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ORIGINAL PAPER

Antimicrobial and antioxidant activities of methanol extracts of *Evax pygmaea* (Asteraceae) growing wild in Tunisia

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Abstract Antimicrobial activity of methanolic extracts obtained from the aerial parts of *Evax pygmaea* was tested against five bacteria and two strains of phytopathogenic fungi using the agar diffusion and broth microdilution methods. Antioxidant properties were evaluated through the ability of the different fractions to scavenge the stable ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (1,1-dip henyl-2-picrylhydrazyl) radicals. The TEAC (Trolox Equivalent Antioxidant Capacity) and IC_{50} values of the fractions were calculated and compared. The experimental data indicated that all fractions exhibit moderate to appreciable antibacterial activities against all Gram-positive cocci and Gram-negative rods except *Pseudomonas aeruginosa*, but no antifungal activity was observed. Ethyl acetate and methanol fractions were found to cause significant free radical-scavenging effects in both assays. These results may suggest that *E. pygmaea* could be used as a natural preservative ingredient in the food and/or pharmaceutical industries.

Keywords ABTS · Antimicrobial activity · Asteraceae · DPPH · *Evax pygmaea* · Radical-scavenging

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Introduction

It has long been recognized that plant polyphenols are an important class of defence antioxidants. These compounds are widespread in virtually all plants, often at high level, and include phenols, phenolic acids, flavonoids, tannins and lignans. Recently, there has been a growing interest in oxygen-containing free radicals in biological systems and their implied roles in the development of degenerative diseases. It is suggested that their damage to cells leads to pathological changes associated with aging. These radicals may also be a contributory factor in the progressive decline in the function of the immune system (Pike and Chandra 1995).

In addition, it has been reported that the use of synthetic antioxidants to prevent free radical damage can involve toxic side effects (Cornwell et al. 1998) and consumers are becoming more conscious of the nutritional value and safety of their food and ingredients. Preference for natural foods and food ingredients that are believed to be safer, healthier and less subject to hazards is increasing compared to their synthetic counterparts. However, the use of natural antioxidants is limited by the lack of knowledge about their molecular composition, the amount of active ingredients in the material source and the availability of relevant toxicity data.

Moreover, the presence and growth of pathogenic microorganisms (bacteria, mould, viruses, fungi) in food may cause its spoilage and result in a reduction in its quality and quantity (Soliman and Badeaa 2002). This microbial contamination still poses important public health and economic concerns for human society.

For these reasons, there has been increasing interest in recent years in healthy lifestyles and healthy aging; correspondingly, search for natural additives, especially of plant origin, has notably increased.

A great number of aromatic, spicy, medicinal and other plants, belonging to the Asteraceae family contain chemical compounds exhibiting antimicrobial and antioxidant properties. Numerous studies have been carried out on some of these plants, e.g. *Achillea millefolium* subsp. *millefolium* (Candan et al. 2003), *Artemisia apiacea* (Kim et al. 2003) and *Carlina acanthifolia* (Đordevic et al. 2007). These plants could provide sources of acceptable natural chemicals in food preservation.

Furthermore, literature survey of the Evax genus revealed that no researchers have reported anti-oxidant and antimicrobial activities so far. This genus is represented by eight species; three of them are found in Tunisia: *Evax pygmaea, E. asteriscifolia* and *E. argentea.* Therefore, the aim of this study is to evaluate the in vitro antimicrobial and antioxidant properties of some extracts of *E. pygmaea* which is also known as *Filago pygmaea* or *Micropus pygmaeus. E. pygmaea* is an annual herb flowering in April–May. It is a small cotton plant reaching 5 cm high, with simple or branched stem, linear, oblong or spatulated leaves surrounding the inflorescences. It is a Mediterranean species distributed in the north-east, centre and south of Tunisia (Pottier-Alapetite 1981). It grows wild on the slopes of the hills, fields and in sandy pastures.

