holding company for biological products and vaccines, VACSERA, Cairo, Egypt) were cultured in 96-well plates at the density of 10^4 cells/well in a final volume of 100 µl cell suspension (RPMI supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin solution). Cells were incubated at 37 °C for 48 h under 5% CO_2 in an incubator, then the supernatant was

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removed and the cells were treated by the T. *polium* extracts at different concentrations (1.5 - 100 µg/ml) for 48 h. Then, 20 µl of MTT solution in DMSO (5 mg/ml) was added to each well, an additional incubation for 4 h was performed. The MTT solution was then separated, and 100 µl of DMSO was added to each well to dissolve the purple formazan formed. The absorbance of the obtained solution was recorded at 570 nm using a microplate reader (EXL 800, USA). The mean of absorbance for each extract was calculated, and then the viability percentage of cells was calculated as follows:

% Cell viability = Mean absorbance of treated sample/Mean absorbance of control sample ×100 the control was the non-treated cultures that contained cells only in the medium.

The inhibition power of the extracts against MCF-7 breast cancer cells was evaluated by the IC_{50} and compared with Doxorubicin as a standard anticancer drug.

Statistical analysis

The results were given as the mean ± SE for three replicates for each sample. The IC_{50} (DPPH, ABTS β-carotene bleaching), A 0.5 (CUPRAC assay, phenanthroline) and EC_{50} (Chelation of the metal ions) values were calculated by linear regression analysis.

Results and discussion

Total phenolics and flavonoids (TP and TF)

The yield of the extraction of *T. polium* leaves was calculated on a dry weight basis as 25%. The content of TP compounds was reported as GAE (gallic acid equivalents) determined from standard curve (y = 0.012x + 0.065, r2 = 0.99). The results revealed that TP was higher in the methanolic extract followed by the ethanolic extract the aqueous extract (Table 1).

Table 1. Polyphenols and flavonoids contents in *T. polium* leave extracts

Extract	Polyphenols (µg EAG/mg of extract)	Flavonoids (µg ER/mg of extract)
Methanolic	134.10 ± 1.96	38.85 ± 1.02
Ethanolic	99.30 ± 1.34	31.64 ± 0.65
Aqueous	74.17 ± 1.15	18.26 ± 0.98

The quantifications of TF were carried out by the $AlCl_3$ method and the amount was expressed as rutin equivalent by reference to the standard curve (y = 0.016x + 0.006, r2 = 0.999). The results also showed that methanolic contains the highest TF content followed by the ethanolic extract then the aqueous extract (Table 1). These results suggested that *T. polium* is mainly rich in TP compounds. The variation between these extracts in the polyphenols may

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probably be owing to the formation of a complex of some phenolic compounds which are soluble in methanol and ethanol and have high molecular weights or more phenolic groups compared to the TP content in the aqueous extract [23]. These authors also suggested that the combination of water and organic solvent facilitates the release of all phenolic compounds which are soluble in both organic solvents and water. Moreover, the results obtained herein seem a bit higher than the results obtained by Stankovic et al. [24] in methanolic extract of *T. polium* with 124.62 ± 1.05 μg EqAG/mg and 41.23 ± 1.12 μg ER/mg for polyphenols and flavonoids, respectively. In this concern, previous reports suggested that the plant matrix, the extracted solvent, the extraction conditions and the number of the hydroxyl group are the main factors affecting the number of polyphenols in the extract [25-28].

HPLC Analysis of phenolic compounds

The obtained chromatographic profile of standards (Fig. 1A) showed the peaks of 15 compounds; however, the methanolic (Fig. 1B), ethanolic (Fig. 1C) and aqueous extracts (Fig. 1D) showed the absence of one or more peak. Identification of the constituents was achieved by comparison with phenolic standards and their abundance was determined through their area peaks that allow us to have an insight into the compounds accountable for the antioxidant and the antitumor properties. The results presented in Table (2) revealed the identification of 9 compounds in methanolic extract and 8 compounds in the ethanolic extract; however, 11 compounds were found in the aqueous extract. Moreover, the methanolic extract contained the highest amount of catechin, ellagic acid, coumaric acid and quercetin compared to the other extracts. Chlorogenic acid, pyrocatechol and 4`.7-Dihydroxyiso flavone was completely absent in methanolic and ethanolic extracts; however, catechin was completely absent in the aqueous and ethanolic extracts. The results of this analysis elucidated that *T. polium* leaves are good sources of the polyphenols such as naringenin and quercetin as natural antioxidant agents. The differences between the three extracts in their phenolic contents may be due to the difference in solubility of the compounds in the extracted solvents as suggested in the previous reports [29-31].

