Chemical Composition and *in vitro* Antimicrobial and Antioxidant Activities of *Citrus aurantium* L. Flowers Essential Oil (Neroli Oil)

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Abstract: Neroli essential oil is extracted from the fragrant blossoms of the bitter orange tree. It is one of the most widely used floral oils in perfumery. In this study chemical composition and *in vitro* antimicrobial and antioxidant activities of neroli oil are investigated. The essential oil of fresh *Citrus aurantium* L. Flowers (Neroli oil) cultivated in North East of Tunisia (Nabeul) were analyzed by GC-FID and GC-MS. About 33 compounds were identified, representing 99% of the total oil. Limonene (27.5%) was the main component followed by (E)-nerolidol (17.5%), α-terpineol (14%), α-terpinyl acetate (11.7%) and (E, E)-farnesol (8%). Antimicrobial activity was determined by Agar-well-diffusion method against 6 bacteria (3 Gram-positive and 3 Gram-negative), 2 yeasts and 3 fungi. Neroli oil exhibited a marked antibacterial activity especially against *Pseudomonas aeruginosa*. Moreover, Neroli oil exhibited a very strong antifungal activity compared with the standard antibiotic (Nystatin) as evidenced by their inhibition zones. Antioxidant activity determined by ABTS assay showed IC_{50} values of 672 mg L^{-1}. Finally, this study may be considered as the first report on the biological properties of this essential oil. The results of this study have provided a starting point for the investigations to exploit new natural substances present in the essential oil of *C. aurantium* L. flowers.

Key words: *Citrus aurantium* L., neroli, antimicrobial activity, antioxidant activity, gas chromatography-mass spectrome

INTRODUCTION

Citrus aurantium L., commonly named sour or bitter orange is a tree that belongs to the Rutaceae family. In Tunisia, *C. aurantium* L. is grown in warm regions shielded from strong winds, mainly in Cap Bon region which covers an area of 1500 ha. Sour orange trees are very resistant to cold, water excess and several diseases. For these main qualities, sour orange is widely used as a rootstock (Lota *et al*., 2001).

C. aurantium L. is among the species most frequently used for both traditional and modern medicinal purposes. Previous studies have underlined that preparations made up from peels, flowers and leaves of *C. aurantium* L. are popularly used for treating gastrointestinal tract disorders, for its diuretic action against tachycardia and rheumatism and for minimizing central nervous system disorders (Moraes *et al*., 2009). In addition, *C. aurantium* L. is a plant most frequently used as sedative at the outpatient clinics (Hernandez *et al*., 1984; Pultrini *et al*., 2006)

Recently, extracts of unripe fruits have gained significant popularity for the treatment of obesity, as an alternative to ephedrine alkaloids (Arbo *et al*., 2009).

These flowers give the orange blossom water, named locally 'zhar'. This floral water is used as food flavoring compounds (syrups, fruit salads, coffee and pastry) as well as corporal and body linen perfumes (Arbo *et al*., 2009). It is also used as therapeutic products (Ayadi *et al*., 2004). Traditionally, floral water was used, in Tunisia, as a cardiac stimulant and for carminative activity in the digestive tract and to calm babies and help them fall

asleep. It is also supposed to be helpful during detoxification programs or when quitting a habit or addiction like smoking (Arias and Ramon-Laca, 2005; Jeannot *et al*., 2005).

In addition to the aromatic waters, the distillation of sour orange flowers gives Neroli, a rare aromatic oil since to extract 1 kg of Neroli, 1000 kg of flowers is required. This volatile oil contains a sensual fragrance and forms the heart of one of the world's most enduring perfumes, 'eau de cologne'. It is also used in pharmacy as a flavouring agent. In fact, the Food and Drug Administration (FDA) has approved bitter orange flowers in small amounts even in some medicines (Jeff, 2002). Neroli oil has many therapeutic properties including sedative, calmative, tonic, cytophylactic, aphrodisiac, anti-depressant and antispasmodic (Jeannot *et al*., 2005).