Materials and methods

Plant material

Aerial parts of *E. pygmaea* were collected randomly from the hills of Monastir in June 2005. A voucher specimen, ASTER-23344, was deposited in the Laboratory of Natural Substances Chemistry and Organic Synthesis, Faculty of Sciences, Monastir, Tunisia.

The plant samples were air-dried for several weeks. Powdered plant tissues were extracted three times by maceration with methanol, the resultant extract was concentrated under reduced pressure. The methanol extract was extracted successively with equal volumes of three organic solvents of increasing polarity (petroleum ether, chloroform and ethyl acetate). Each fraction was taken to dryness under vacuum and stored at 4°C until tested.

Antibacterial activity

The antibacterial activity of methanolic extracts was evaluated by paper disc diffusion and dilution methods against five selected Gram-positive and Gram-negative species: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* NCIMB 8853, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Micrococcus luteus* NCIMB 8166.

Diffusion method

The qualitative antibacterial assay of the methanolic extracts of *E. pygmaea* was carried out by the disc diffusion method (Marmonier 1987). All the microorganisms mentioned above were incubated at 37°C for 24 h by inoculation into Mueller–Hinton (M–H) broth. The culture suspensions were prepared and adjusted to approximately 10^5 c.f.u. of bacteria/ml.

Five hundred microliters of the inocula were spread over plates containing sterile Mueller–Hinton agar (pH 7.2) and paper filter discs (5 mm) impregnated with 0.2, 0.4 and 1 mg/disc of each fraction (petroleum ether, chloroform, ethyl acetate and methanolic fractions) were placed on the surface of the media. The plates were left 30 min at room temperature to allow the diffusion of the extract and incubated at 37°C for 18 h. At the end of that period, the inhibition zone around the disc was measured. Two controls were also included in the test: a positive control without extracts and a reference control using two standard antibiotics (Gentamicin and Tetracycline), to evaluate the susceptibility of tested strains. The experiments were run in triplicate, and the developing inhibition zones were compared with those of reference discs.

Dilution method

The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of tested fractions were determined using the Mueller–Hinton Broth (MHB) dilution method (Burt 2004). All tests were performed in MHB supplemented with dimethylformamide (DMF) (5%). Dilutions of the fractions were prepared as follows: 0.2, 0.4 and 1 mg/ml. Each extract was dissolved in 5% dimethyl sulfoxide (DMSO) and added to MHB. A negative control with 0.5% DMSO was used to ensure that DMSO did not affect growth.

The MIC was defined as the lowest concentration preventing visible growth. The complete absence of growth was considered as the MBC (Burt 2004). To confirm the results of MBC, 10 µl of the experimental suspensions were sub-cultured on TCSA plates (Tryptone-Casein-soya agar) which were incubated at 37°C during 18–24 h (Ronda and Rybak 2001). All samples were tested in triplicate.

Antifungal activity

Antifungal studies were conducted against two phytopathogenic fungi obtained from the laboratory of phytopathology at the Regional pole of Agricultural

Research-development in the eastern center of Chott Mariem, Sousse, Tunisia. They were: *Fusarium oxysporum* and *Aspergillus niger*.

The disc diffusion method was used for antifungal screening. Briefly, a suspension of the tested fungi was prepared (10^4–10^5 c.f.u./ml), added to Potato Dextrose Agar (PDA) medium and dispensed uniformly into Petri plates of 90 mm diameter. Different dilutions of the extracts were made with their appropriate solvents. Sterilized paper discs (6 mm, Whatman No. 1 filter paper) were impregnated with tested extracts at different concentrations (1, 2 and 5 mg/disc). The positive control used was Carbendazine, while petroleum ether, chloroform, ethyl acetate and methanol were used as a negative control. The diameter of the inhibition zone (mm) around the disc was measured after incubation at 28°C for 4 days and compared with control. The experiments were run in triplicate.

