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Antioxidant and anti-listerial activities of selected Egyptian medicinal plants

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This work investigates the phenolic, antioxidant capacity of crude extracts of eight Egyptian medicinal plants (Syrian oregano, marjoram, rosemary, lemongrass, thyme, yarrow, marigold and sweet wormwood) and estimates their activity against *Listeria monocytogenes,* one of the most virulent food borne pathogens. Antioxidant activity of Rosemary (70.6±1.65%) and thyme (70.8±1.72%) based on TBA assay was significantly higher compared to other plants and ascorbic acid. Rosemary was found to possess the best antilisterial activity with lowest MBC (8 µl/ml); while its total phenolic content (TPC) represented 69.73 ± 0.47 mg/g GAE. Thyme showed MBC of 46 µl/ml with TPC 96.85±0.56 mg/g GAE. Lemon grass and marigold showed considerable antilisterial activity (MBC 31, 46 µl/ml respectively), although they had lower phenolic contents and low thiobarbituric acid inhibition. Sweet wormwood, marjoram and yarrow were inactive against listeria. Rosemary and thyme appeared as possible alternatives for synthetic food additives and preservatives.

Key words: Medicinal plants, anti-listerial, antioxidant, rosemary, thyme.

INTRODUCTION

Listeria monocytogenes is the causative agent of listeriosis. In the 1980s, a number of outbreaks of listeriosis occurred, in which contaminated foods were identified to be the source of transmission. Fresh and frozen meat, poultry, seafood products, fruits and vegetable products have also been involved in *Listeria monocytogenes* outbreaks (Schlech and Acheson, 2000).

L. monocytogenes is one of the most virulent food borne pathogens, with 20 to 30% of clinical infections resulting in death (Ramaswamy et al., 2007); its fatality rates even exceed that caused by *Salmonella* and *Clostridium botulinum* (Dharmarha, 2008). It is resistant to different environmental conditions, including acid pH, high NaCl concentration, and refrigeration temperatures. It can grow in many foods when stored at refrigeration temperatures (Embarek and Huss, 1993).

Listeria species are susceptible to antibiotics active against Gram (+) bacteria, but more recently, reports on antibiotic resistance in *Listeria* species have been published. Current therapy of choice for all forms of listeriosis is a combination of ampicillin - gentamicin (Moellering et al., 1982). Herbs and spices are widely used components

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in food preparation and as natural and safe food preservatives for preventing bacterial and fungal growth (Zheng and Wang, 2001; Lanciotti et al., 2004). Plants synthesize many compounds with complex molecular structures and some of them have been related with antimicrobial properties found in plant and their derivatives. Among these secondary metabolites are alkaloids, flavornoids, isoflavonoids, tannins, coumarins, glycosides, terpenes and phenolic compounds (Simoes et al., 1999).

Recently, there has been increasing interest in discovering new natural antimicrobials (Sagdic et al., 2003) due to the great concern of consumers about food free or with lower level of chemical preservatives because these could be toxic to humans (Bedin et al., 1999).

Several plants are known in the Egyptian market as palatable and having culinary uses or used as herbal tea. The aim of this study was to (i) determine the phenolic content of the crude extract of eight medicinal plants widely cultivated in Egypt [oregano (*Origanum syriacum*), marjoram (*Majorana hortensis*), rosemary (*Rosmarinus officinalis*), lemongrass (*Cymbopogon citratus*), thyme (*Thymus vulgaris*), yarrow (*Achillea millefolium*) and sweet wormwood (*Artemisia annua*)]; (ii) to determine the antioxidant activity of the Egyptian crude extracts using two different antioxidant tests; and (iii) to determine their effectiveness against *L. monocytogenes*.

MATERIALS AND METHODS

Materials

Fresh plant materials: Oregano (*Origanum syriacum*), marjoram (*Majorana hortensis*), rosemary (*Rosmarinus officinalis*), lemon grass (*Cymbopogon citratus*), thyme (*Thymus vulgaris*), yarrow (*Achillea millefolium*), marigold (*Calendula officinales*) and sweet wormwood (*Artemisia annua*) were collected in April 2008 from Sekem Company Farm located at Bilbeis City, Sharkea Governorate, 85 km East north of Cairo, Egypt. The cultivation is certified for organic biodynamic agriculture by COAE (Center of Organic Agriculture in Egypt). The identification of the plant material was performed by Prof. Dr. Kamal Zayed, Botany Department, Faculty of Science, Cairo University (Egypt). Voucher samples (No.: RS-4, RS-10) were kept in the Herbarium, Pharmacognosy Department, Faculty of Pharmacy, October University for Modern Sciences and Arts, Giza, Egypt.

Chemicals

All chemicals used, including solvents, were of analytical grade. DPPH, folin Ciocalteu's phenol reagent and gallic acid were purchased from Sigma-Aldrich Chemie, Steinheim, Germany.