Although, Neroli oil is produced in many countries such as Algeria, Morocco, Egypt, France and Spain, the oils produced in Tunisia have always been considered to be the finest and the most expensive ones.

Distillation of *C. aurantium* L. flowers activity represents a considerable value for Tunisian economy since it releases revenue between three and four million dinars per year in terms of exportation. Neroli is mainly exported to France, especially to Grasse region (NIS, 2008).

Inspite of the economic importance of the Tunisian Neroli oil, no attempt has been made to study their biological activities. To the best of our knowledge, only the chemical composition of the sour orange flower oil has been reported (Boussaada *et al*., 2007). This prompted us to investigate flowers of *C. aurantium* L. for Neroli oil constituents and *in vitro* antimicrobial and antioxidant activities.

MATERIALS AND METHODS

Chemicals: All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

Plant material: Bitter orange flowers (*C. aurantium* L.) were collected by hand in the beginning of May 2009 from the Cap Bon of Tunisia, precisely in the surroundings of Nabeul localized in 5 m of altitude and 1044 of longitude. In May 2009, Nabeul average rainfall was 31 mm, the average of Temperature was 20.5°C and humidity was 79%. These data were provided by National Institute of Meteorology of Tunisia.

Isolation of essential oils: Fresh flowers were subjected to hydrodistillation using a Clevenger-type apparatus (European Pharmacopeia, 1975). Extraction was performed in triplicate. During every experiment, a portion (100 g) of plant material was submitted for 3 h (after the falling of the first drop of distillate) to water distillation. The essential oil yield (R) is defined as follows:

$$R\,(\%) \quad \frac{\text{Essential oil mass}}{\text{Fresh flowers mass}} \times 100$$

The obtained essential oils were dried over anhydrous sodium sulfate, measured (yield) and stored at 4°C in dark glass bottles until tested and analyzed.

Analysis of the essential oil

Gas chromatography: Essential oils were analyzed using a Thermo Electron (Courtaboeuf, France) gas chromatograph equipped with flame ionization detection (FID) detector and DB-5MS capillary column (30 m×0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 200 and 270°C, respectively. Oven temperature gradually raised from 60 to 260°C at 5°C min^{-1}, held for 15 min and finally raised to 340°C at 40°C min^{-1}. Helium (purity 99.999%) was the carrier gas, at a flow rate of 1 mL min^{-1}. Total analysis time was 57 min. Diluted sample (1/100 in petroleum ether, v/v) of 1.0 μL was injected in the split mode (ratio 1:10). Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

Gas chromatography/mass spectrometry (GC-MS): Analysis of essential oils was performed under the same conditions used for GC-FID analysis (column, oven temperature, flow rate of the carrier gas) using a Thermo Electron (Courtaboeuf, France) DSQ II GC-MS single quadrupole mass selective detector in the electron impact mode (70 ev). Injector and MS transfer line temperatures were set at 200 and 300°C, respectively. MS was adjusted for an emission current of 10 μA and electron multiplier voltage at 1500 v. Trap temperature was 250°C and mass scanning was from 40 to 650 amu.

The components were identified based on the comparison of their KI (Kovats indices) and mass spectra with those of standards, Wiley 2001 library data (NIST 02 version 2.62) of the GC-MS system and literature data (Adams). Alkanes (C5-C24) were used as reference points in the calculation of KI. All determinations were performed in triplicate and then averaged.

Microbial strain: Neroli essential oil was tested against a panel of microorganisms. Seven bacteria including 3 Gram-positive (*Bacillus subtillis* ATCC 6633, *Staphylococcus aureus* CIP7625, *Listeria monocytogenes* Scott A 724) and 4 Gram-negative (*Escherchia coli*

ATCC10536, *Salmonella enteric* ATCC14028, *Pseudomonas aeruginosa* CIPA22, *Klebsiella pneumoniae* CIP8291), 2 yeasts (*Saccharomyces cerevisiae* ATCC 4226 A, *Candida albicans* IPA 200) and 3 fungi (*Aspergillus parasiticus* NRRL 3174, *Mucor ramamnianus* ATCC 9314, *Fusarium culmorum*) were used.