Antioxidant activity

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH); 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma and potassium persulfate (dipotassium peroxodisulfate) from Merck. All other solvents and chemicals were of analytical grade purity.

DPPH radical-scavenging assay

The hydrogen-donating abilities of the tested extracts were examined on the basis of the method described by Cuendet et al. (1997) with some modifications. Used as reagent, DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) obviously offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants.

About 0.5 ml of 60 μM solution of DPPH in ethanol was thoroughly mixed with an equal volume of test extracts at various concentrations (10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml) and kept in the dark for 30 min. Absorbance was read at 520 nm using ethanol as blank. 0.5 ml of DPPH solution mixed with 0.5 ml of ethanol was used as control. Inhibition of DPPH radical was calculated using the equation: $I\,(\%) = 100 \times (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the tested sample. The actual decrease in absorbance induced by the tested sample (change of colour from deep-violet to light-yellow) was compared to that of the positive control trolox. The IC_{50} value, represented the concentration of extract that causes 50% inhibition, was determined. Experiments were carried out in triplicate and the mean value was recorded.

ABTS radical cation decolorization assay

The standard TEAC (Trolox Equivalent Antioxidant Capacity) assay described by Re et al. (1999) and Tiwari and Tripathi (2007) has been used with minor modifications for determination of the TEAC value. This assay assesses the total radical-scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical ($ABTS^{\cdot}$) in 6 min. The blue green $ABTS^{\cdot}$ was produced through the reaction between 7 mM ABTS and 2.45 mM potassium persulfate in water. This solution was stored in the dark for 12–16 h before use. The concentrated $ABTS^{\cdot}$ solution was diluted with phosphate buffered saline (PBS°, pH 7.4) to yield a final absorbance of 0.7 ± 0.02 at 734 nm at 37°C. Stock solutions of Trolox were prepared in ethanol. The final reaction mixture of the standard group was made up to 1 ml with 990 μl of ABTS solution and 10 μl of Trolox (used as reference antioxidant compound). Similarly, in the test group 1 ml reaction mixture comprised 990 μl of ABTS solution and 10 μl of the extract solutions. The reaction mixture was vortexed for 10 s and reduction in absorbance (change of colour from blue-green to colourless) was recorded at time intervals of 5, 10, 15 and 20 min and compared with the control ABTS solution.

The TEAC of each extract was compared to that of the positive control Trolox solution. The amount of the samples (mg) reducing the absorbance by 50% was determined. All experiments were carried out in triplicate.

Results and discussion

Various fractions obtained from the methanol extract of *E. pygmaea* were tested for their antimicrobial activity and free radical-scavenging effects.

Antimicrobial activity

The antibacterial activity of *E. pygmaea* extracts was tested in vitro by using disc diffusion and liquid dilution method. According to the results given in Table 1, these extracts exhibited moderate to appreciable antibacterial activities against all Gram-positive cocci and Gram-negative rods except *P. aeruginosa*. According to several authors, this gram-negative bacteria appeared to be the least sensitive to the action of many other plant extracts and tested compounds (Boukamcha et al. 2004). Moreover

all fractions appeared more active against the tested Gram-positive than Gram-negative bacteria. This result is in agreement with many studies realized on other plant species belonging to the Asteraceae family such as those of Boukamcha et al. (2004) and Oueslati et al. (2004). This higher resistance among Gram-negative bacteria could be due to the differences in the cell membrane of this bacterial group. Indeed, the external membrane of Gram-negative bacteria renders their surfaces highly hydrophilic whereas the lipophilic ends of the lipoteichoic acids in the cell membrane of Gram-positive bacteria may facilitate penetration by hydrophobic compounds.

