SUPPLEMENTARY MATERIAL

Phytochemicals, Antioxidant, Anti-Acetyl-cholinesterase, and Antimicrobial Activities of Decoction and Infusion of *Pelargonium graveolens*

Malek Ennaifer[a,b*],

Taroub Bouzaiene[a],

Chokri Messaoud[a] and Moktar Hamdi[a]

[a]University of Carthage, Institut National des Sciences Appliquées et de Technologie (INSAT), 1080 Tunis, Tunisia_LETMI – INSAT ; [b]High Institute of Food Industry, Tunis (ESIAT)

*Corresponding Author: MalekEnnaifer, e-mail: malekebm@gmail.com

Abstract: In Tunisia, *Pelargonium graveolens* is widely consumed as a food aromatizing hydrosol. Recent studies have shown the potential of plant solvent-free extracts as food and pharmaceutical natural additives. Accordingly, in this study, we investigate the phenolic content, the volatile fractions of green *P.graveolens* extracts such as infusion and decoction, and we evaluate their biological activities. The total phenolic content of the infusion (27.05 mg GAE/gDM) is significantly different from that of decoction (31.2 mg GAE/gDM). The GC-MS analysis identified about twenty volatile components in both extracts. The DPPH inhibition and the β-carotene bleaching tests of the infusion and the decoction had considerable results. Besides, infusion and decoction exhibited a relatively high anti-acetyl-cholinesterase activity and a considerable antimicrobial activity against *S. aureus*, among three tested pathogenic bacteria.

Keywords: anti-acetyl-cholinesterase, antimicrobial, antioxidant, decoction, infusion, phenolic and volatile compounds.

Experimental section

All chemicals used in this work were purchased from Sigma (Tunisia)

Plant material

Geranium aerial parts were harvested from a random sample of a plant growing in Ariana (North of Tunisia: latitude 36°51′36″ N, longitude 10°11′36″ E, altitude 10 m) in April 2014. The plant was identified by Professor ChokriMessaoud and a voucher specimen (PG2014) was deposited in the herbaria of National Institute of Applied Science and Technology of Tunisia for future reference. *Pelargonium graveolens* leaves, flowers and stems were isolated manually from the roots in our laboratory to obtain a weight of 1.00 kg and dried at room temperature for 2 weeks.

Decoction and Infusion preparation

Infusion preparation. 10.00 g of the dried plant material, finely ground, were infused into boiling water (10%, w/v). After 10 min the mixture was filtered over the Büchner funnel. The extraction was made in triplicate. The resulting infusions were kept at 4 ◦C for further use.

Decoction preparation. 10.00 g of the ground powder were boiled for ten minutes into distilled water (10%, w/v). The flask was then allowed to cool and the mixture was then filtered over the Büchner funnel. The decoction was prepared in triplicate. The resulting decoctions were kept at 4 ◦C.

Phytochemical composition

Total phenolic. Total phenolic contents were determined using theFolin–Ciocalteu method(Messaoud et al. 2012a)

Flavonoid contents. Flavonoid contents were determined according to Djeriidane and al. (2006). Briefly, 0.5 mL of each diluted extract was mixed with 0.5 mL of 2% $AlCl_3$methanolic solution. After 30 min, the absorbance was read at 430 nm. Flavonoid contents were calculated from a calibration curve of rutin and expressed as milligrams of rutin equivalent per gram of dry matter (mg RE/gDM). The results are presented as means of 3 determinations.

Condensed Tannins. The content of condensed tannins was estimated according to (Sun et al. 1998) with modifications. To 100 µl of each extract (diluted 40 times), 600 µl of vanillin (4% in methanol) and 300 µl of concentrated hydrochloric acid (37%) are added. the mixture is well mixed and left for 15 min before measuring the absorbance at 500 nm. The content of condensed tannins, expressed in mg catechin equivalent per gram of plant material (EC mg / g DM), is determined from a standard range of catechins. For each sample, the experiment was run 3 times.

Volatile compounds. The volatile compounds of the *Pelargonium graveolens* aqueous extracts (decoction and infusion) were isolated by liquid-liquid extraction with hexane according to Radulescu and al. (2004). Briefly, 30 mL of each extract were put in a glass vial and mixed with 30 mL n-hexane. After agitation for 24 h, 50 mL of the supernatant was separated by decantation, dried over anhydrous sodium sulfate, and then concentrated to 5 mL by evaporation under vacuum at 40°C. GC-MS analyses were performed with an Agilent 7890A gas chromatograph (HP-5MSfused silica column (30 m × 0.25 mm; 0.25 µm film)), interfaced with an Agilent mass selective detector.