Well-diffusion assay: Agar-well-diffusion method (Kalemba and Kunicka, 2003; Oke *et al*., 2009) was used for the determination of antimicrobial activity of Neroli essential oil. Ampicillin and nalidixic acid were used as positive reference standards to, respectively determine the sensitivity of Gram-positive and Gram-negative bacterial. Nystatin was used as positive reference standard to determine the sensitivity of yeast and fungi species. These plates, after staying at 4°C for 2 h to allow dispersal, were incubated at 37°C (bacteria) and at 30°C (yeast and fungi) during 48 h. Antimicrobial activity was evaluated by measuring the diameter of the growth of the inhibition zone, around the disc, against the test organism. The diameter of the inhibition zones was measured in millimeters. All the experiments were conducted in triplicate and the mean diameter of the inhibition zone was recorded.

Antioxidant activity by ABTS radical-scavenging assay: The radical scavenging capacity of antioxidants for the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate) radical cation was determined as described by Re *et al*. (1999). For each sample, diluted methanol solution of the essential oil (100 µL) was allowed to react with fresh ABTS solution (900 µL) and then the absorbance was measured 6 min after initial mixing and the absorbance was recorded as $A_{(sample)}$. A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(blank)}$. The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\text{Inhibition} = \frac{A_{(blank)} - A_{(sample)}}{A_{(blank)}} \times 100$$

Antioxidant activity of standard or essential oils was expressed as IC_{50} (mg L^{-1}), defined as the concentration of the test material required to cause a 50% decrease in initial ABTS concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

Statistical analysis: All data were expressed as Mean±Standard deviations of triplicate measurements. The confidence limits were set at $p<0.05$. Standard Deviations (SD) did not exceed 5% for the majority of the values obtained.

RESULTS

The Tunisian Neroli essential oil obtained by hydrodistillation from flowers of *C. aurantium* L. has a light yellow color and a very pleasant soft odor characteristic of the orange tree flower that is "fresh", "zesty" and "citral". The extraction yield of hydrodistilled *C. aurantium* L. essential oil was 0.12%.

Chemical composition: Neroli oil was analyzed by GC-FID and GC-MS. Thirty three components were identified, representing 99% of the total components in the essential oil from the flowers of *C. aurantium* L. The constituents identified by GC-MS analysis, the retention indices and area percentages are summarized in Table 1. The Neroli oil

Table 1: Chemical composition (percent) of Neroli essential oil

No.	RI	Compounds	%
1	878	Ethylbenzene*	0.1
2	936	α-pinene	0.2
3	974	Sabinene	0.5
4	985	β-myrcene	0.3
5	1011	δ-3-carene	2.4
6	1025	p-cymene	0.2
7	1028	Limonene	27.5
8	1037	(E)-β-ocimene	4.3
9	1050	Z-β-ocimene	0.8
10	1057	γ-terpinene	0.3
11	1145	Isopulegol*	1.0
12	1178	Terpinen-4-ol	0.4
13	1185	α-terpineol	14.0
14	1278	Geraniol	0.3
15	1291	Trans-sabinyl acetate	0.1
16	1322	Geranial	0.3
17	1338	Methyl anthranilate	1.2
18	1352	α-terpinyl acetate	11.7
19	1359	β-terpinyl acetate	1.7
20	1389	β-elemene	0.3
21	1428	γ-elemene	3.4
22	1436	Geranyl acetone	0.1
23	1480	Germacrene D	0.7
24	1540	(E)-nerolidol	17.5
25	1565	Z-nerolidol	0.2
26	1580	β-caryophyllene oxide	0.1
27	1652	α-cadinol	0.1
28	1720	(E,E)-farnesol	8.0
29	1733	E,E-farnesal	0.9
30	2202	Docosane*	0.1
31	ND	α-terpinyl formate*	0.3
32	ND	Nerolidyl propionate*	0.1
33	ND	Tetratetracontane*	0.1
Total			99.0
		Monoterpene hydrocarbons	36.2
		Monoterpenes oxygenated	14.1
		Sesquiterpenes hydrocarbons	4.1
		Sesquiterpenes oxygenated	26.2
		Others	18.4