The present study revealed that petroleum ether extract of *E. pygmaea* was more active against *S. aureus*, presenting an important growth inhibition at lower concentrations (MIC: 0.2 mg/ml and MBC: 1 mg/ml) (Table 2). Chloroform extract was less potent but effective against *S. epidermidis* and *M. luteus* (MIC: 0.4 mg/ml). Ethyl acetate extract showed high antibacterial activity against all Gram (+) bacteria (MIC: 0.4 mg/ml). It was the sole active extract against Gram (−) bacteria *E. coli* (MIC: 0.4 mg/ml). Activity of the methanol extract against *S. epidermidis* was also appreciable (MIC: 0.2 mg/ml).

The screening of the chemical groups found in the powder material of this species done as described by Bruneton (1993), showed the presence of tannins, flavonoids and coumarins. This activity may be attributed to the presence of the high concentration of tannins, flavonoids, heterosides and volatile oils, known to possess antimicrobial properties (Scalbert 1991), in the different active extracts.

According to Scalbert (1991) the antimicrobial effects of tannins take place by involving different mechanisms such as inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation. Tsuchia et al. (1996) reported that the antimicrobial effects of flavonoids are due to their capacity to form complex with the extracellular and the soluble protein and with the cell membrane. Accordingly, the presence of these molecules in the active fractions might play a role in the observed antibacterial activity. Their mode of action probably depends on the individual microorganism which could explain the large differences in MIC values between bacteria.

The different extracts of *E. pygmaea* failed to show antifungal activity when applied at 0.2, 0.4 and 1 mg/disc against all the tested fungi. This result could be due to the low concentration of extract used. Therefore, the use of higher concentrations of fractions in order to obtain a more potent effect against all microorganisms could be recommended.

Antioxidant activity

For evaluation of free radical-scavenging properties of *E. pygmaea* extracts two assays have been used: DPPH radical and ABTS radical cation assays.

DPPH assay

The results of DPPH° inhibition by different fractions are shown in Fig. 1. DPPH was reduced with the addition of all fractions in a concentration-dependent manner. In all applied concentrations, inhibition was above 40%.

Table 1 Antimicrobial activity of *E. pygmaea* extracts

Name of microorganism	Inhibition zone (mm)														
	PE			$CHCl_3$			EtOAc			MEOH			NC	TE	GM
	Charge/disc (mg)													(30 µg/disc)	(10 µg/disc)
	0.2	0.4	1	0.2	0.4	1	0.2	0.4	1	0.2	0.4	1			
Gram-positive bacteria															
Staphylococcus aureus ATCC 29213	6.5	7	12.5	(−)	(−)	(−)	(−)	6	6.5	(−)	(−)	6	(−)	20	14
Staphylococcus epidermidis NCIMB 8853	(−)	6	7	(−)	6	6	(−)	6	6.5	6	7	8	(−)	5	20
Micrococcus luteus NCIMB 8166	(−)	6	6	(−)	6	6	(−)	6	7	(−)	(−)	(−)	(−)	21	19
Gram-negative bacteria															
Escherichia coli ATCC 35218	(−)	(−)	(−)	(−)	(−)	(−)	6.5	7	7.5	(−)	(−)	(−)	(−)	17	16
Pseudomonas aeruginosa ATCC 27853	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	15	12
Fusarium oxysporum	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)			
Aspergillus niger	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)			

ATCC: American Type Culture Collection; NCIMB: National Collections of Industrial Marine and Food Bacteria; TE: Tetracyclin; GM: Gentamicin; (−): No activity detected; NC: Negative control; PE: Petroleum ether fraction; $CHCl_3$: Chloroformic fraction; EtOAc: Ethyl acetate fraction; MEOH: Methanolic fraction

Table 2 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Evax pygmaea* extracts

Microorganisms	PE		$CHCl_3$		EtOAC		MEOH	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria								
Staphylococcus aureus ATCC 29213	0.2	1	>1	n.d.	0.4	n.d.	1	n.d.
Staphylococcus epidermidis NCIMB 8853	0.4	1	0.4	n.d.	0.4	n.d.	0.2	n.d.
Micrococcus luteus NCIMB 8166	0.4	n.d.	0.4	n.d.	0.4	n.d.	>1	n.d.
Gram-negative bacteria								
Escherichia coli ATCC 35218	>1	n.d.	>1	n.d.	0.2	<1	>1	n.d.
Pseudomonas aeruginosa ATCC 27853	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