Preparation of plant extract

Three hundred grams of dried, ground plant materials were exhaustively extracted with 70% ethanol at room temperature. The combined hydroalcoholic extracts were then filtered, evaporated under vacuum (45°C) and stored at 4°C.

Total phenols

The total phenolic content (TPC) in the extracts was investigated by the Folin-Ciocalteu method, using gallic acid as the standard and the results were expressed as gallic acid equivalent (GAE) (Perry and Shetty, 1999). The assay was developed by Chandler and Dodds (1983) and modified by Shetty et al. (1995). Stock solution of plant extract (0.1 g) was dissolved in 1 ml methanol, and then 1ml of the above extract was transferred to a test tube. 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% (v/v) Folin-Ciocalteu phenol reagent (Sigma Chemical Co., St. Louis, MO) were added. After an incubation period of 5 min, 1 ml of 5% Na_2CO_3 was added, mixed well and kept in the dark for one hour. Then the samples were vortexed and the absorbance was measured at 765 nm using a UV spectrophotometer (SpectonicGenesys5; Milton Roy Company, Rochester, NY) and compared to a gallic acid calibration curve. Total phenols were calculated as GAE and the values are presented as means of triplicate analyses.

1, 1-Diphenyl-2-picrylhydrazyl radical-scavenging

The ability of the extracts to scavenge 1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH) was estimated using the method modified by Tagashira and Ohtake (1998). Total extracts were screened at 100 µg/ml (200 µl methanolic extract was added to 2 ml (6×10^{-5} M) methanolic solution of DPPH) and the absorbance was measured at 517 nm using an HP 8451 spectrophotometer (Hewlett-Packard). Ascorbic acid was used as positive control. Percentage inhibition (%) was calculated using the following equation,

$$\% I = [(A_B - A_S)/A_B] \times 100 \quad (1)$$

Where, I is the DPPH inhibition (%); A_B is the absorbance of control sample (t = 0 h) and A_S is the absorbance of a tested sample at the end of the reaction (t = 1 h). The values were presented as the mean of triplicate analyses.

Thiobarbituric acid assay

The potential of plant extracts to inhibit peroxidation of linoleic acid was assessed based on a procedure described by Ottolenghi (1959) and Kikuzaki and Nakatani (1993). Ascorbic acid was used as reference compound. The Thiobarbituric acid Assay (TBA) measures the total peroxide content at a later stage of lipid oxidetion, involving the quantitation of the secondary products formed from oxidation. Samples (4 mg) were dissolved in 4 ml of 99.5% ethanol, 4.1 ml of 2.51% linoleic acid in 99.5% ethanol, 8.0 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water contained in screw cap vial (final concentration of 0. 02% w/v). 1.0 ml of 20% aqueous trichloroacetic acid and 2.0 ml of aqueous thiobarbituric acid solution (0.67%) were added to 2.0 ml of the sample solution. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant (at 532 nm) was measured every 24 h until a day after the absorbance of the control reached maximum value (day seven). Antioxidant activity was recorded based on absorbance on the final day (7^{th} day) (L1). A control containing linoleic acid and other additives without antioxidants, representing 100% lipid peroxidation, was also prepared (L2). The blank L1 and L2 solutions were prepared as described above but without linoleic acid. Percent of inhibition was calculated using equation 1.

$$\text{Percentage of peroxidation inhibition} = [1- (L1/L2)] \times 100 \quad (2)$$

Table 1. Total phenolics, DPPH and TBA % inhibition of different hydroalcoholic extracts.

Parameter	Marigold	Lemon-grass	Oregano	Thyme	Sweet wormwood	Rosemary	Yarrow	Marjoram	Ascorbic acid
Total Phenolics (mg/g GAE)	22.08 ±0.32	24.79±0.45	81.35±0.34	96.85±0.56	37.97±0.21	69.73±0.47	58.11±0.31	69.73±0.8	-
DPPH % inhibition	58.17±0.92	54.18±0.75	55.78±0.64	52.19±0.75	40.50±0.53	66.80±0.53	61.49±0.23	60.16±0.5	72.54±0.25
TBA % inhibition	2.6±0.76	2.7±0.54	25.39±1.1	70.8±1.72	49.6±1.3	70.6±1.65	51.2±0.98	63.6±0.45	51.2±0.74

Antimicrobial activity

Inhibitory effect by disc diffusion method

Listeria monocytogenes Z7 serotype 1 was obtained from Dr. Aza Abo Elnaga. The antilisterial activities of the crude extracts were individually assayed by the standard disc diffusion method (Perez et al., 1990). Sterile filter paper disks (9 mm in diameter) (Schlinder and Schuell, Dassel, Germany) were impregnated with 0.1 ml (100mg/1ml dissolved in dimethylsulphoxide (DMSO) plant extracts. *L. monocytogenes* (0.1 ml of 10^6 CFU/ml) was inoculated into Trypticase soya agar (TSA, Oxoid) media by spreading the bacterial inoculums on the media. Control disc containing neat solvents (negative control) was also run parallel in the same plate. The plates were incubated at 37°C for 18 h. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition for the respective drug in millimeters (Perez et al., 1990). All tests were performed in triplicate.