Antioxidant activities

DPPH radical scavenging activity

The antiradical efficacy of the three *T. polium* leaves extracts was evaluated using DPPH radical scavenging method. The results presented in Fig. (2A) showed that the activity increased as the extract's concentration increases. The best activity was observed for the methanolic extract

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(IC_{50} value of 8.27 ± 1.92 µg/ml) which is lower than BHT (12 µg/ml) and less high than the BHA (6.14 ± 0.41 µg/ml). The ethanolic extract was less active (IC_{50} of 21.92 ± 2.28) and the aqueous extract was the weakest (IC_{50} of 61.84 ± 3.52). These results suggested that the hydro-alcoholic extracts (methanolic and ethanolic) of *T. polium* leaves have a potent antioxidant activity which is probably attributed to their high content of the TP. Effectively, it was established that the radical scavenging property of the plant extracts depends mainly on their content of phenolic components [32].

Table 2. HPLC analysis of the total polyphenolic compounds in *T. polium* leaves extracts.

Extracts	Methanolic	Ethanolic	Aqueous
Compounds	Concentration (µg/g)		
Chlorogenic acid	0.00	0.00	1737.97
Catechin	7182.02	0.00	0.00
Caffeine	455.23	455.54	409.06
Pyrocatechol	0.00	0.00	435.18
Ellagic acid	2531.44	2286.94	946.91
Coumaric Acid	975.46	958.28	458.43
Vanillin	325.05	693.48	328.58
Naringenin	12286.67	7351.81	13650.05
Propyl Gallate	423.72	404.18	891.48
4`.7-DihydroxyisoFlavone	0.00	0.00	275.68
Querectin	3225.49	2498.23	1457.64
Cinnamic Acid	148.65	443.54	56.24

The antioxidant capability of phenolic components is modulated by the donation of hydrogen atoms. Molecular structure, mainly the positions and number of the hydroxyl groups, and their substitute's nature on the aromatic rings confer to phenolic compounds the capacity of inactivating free radicals [33]. These findings are consistent with the study of Chaouche et al. [34] who reported that the *T. polium* induced a significant antiradical activity in DPPH assay and the recorded IC_{50} was 20.1 ± 1,7 µg/ml for methanolic extract and 40.6 ± 4.0 µg/ml for aqueous one.

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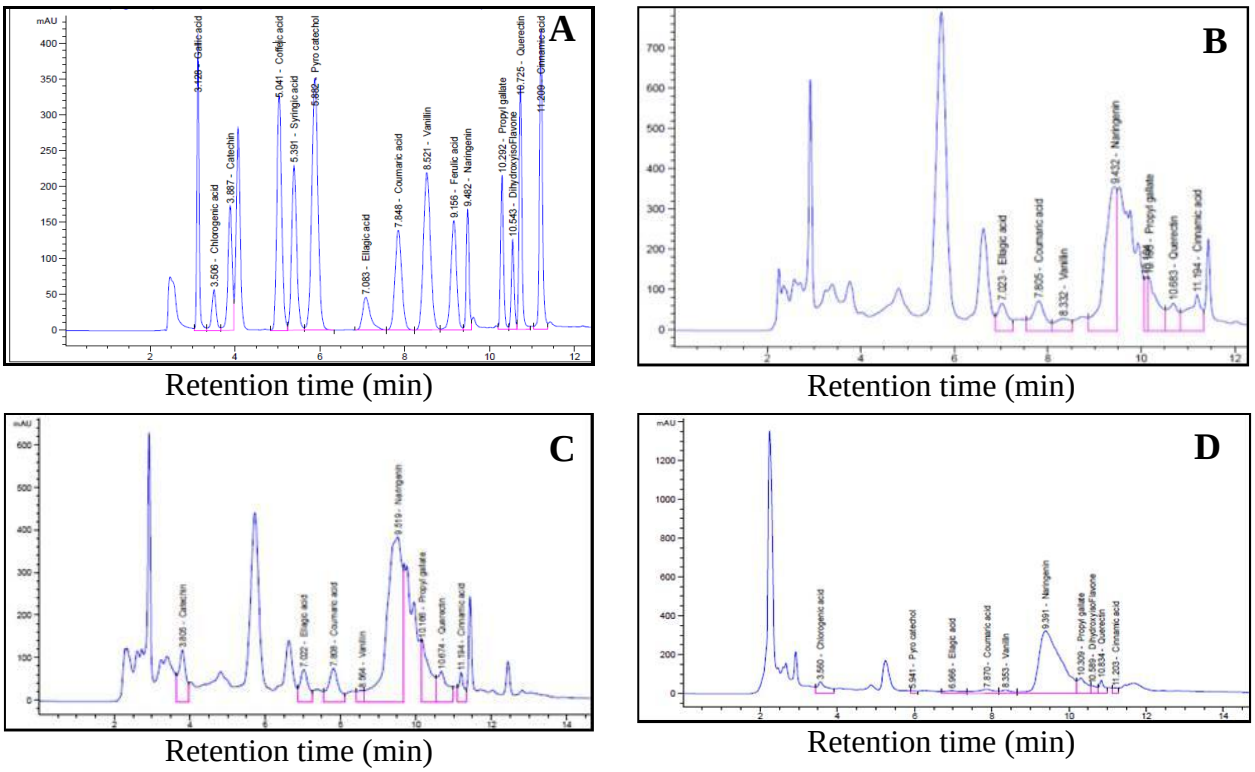