ATCC: American Type Culture Collection; NCIMB: National Collections of Industrial Marine and Food Bacteria; n.d.: Not determined; PE: Petroleum ether fraction; $CHCl_3$: Chloroform fraction; EtOAc: Ethyl acetate fraction; MEOH: Methanolic fraction

Ethyl acetate fraction was the most effective DPPH radical scavenger. It almost removed DPPH absorbance (84.3% at 0.156 mg/ml). This percentage can be considered as a full absorbance inhibition of DPPH, because after completing the reaction, the final solution always possesses some yellowish colour. Methanolic fraction was also a good radical scavenger with the inhibition of 78% at a concentration of 0.156 mg/ml whereas the chloroform fraction showed a somewhat lower activity in comparison to ethyl acetate and methanolic fractions.

The maximum of inhibition was achieved by using higher concentrations of petroleum ether fraction (71.1 and 72.5% at 5 and 10 mg/ml respectively).

ABTS assay

Another antioxidant activity screening method applicable for these fractions was ABTS radical cation decolorization assay. This latter is widely used to assess the total amount of radicals that can be scavenged by an antioxidant, i.e. the antioxidant capacity. In the present investigation, this method showed quite similar results compared to those obtained in DPPH reaction (Fig. 2). The ethyl acetate fraction was the strongest radical scavenger in both tests: it nearly fully scavenged $ABTS^+$ (the absorption after 6 min was close to 0). The activity of petroleum ether fraction in the ABTS test was remarkably lower than in the DPPH test.

It should be noted that, in most cases, the reaction with $ABTS^+$ was quite fast and completed in 6 min (except for petroleum ether fraction). During the remainder of the reaction time, the changes in absorbance were negligible.

IC_{50} values concentration of sample required to scavenge 50% of free radicals were calculated from the regression equations of graphics prepared from fraction concentrations and percentage inhibition of free radicals in the DPPH and ABTS assays (Table 3).

Compared to IC_{50}, the free radical-scavenging potency of the ethyl acetate and methanol fraction was 0.090 and 0.096 mg/ml respectively, which were very near to that of the Trolox, as a positive reference drug, demonstrating the strong ability of these extracts to act as a donor for hydrogen atoms.

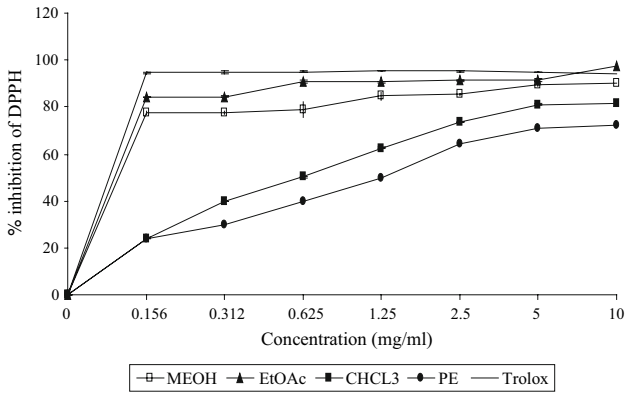

Fig. 1 The percentage bleaching of free DPPH radical in the presence of different concentrations of antioxidant fractions. *Abbreviations*: PE: Petroleum ether fraction, $CHCl_3$: Chloroform fraction, EtOAc: Ethyl acetate fraction, MEOH: Methanolic fraction