Biological activities

Antioxidant activity tests

Free radical scavenging activity. The DPPH radical scavenging capacity was assessed according to Anton and al. (2008). Percentages of radical inhibition (% I)were estimated as:

$$\%I = 100 \times \frac{(A0 - A1)}{A0}$$

where A_0 is the control absorbance and A_1 is the test sample absorbance. The scavenging activity was determined from a standard range of Trolox (from 12.5 to 100µg/mL) as milligram equivalent Trolox per gram of dried matter (mgTX/gDM). All tests were carried out in triplicate.

*Inhibition of β-carotènebleaching.*According to Messaoud et al. (2012b), 2 mg of β-carotene were dissolved in chloroform (20 mL). Four milliliters of this solution were mixed with linoleic acid (20 mg) and Tween 40 (200 mg). After the chloroform was removed at 40 ◦C under vacuum, 60 mL of oxygenated ultra-pure water was added, and then the emulsion was vigorously shaken.Aliquots (0.75 mL) of this preparation were poured into tubes containing different extract concentrations(0.05 mL). Once the emulsion was added, the initial

absorbance (t0) was measured at 470 nm and the test emulsion was incubated in a water bath at 50 ◦C for 120 min when the absorbance was measured one more time. In the negative control, the same volume of methanol replaced the infusion. The corresponding activity was evaluated using the following formula:

$$\%\text{Inhibition} = 100 \times \left[\frac{(\text{At} - \text{Ct})}{(\text{C0} - \text{Ct})}\right]$$

where A_t and C_t are the absorbance of the test sample and control, respectively, after incubation for 2 hours, and C_0 is the absorbance of the control measured at zero time. Tests were carried out 3 times.

Acetylcholinesterase inhibitory activity assay

The anti-acetylcholinesterase activity of each sample was determined according to the method of Kiendrebeogo and al. (2011) with a slight modification. In this method, 25 µL acetylcholinesterase solution (0.28 U/mL), 100 µL sample diluted in275µL of Tris-HCl buffer (50 mMTris-HCl, pH 8) and 0.1% of BSA were mixed and incubated at 37°C for 15 min. Then, 500µL DTNB (0.15 mM) and 100µL ATCI (0.3mM) were added. The reaction mixture was incubated at 37°C for 30 min. Then, the absorbance was measured at 405 nm using a UV-visible spectrophotometer. The blank contained the acetylcholinesterase enzyme instead of the buffer. The control was prepared in the same manner by replacing the sample with Tris-HCl buffer. The percentage of inhibition was calculated using the following formula:

$$\%\ \text{inhibition} = 100 \times \left[\frac{\left(\text{A Control} - \left(\text{A Sample} - \text{A Sw}\right)\right)}{\text{A Control}}\right]$$

Where$A_{Control}$ and A_{Sample} are the absorbance values of the control and the sample, respectively. The A_{Sw} is the absorbance of the sample without the enzyme. Results were expressed as equivalent Donepezil (a drug used for the treatment of mild to moderate dementia of the Alzheimer's disease type).

Antimicrobial activity

The antibacterial activity of the aqueous extracts was determined according to Zaouali et al. (2010). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined. All tests were repeated twice.

Microbial strains. Antibacterial activities of the aqueous extracts were tested against *Escherichia coli* (ATCC10536), *Staphylococcus epidermis* (ATCC12228), and *Staphylococcus aureus*(ATCC6538). All the strains tested were collected at the microbiology laboratory in the National Institute of Applied Science and Technology of Tunisia (INSAT)

Well diffusion method. The agar well diffusion method (AWD)was used for the inhibition zones determination. Inoculums were prepared with fresh cultures of microbial strains, cultured on Trypticase soy broth at 37°C (18 h). The AWD method was used to determine the inhibition zones. One hundred microliters of a suspension of each tested bacteria were mixed with 5mL of technical agar (the final concentration of bacteria was 5.10^5 CFU/mL). After spreading the inoculated gelose on the surface of Tryptic soy agar plates(TSA), wells(d = 6 mm) were made in the Petri plates. Seventy microliters of each filter-sterilized extract, at a concentration of 100mgDM/mL, were dropped into each well. mg mL?1 Then, plates were kept at 4°C for 2 h followed by incubation at 37°Cfor 24 h. The results of the AWD method were presented as means of the inhibition zone measures (including well diameter). Gentamycin (30 µg) was used as a positive and negative control.

*MIC and MBC.*Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined in Tryptic soy broth (TSB) (Zaouali et al., 2010). The final concentration of bacteria was 10^5 CFU/mL of TSB. Serial dilutions of filter-sterilized infusions (100 to 5 mg DM/mL**)** were used. Therefore, each strain was tested on a range of extracts' concentrations from 1 to 0.05 mg/mL of the final medium. of The MIC was defined as the lowest concentration of extract that resulted in no visible growth after 24 h of incubation at 37°C(Okeke et al., 2001). A total of 100 µL from clear tubes were plated on Tryptic soy agar (TSA) plates. MBCs were determined as the highest dilution extract that caused no growth.