Fig. 1. HPLC chromatograms of the total polyphenols of (A) standard, (B) methanol (C) the ethanol and (D) the aqueous extracts of *T polium*

Reducing power

Reducing power assay is connected with the antioxidant efficacy and serves as an acceptable indicator of the antioxidant capacity [35]. The reductive activity of the extracts was based on the reduction of the potassium hexacyanoferrate (K_3FeCN_6) complex to the ferrous form depending on the reducing power of each compound [36]. This method is well known as sensitive for the semi-quantitative assay of the dilute concentrations of polyphenolics that participate in redox reaction [37]. In the present approach, the reducing power of *T. polium* extracts was developed in a dose-dependent manner (Fig. 2B). It can be seen that methanolic extract exhibited high reducing power and the recorded IC_{50} was 268 µg/ml; which was increased and correlated well with the increasing concentration. However, ethanolic and aqueous extracts showed moderate activity with IC_{50} =398 and 896 µg/ml, respectively. This difference in reducing power correlated well with the TP content in each extract which would have converted the Fe_{3+}/ferricyanide complex to the ferrous form. Based on this result, polyphenols in *T. polium* leaves appear to be able to act as good electron donors and therefore, the radical chain

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reactions can be terminated by converting free radicals into more stable, non-reactive species. In a previous study conducted on two wild *Teucrium* species, a good correlation was found between their phenolic contents and the ferric reducing power [38]. The obtained results were close to those recorded in our study and methanolic extract showed the most powerful with an IC_{50} of 280 ± 0.014 µg/ml followed by ethanolic and aqueous extracts with IC_{50} of 320 ± 0.01 and 480 ± 0.01, respectively.