The minimum inhibitory concentration (MIC)

MIC was calculated according to the method modified by Hamza et al. (2006). Stock solutions of dried methanolic extract were prepared by dissolving 100 mg of each extract in 1 ml of DMSO. Solution was sterilized by filtration through 0.22 µm Millipore filter. Three milliliter of media (nutrient broth) was inoculated with 100 µl of the inoculum. 25, 50, 75, 100, 150 µl of plant extracts were added to the previously prepared media to reach a final concentrations of 8, 16, 23, 31, 46 µg/ml. The test solutions were incubited for 24 h. DMSO was used as the control, after which readings were performed by comparing growth in the control, test solution and blank (extract, uninoculated plate) and measured spectrophotometrically at 405 nm. Inhibitory concentration (I) was calculated using equation 3.

I% = (1- Absorbance of sample / absorbance of control) x 100 (3)

MIC was determined by sub-culturing each clearly, optically (no growth seen). Fifty microliter (50 µl) was removed from clear solution and inoculated onto TSA media and incubated for 24 h at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth. The lowest concentration of extract required to completely destroy test microorganisms (no growth on the TSA plate) after incubation at 37°C for 24 h was reported as minimum bactericidal concentration (MBC) according to the study of Hawser and Islam (1990).

RESULTS

Total phenol

Results of TPC are shown in Table 1. The TPC varied from 22.08±0.32 to where thyme showed the highest content (96.85 ± 0.56 mg/g GAE) > Syrian oregano > rosemary and marjoram > Yarrow > sweet wormwood > lemongrass. The lowest content was found in marigold.

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging

As shown in Table 1, Rosemary showed the highest DPPH free radical scavenging activity followed by yarrow and marjoram compared to the other extracts.

Thiobarbituric acid assay

The potency of thiobarbituric acid reactive substances of different plant extracts with inhibitory activity is presented in Table 1. Rosemary (70.6 ± 1.72%) and thyme (70.8 ± 1.3%) have nearly the same activity followed by marjoram with higher potency and then ascorbic acid; while yarrow had the same potency as ascorbic acid (51.2 ± 0.74%). Marigold and lemongrass showed the lowest inhibitory activity.

Antilisterial activity

The antilisterial activities of the eight plant extracts estimated by the disc diffusion method are shown in Table 2. Rosemary, lemongrass, marigold and thyme showed considerable antilisterial activity. The lowest MBC concentration was observed with Rosemary (<8 µg/ml) followed by lemongrass (31 µg/ml), thyme calendula (46 µg/ml) and then oregano (>46 µg/ml).

DISCUSSION

Nowadays, plant derived food additives have become a great concern. According to the study of Dimitrijević et al. (2007), the increased demand of consumers for additive-free, fresher, more natural tasting foods, that impact the environment a little and maintain microbiological safety has provoked many researchers to investigate the antimicrobial effects of natural compounds.

Through this study, there was remarkable difference in phenolic contents, antioxidant activity as well as antilisterial potency of the different plants. Thyme had the highest phenolic content

Table 2. *In vitro* antilisterial activity of the different extracts.

Plant	Inhibition zone (mm)	inhibitory concentration %					MBC µg/ml
		8 µg/ml	16 µg/ml	23 µg/ml	31 µg/ml	46 µg/ml	
Thyme	17	50	51.34	65.6	93	100	46
Syrian oregano	9	27.3	36.6	45.8	63.56	76.34	>46
Sweet wormwood	0	-	-	-	-	-	-
Marjoram	0	-	-	-	-	-	-
Rosemary	27	100	100	100	100	100	<8
Lemongrass	23	76.34	82.5	87.2	100	100	31
Marigold	18	51	52.2	70	94.8	100	46
Yarrow	0	-	-	-	-	-	-

(96.85 ± 0.56 mg/g GAE) while marigold had the lowest (22.08 ± 0.32 mg/g GAE). Phenolics represent an important class of phytochemicals present in almost all plants and contribute to the development of colour, taste and palatability, as well as the defense system of plants (Tarnai et al., 1994). Phenolics are commonly found in both edible and inedible plants; extracts of spices are rich in phenolics (Wojdylo et al., 2007).