*Tentatively identified supported by good match of MS, ND: Not determined

Table 2: Antibacterial activity of Neroli oil

	Diameter of inhibition zones (mm)					
Samples	*B. subtilis*	*S. aureus*	*L. monocytogenes*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
Essential oil Neroli	12		16	18	19	11
References standard Ampicillin	54	20	31			
Nalidixic acid				30	19	28

---: Absence of inhibition zone detected

Table 3: Antifungal activity of Neroli oil

	Diameter of the inhibition zones (mm)				
Samples	*S. cerevisiae*	*C. albicans*	*A. parasiticus*	*M. ramannianus*	*F. culmorum*
Essential oil Neroli	20	22	27	35	35
References standard N° statin	29	25	24	31	31

was dominated by monoterpenes which accounted for 50.3% of the oil. Limonene, one of the most common terpenes in nature and the majority constituent of an essential oil series (Sun, 2007), was the main component of this fraction (27 5%) followed by α-terpineol (14%) Other monoterpenes present in fairly good amounts were (E)-β-ocimene (4.3%) and δ-3-carene (2.4%). The sesquiterpenes compounds constituted 30.2% of the total essential oil. (E)-nerolidol (17.5%) was the main component of this fraction followed by (E,E)-farnesol (8%) and γ-elemene (3.4%)

Antimicrobial activity: The *in vitro* antimicrobial potential of Neroli essential oil (*C. aurantium* L.) was evaluated against 3 Gram positive bacteria, 3 Gram negative bacteria, 2 yeasts and 3 fungi by zone diameter. These morphologically and physiologically different microorganisms were selected on the basis of their relevance as food contaminants. As it can be seen in Table 2 and 3, the essential oil was found to have good to excellent antimicrobial activities against all microorganisms tested except for *Staphylococcus aureus* since no inhibition zone was observed.

Neroli oil showed a reasonable *in vitro* antibacterial activity compared with references against all the tested bacteria including Gram-positive and Gram-negative with inhibition zones diameter 11 to 19 mm (Table 2). Among the Gram-negative bacteria, the oil was very active against *P. aeruginosa* which the response was else the same as that of the standard antibiotic (Nalidix acid) (19 mm).

Marked antimicrobial activity of Neroli essential oil was observed against the 2 yeasts *Saccharomyces cerevisiae* (20 mm) and *Candida albicans* (22 mm) (Table 3). In addition, Neroli oil exhibited a great potential for antifungal activity against the 3 tested fungi more as that of the standard antibiotic (Nystatin), as evidenced by their inhibition zones which are 27 mm against 24 mm for *Aspergillus parasiticus*, 35 mm against 31 mm for *Mucor ramannianus* and 35 mm against 31 for *Fusarium culmorum*.

Table 4: Free radical scavenging capacity of Neroli essential oil

Samples	ABTS IC_{50} (mg L^{-1})
Essential oil	
Neroli	672.0±4.0
Reference standard	
Vitamin C	1.9±0.1

Standard Deviations (SD) did not exceed 5%

Antioxidant activity: Free radical activity of Neroli oil was investigated by ABTS assay. A moderate antioxidant activity was obtained to scavenge the ABTS radical cation (IC_{50} of 672±4 mg L^{-1}) (Table 4).

DISCUSSION

Chemical composition: Results showed some differences in composition with respect to data in literature. Anonis (1985), Boelens and Jimenez (1989), Lin *et al.* (1986) and Zhu *et al.* (1993) found that the Neroli oil is characterized by the dominance of linalool (37.5-74%) while no significant quantity was detected in the Tunisian Neroli oil. Conversely, limonene, the main component of the Tunisian Neroli oil (27%), has been found in very weak quantities except in the Spanish Neroli oil (16.6%) (Boelens and Jimenez, 1989).