Data analysis

Determinations were conducted three times and results for each measured parameter were expressed as mean of Standard Deviation. Quantitative differences were assessed by ANOVA procedure (at $P<0.05$). Calculations were run using the SAS v.9.1.3 program. The correlation analysis between phenol contents and antioxidant activity values was carried out using the same program.

Table S1: Chemical composition (%) of the lipophilic fraction of *P. graveolens*extracts

Compound	Rt (min)	Decoction	Infusion
cis- Linalooloxide	9.347	5.19	2.42
Linalool	9.833	2.64	<0.1%
Silane, cyclohexyldimethoxymethyl	12.070	4.27	<0.1%
α-terpinolene	13.335	4.00	4.03
β-Citronellol	14.514	5.62	5.62
Geraniol	15.441	<0.1%	1.40
1-Iodononane	15.950	3.65	2.78
Tetradecane	19.864	3.85	3.85
1,2-benzenedicarboxylicacidDimethyl ester	21.781	5.21	5.45
Eicosane	22.645	<0.1%	2.27
N- Heneicosane	22.793	4.48	4.61
Methyldi-Tert-Butylphenol	23.783	7.95	7.41
DihydroActinidiolide	24.150	4.76	5.08
2-phenylethylcyclohexanoate	25.694	<0.1%	3.89
β-cadinene	26.782	<0.1%	1.04
Widdrolhydroxyether	27.709	9.32	12.05
Untriacontane	28.882	1.75	2.4
Heneicosane	28.956	6.76	7.25
1-Octadecanesulfonyl Chloride	29.219	8.37	9.70
Pentacosane	30.129	9.27	6.02
Docosane	34.489	3.96	4.90
2,5-Cyclohexadien-1-one, 2,6-di-tert-butyl-4-ethylidene-	34.649	7.64	8.63

Rt: Retention time, min.

Table S2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *P. graveolens* infusion and decoction

		MIC*		MBC*	
Testedbacteria	Source no.	Decoction	Infusion	Decoction	Infusion
E. coli	*ATCC10536*	R	R	_	_
S. epidermis	*ATCC 12228*	R	R	_	_
S. aureus	*ATCC 6538*	50	50	100	100

*Values of MIC and MBC were expressed as mg DM per mL of extract. R: the strain was resistant to the extract.

References

Djeridane A, YousfiiM, Nadjemi B, Boutasouna D, Stocker P, Vidal N. 2006. Antioxidant activity of some Algerian medicinal.plants extracts containing phenolic. compounds. Food Chem. 97:654–60.

Kiendrebeogo M, Coulibaly AY, Nebie RCH, Zeba B, Lamien CE, Lamien-Meda A, Nacoulma OG. 2011. Antiacetylcholinesterase and antioxidant activity of essential oils from six medicinal plants from BurkinaFaso. Rev Bras Farmacogn. 21:63–69.

Messaoud C, Chograni H, Boussaid M. 2012a. Chemical composition and antioxidant activities of essential oils and methanol extracts of three wild Lavandula L. species.Nat ProdRes. 21:1976–84.

Messaoud C, Labidi A, Boussaid M. 2012b. Myrtuscommunis L. infusions: the effect of infusion time on phytochemical composition, antioxidant., and antimicrobial. activities. J Food Sci. 77: C941-7.

Okeke, M.I., Iroegbu, C.U., Eze, E.N., Okoli, A.S., Esimone, C.O., 2001. Evaluation of the root of Landolphiaowerrience for antibacterial activity. J Ethnopharm 78, 119–127

Radulescu V, Chiliment S, Oprea E. 2004. Capillary gas chromatography-mass spectrometry of volatile and semi-volatile compounds of Salviaofficinalis. J Chromatogr A. 1027: 121–6.

Rodriguez Vaquero, M.J., Alberto, M.R., MancadeNadra, M.C., 2007. Antibacterial effect of phenolic.compounds from different wines. Food Control 18, 93–101

Singleton V. L., Rossi J. A. 1965.Colorimetry of total phenolics.withphosphomolybdic-phosphotungstic. acid reagents. Am J EnolVitic. 16: 144–158.

Sun B, Riicardo-da-Silva JM, Spranger I. 1998. Critical Factors of Vanilin Assay for Catechins.andProanthocyanidins. J Agric Food Chem. 46:4267–4274.

Zaouali Y, Bouzaine T, Boussaid M. 2010. Essential oils composition in two RosmarinusofficinalisL .varieties and incidence for antimicrobial. and antioxidant. activities. Food ChemToxicol. 48:3144–3152.