They are able to act as antioxidants in a number of ways. Phenolic hydroxyl groups are good hydrogen donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of generation of new radicals (Valentão et al., 2003; Pereira et al., 2009).

Plant extracts might substitute synthetic food antioxidants, which may influence human health when consumed chronically (Martínez-Tomé et al., 2001). There are many different methods for determining antioxidant function and each depends on a particular generator of free radicals, acting by different mechanisms (Huang et al., 2005). Antioxidant activity was determined by two spectrophotometric methods: DPPH and TBA assay.

The DPPH radical is commonly used for the assessment of antioxidant potency *in vitro* (Zhou and Yu, 2004). By increasing the concentration of phenolic compounds or degree of hydroxylation of the phenolic compounds, their DPPH radical scavenging activity also increases (Sanchez-Moreno et al., 1999).

DPPH assay revealed Rosemary as the highest radical scavenger (66.80±0.53) followed by yarrow (61.49 ± 0.23) and marjoram (60.16 ± 0.5) compared to the other extracts (Table 1). Rosemary has been widely accepted as one of the spices with the highest antioxidant activity (Peng et al., 2005). The TBA method measured the amount of peroxide produced at a later stage of lipid oxidation when peroxide decomposes to form carbonyl compounds. It is a sensitive method and achieves reproducible results. This method is preferable in order to obtain useful data in an environment similar to real-life situation (Kulisic et al., 2004). Concerning the TBA inhibitory activity, rosemary, thyme and marjoram were superior to ascorbic acid while marigold and lemongrass had the lowest inhibitory activity (Table 1).

Several reports revealed the significant antioxidant and antimicrobial activities of Rosemary (Genena et al., 2008; Tavassoli and Djomeh, 2011) in addition to a protective effect against kidney injury induced by CCl_4. This effect may be attributed to its antioxidant activity (Sakr and Lamfon, 2012). In addition, thyme extract was reported to have tremendous potential to prevent or reverse the changes induced by paracetamol toxicity back to normal via its antioxidant activity (Abd El Kader and Mohamed, 2012).

In this study, there was weak correlation between total phenolic content and antioxidant activity, which is similar to the study of Yang et al. (2002), Bajpai et al. (2005) and Sengul et al. (2009). Total phenolic content determined according to the Folin-Ciocalteu method is not an absolute measurement of the amount of phenolic materials. Different types of phenolic compounds have different antioxidant activities, depending on their structure (Sengul et al., 2009).

Koleva et al. (2002) revealed that the difference in antioxidant activity observed in both DPPH and TBA assays depends on the chosen concentration, nature and physicochemical properties of the studied antioxidants. In the present study, different antioxidative values were observed while comparing both methods. The radical scavenging activity was not similar to lipid peroxidation inhibition showing that radical scavenging activity is a not unique factor to suppress lipid peroxidation (Özgen et al., 2011).

The antibacterial effects of phenolic compounds on pathogenic microorganisms in food were previously confirmed by several authors (Wen et al., 2003; Puupponen-Pimiä et al., 2005); therefore, plants with high phenolic contents are expected as potent antimicrobials.

The potent antilisterial action of Rosemary shown in Table 2 with the highest inhibition zone (27 mm) may be attributed to the high phenolic content (69.73 ± 0.47 mg/g), the highest percentage of radical scavenging activity (66.80 ± 0.53%) and high TBA % inhibition. The high antilisterial activity of rosemary is in agreement with the study of Bubonja-Sonje et al. (2011).

Lemongrass follows marigold in activity with MBC 31 µl/ml. The antimicrobial effect of lemongrass was previously studied by Sikkema et al. (1994) who declared that the mechanism of action of monoterpenes had toxic effects on the structure and function of the cell membrane. As a result of their lipophilic character, the monoterpenes will preferably dislocate from the aqueous phase towards the membrane structures. This would justify the potent antilisterial effect of lemongrass, although with low phenolic content (24.79 ± 0.45 mg/g GAE).

Marigold and thyme showed a MBC of 46 µl/ml in agreement with the broad spectrum antimicrobial activity revealed by Nand et al. (2012). This may be attributed to the previously reported sitosterol, stigmasterol, taraxasterol, lupeol, faradiol 3-O-Laurate in addition to quercetin isoquercetin and calendoflaside (Vidal-Ollivier et al., 1989).

Thyme antilisterial activity is mainly attributed to the high phenolic contents in addition to terpenes which account for their antimicrobial activity (Farag et al., 1989; Viuda-Martos et al., 2010).

These results showed that rosemary and thyme are potent antioxidant, while rosemary and lemongrass are potent antilisterial. These results are promising and further studies may be helpful for applying them in industrial field as food preservatives.

Conclusion

Rosemary, thyme and lemongrass appeared as possible alternatives for synthetic food additives and preservatives due to their potent antilisterial activity and their high antioxidant content.

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