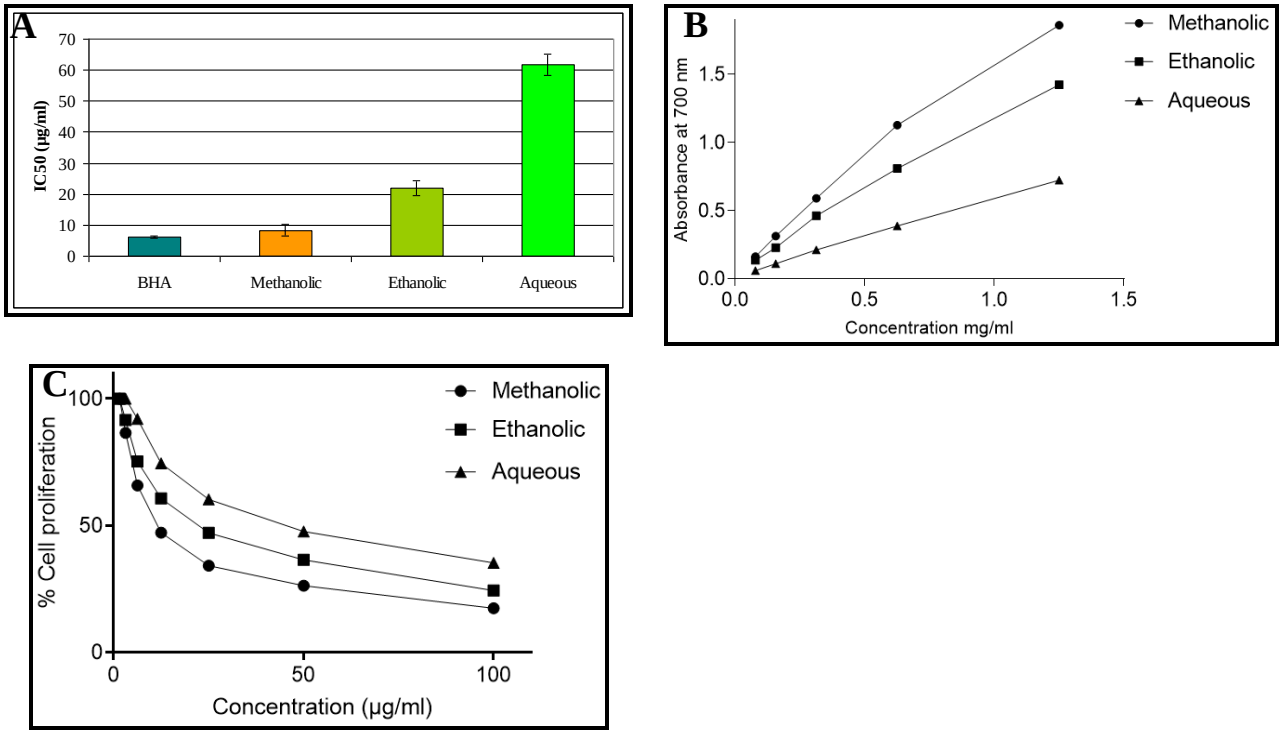

Fig. 2. (A) DPPH radical scavenging activity of *T polium* leaves extracts; (B) Reducing power of *T. polium* leaves extracts**.** Values represent the mean of three experiments and (C) Effects of different concentrations of *T. polium* leaves against MCF-7 cell line. Values represent the mean of three experiments

Cytotoxic activity

The MTT assay was applied to estimate the antitumor activity using MCF-7breast cancer cells exposed to different concentrations of *T. polium* extracts compared to the positive control exposed to Doxorubicin for 72 h. As presented in Fig (2C), the tested extracts inhibited the proliferation of MCF-7 cells in a dose-dependent manner. Among the tested extracts, the highest antiproliferative action was observed with the methanolic extract which showed strong inhibition of breast cancer cell growth with an IC_{50} of 14.20 ± 1.3 µg/ml. However, ethanolic and aqueous extracts showed a moderate cytotoxic action where their IC_{50} was in the order of 24.38 ± 2.1% and 45.68 ± 3.2%, respectively (Table 3). The viability of the breast cancer cell line was

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decreased by increasing TP extracts concentration which was observed to be the highest using a concentration of 100 µg/ml with 17.4%, 24.3%, and 35.2% for the methanolic, the ethanolic and the aqueous extracts, respectively.

Table 3. Cytotoxic activity of some compounds against different *T. polium* extracts against MCF-7 breast cancer cells

Extract	IC_{50} (µg/ml) *
Methanolic	14.20 ± 1.3
Ethanolic	24.38 ± 2.1
Aqueous	45.68 ± 3.2
Dox (standard)	4.17 ± 0.2

* IC50 (mg/ml): 1-10 (very strong), 11-20 (strong), 21-50 (moderate), 51-100 (weak) and above 100 (non-cytotoxic).

The beneficial effects of phenolic compounds in cancer chemoprevention often correlated with their antioxidant power including reducing capacities and ROS-scavenging properties [39]. However, the pro-oxidant activity of these natural compounds rather than their antioxidant action may be an important mechanism for their anticancer and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation [40]. It was reported that polyphenols participate by their antioxidant power in the initiation step of carcinogenesis against ROS and carcinogens that oxidize DNA and in the regulation of cell cycle, the induction of apoptosis, and the inhibition of angiogenesis [41,42]. As previously mentioned in the HPLC study, *T polium* extracts are rich in polyphenols such as naringenin, quercetin, and ellagic acid, which may be accountable for the anticancer action of these extracts. Different mechanisms by which TP act against breast cancer cells have been revealed such as induction of apoptosis increase in connexin 43, inhibition of fatty acid synthase, and reduction in the expression of cyclins D3, E, and A [43-45]. Indeed, naringenin, a major flavanone presented in *T. polium* extracts, was reported to exert an inhibitory effect on MCF-7 cells proliferation through the impairment of glucose uptake. Furthermore, it was also reported that this flavonoid induced 50% growth inhibition of MCF-7 cells at 240 ± 23 µM after 48 hours [46]. The antiproliferative action of quercetin was studied in human breast cancer cell growth *in vivo* and *in vitro*. Hashemzaei et al. [47] showed that quercetin flavonoid administration in mice bearing MCF-7 tumors significantly decreased the tumor volume interfering with the cell cycle by causing cell cycle arrest in G2/M or at the G1/S transition. The

combination of this flavonoid with ellagic acid acts synergistically and was able to reduce proliferation and viability and to induce apoptosis in MOLT-4 leukemia cells [48]. From this study, it is evident that *T. polium* has a strong cytotoxic activity *in vitro* against human breast cancer. This activity is possibly due to the polyphenols and flavonoids which are demonstrated to be presented with significant quantities and act in synergy to provide significantly better results as chemopreventive agents. The decrease in the viability of cancer cell by increasing the concentration of the *T. polium* extracts suggested that these extracts can act as an effective anti-cancer therapy.

Conclusion

The current results revealed that *T. polium* leaves are rich in natural polyphenolic compounds and possess significant antioxidant and reducing power properties. Additionally, these *in vitro* findings demonstrated that the extracts of *T. polium* had a potential antioxidant and radical scavenging activity and were able to inhibit significantly the MCF-7 cell line proliferation. Therefore, the leaves of *T. polium* are highly promising to be used in antioxidant treatments and breast cancer therapy.

Conflict of interest

The authors declare no competing interests.

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