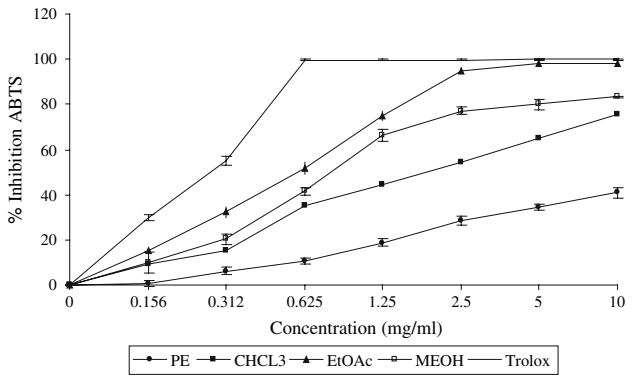

Fig. 2 The percentage bleaching of ABTS radical cation in the presence of different concentrations of antioxidant fractions (6 min). *Abbreviations*: PE: Petroleum ether fraction, $CHCl_3$, Chloroform fraction, EtOAc: Ethyl acetate fraction, MEOH: Methanolic fraction)

Petroleum ether fraction improved 50% inhibition at higher concentrations, indicating less antioxidant capacity than the positive control.

The TEAC of each fraction at each time interval was determined and plotted (Fig. 3). Since TEAC is a measurement of the effective antioxidant activity of the extract, a higher TEAC would imply greater antioxidant activity of the sample. It was observed that at 6 min point, ethyl acetate fraction had the highest TEAC of 2.27 mM (Table 3), followed by methanolic (1.85), chloroformic (1.31) and petroleum ether fraction (0.69).

The dose–response curve obtained by analysis of a range of Evax extracts concentrations and of Trolox at different time points (5, 10, 15 and 20 min) was plotted as the percentage inhibition of the $ABTS^{+}$ solution absorbance as a function of time at 0.156 mg/ml and at 2.5 mg/ml (Fig. 4). For all tested fractions and in both concentrations, the increase in percentage inhibition is very important within the first 5 min, then it slows down till 20 min.

These findings suggested that there can be a correlation between the antioxidant activity and phenolic compounds. Their antioxidant properties have been well and widely studied by several authors (Pietta 2000; Candan et al. 2003). Flavonoids are one of the most numerous and widespread group of phenolics in higher plants (Czinner et al. 2000; Tepe et al. 2005). Pietta (2000) suggested that plant flavonoids, which show potent activity in vitro, also function as antioxidants in vivo, and their protective effects may be attributed to their scavenging ability towards free radicals. However, further investigation is required to correlate the antioxidant activity and polyphenol contents and to clarify the mechanism of plant flavonoids action in vivo.

In a previous study, methanol extract of *Helichrysum plicatum* subsp. *Plicatum*, another species of the Asteraceae family and belonging to the same subtribe Gnaphaliinae, has been reported to have antioxidant activity using two in vitro methods: DPPH and β-carotene-linoleic acid assays (Tepe et al. 2005).

Moreover, it has been recently reported that flavonols are known as important compounds in terms of radical-scavenging properties (Miliauskas et al. 2004). Phenolic coumarins could account for the protection of the plant against oxidative stress (Torres et al. 2006).

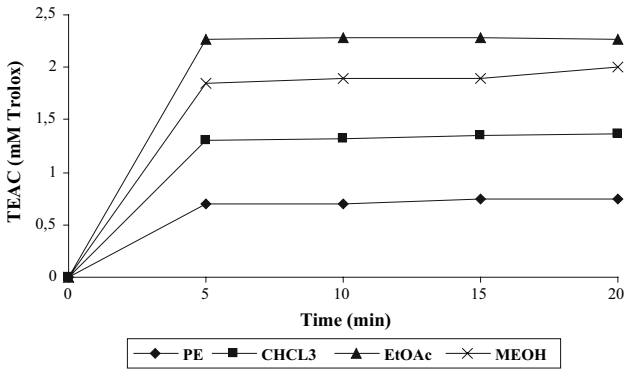

Fig. 3 The variation of the Trolox Equivalent Antioxidant Capacity of each fraction with time. *Abbreviations*: PE: Petroleum ether fraction, $CHCl_3$, Chloroform fraction, EtOAc: Ethyl acetate fraction, MEOH: Methanolic fraction

Piao et al. (2004) reported that DPPH radical-scavenging activity in furanocoumarins was correlated with the number of phenolic hydroxyl groups present in their structures.

The biological and pharmacological effects of antioxidants have attracted much interest with regard to their protective role against free radical damage that may be the cause of many diseases including cancer and diabetes (Czinner et al. 2000). Furthermore, Ivanova et al. (2005) reported that the roles of herbal tea in disease prevention and cure have been partly attributed to the antioxidant properties of phenolic compounds present in the extracts.

Conclusion

This preliminary screening is an interesting evaluation of the potential antimicrobial and antioxidant activity of *E. pygmaea*. On the light of these experiments, it could be concluded that the different fractions of *E. pygmaea* aerial parts exhibited an interesting antibacterial activity against all strains tested except *P. aeruginosa*. The ethyl acetate and methanolic extracts possess strong antioxidative activity. The presence of these antioxidants is a desirable feature which may have beneficial health effects on prevention of many diseases. Further studies need to be carried out to define active principle(s) of fractions and to study the

Table 3 Effects of methanolic extracts and positive control on the in vitro free radical (DPPH, ABTS)

Antioxidant	IC_{50} (mg/ml)		TEAC (mM)
	DPPH°	ABTS° (6 min)	(6 min)
Methanolic fraction	0.096	0.801	1.85
Ethyl acetate fraction	0.090	0.572	2.27
Chloroform fraction	0.624	1.875	1.31
Petroleum ether fraction	1.250	19.985	0.69
Trolox	0.078	0.273	

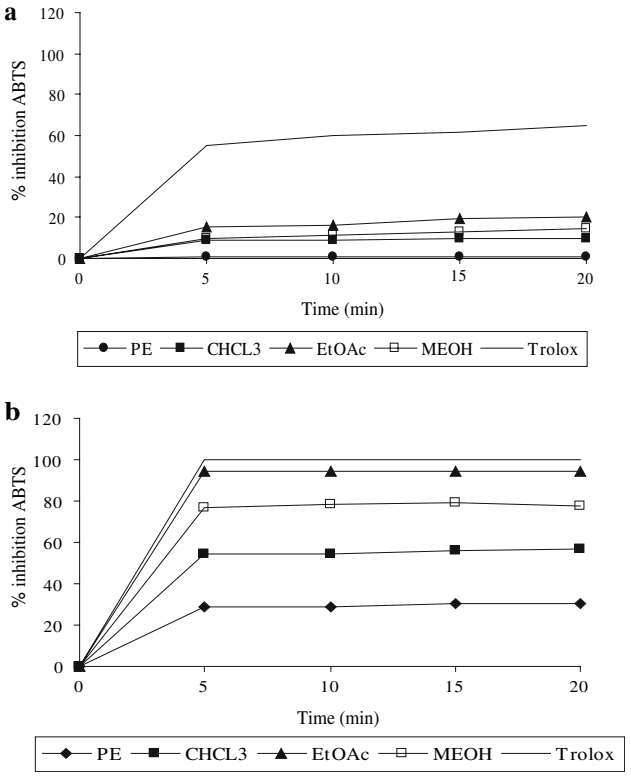

Fig. 4 The percentage inhibition of free ABTS at the individual time points in the presence of 0.156 mg/ml (**a**) and 2.5 mg/ml (**b**) of fractions. *Abbreviations*: PE: Petroleum ether fraction, $CHCl_3$, Chloroform fraction, EtOAc: Ethyl acetate fraction, MEOH: Methanolic fraction

relation between chemical structure and antioxidant activity in vitro and in vivo and the mechanisms by which they may exhibit pharmacological actions.

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