In the present study, much higher concentration of (E)-nerolidol (17.5%), α-terpineol (14%) and α-terpinyl acetate (11.7%) were found. However, several components such as β-pinene and citronellol, mentioned in the literature, were not detected in our oil (Anonis, 1985; Boelens and Jimenez, 1989). These changes in the essential oil composition might arise from several environmental (climatical, seasonal, geographical) and genetic differences (Perry *et al.*, 1999).

Antimicrobial activity: Results obtained in this study show that Neroli oil does not have selective antibacterial activity on the basis of the cell-wall differences of bacterial microorganisms (Gram-positive or Gram-negative bacteria) as reported previously (Cosentino *et al*., 1999; Karaman *et al*., 2003). In fact, a general observation derived from studies with essential oils from many other plant species (Nostro *et al*., 2000; Ouattara *et al*., 1997) showed that the antimicrobial activity of the tested essential oil was more pronounced against Gram-positive than against Gram-negative bacteria. This generally higher resistance among Gram-negative bacteria could be ascribed to the presence of their phospholipidic membrane, almost impermeable to lipophilic compounds (Nikaido and Vaara, 1985). The absence of this barrier in Gram-positive bacteria allows the direct contact of essential oil's hydrophobic constituents with the phospholipid bilayer of the cell membrane, where they bring about their effect, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems (Cowan, 1999; Wendakoon and Sakaguchi, 1995). The antimicrobial activity of an essential oil is related in most cases to its major components. Referring to the literature, the most frequently occurring constituents in Neroli oil showing high antifungal activity were limonene, (E)-nerolidol and (E,E)-farnesol (Lee *et al*., 2007; Wilson *et al*., 1997; Ramage *et al*., 2002).

Although limonene (27%) and (E)-nerolidol (17.5%), two major components of Neroli oil, presented a strongly positive antifungal activity, they are inactive as antibacterial agents (Kim *et al*., 1995).

α-terpineol which was found to be in appreciable amounts in the oil of this study (14%), has been reported to inhibit the growth of a quite number of bacteria and fungi that include *E. coli*, *S. epidermis* and *C. albicans* (Carson and Riley, 1995; Raman *et al*., 1995).

It is necessary to signal that other compounds in lower amount may also contribute to improve this stronger activity. In fact, the synergistic effects of the diversity of major and minor constituents present in the essential oils should be taken into consideration to account for their biological activity.

In this context, terpinen-4-ol, a minor constituent in the oil under study (0.4%), is reported (Carson and Riley, 1995) to show activity against these organisms. It is also said to be responsible for the broad spectrum activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) (Sean *et al*., 2001). Another minor constituent, β-caryophyllene oxide (0.1%), is known to have very efficient antibacterial properties (Magiatis *et al*., 2002). Although, these compounds are not abundant in the essential oil, their activity could be important.

Antioxidant activity: Antioxidant activity of Neroli oil is largely depending on its chemical composition. In the present study, the major component of Neroli oil, limonene, had low ABTS free radical scavenging activity. This result was confirmed by previous studies in which commercially obtained limonene displayed negligible antioxidant activity (Ruberto and Baratta, 2000). Although, γ-terpinene, a minor component in Neroli oil, is known to show high antioxidant activity, no activity was observed here, since its concentration was too low (0.3%).

Essential oils are quite complex mixtures constituted by several tens of components and this complexity makes it often difficult to explain the activity pattern. For this reason, many reports on the antioxidant potentials of the essential oils often refer to concepts such as synergism, antagonism and additivity.

From a general point of view, phenols were confirmed to possess strong antioxidant activity (Ruberto and Baratta, 2000). In particular, oxygenated monoterpenes (especially two well known phenolic compounds, thymol and carvacrol) are mainly responsible for the antioxidant potential of the plant oils which contain them (Aeschbach *et al*., 1994; Baratta *et al*., 1998; Lagouri *et al*., 1993). Monoterpene hydrocarbons; particularly terpinolene, α-and γ-terpinene, could also be taken into account for the antioxidative activity observed, but obviously, none has stronger than that of oxygenated monoterpenes. The presence of strongly activated methylene groups in these molecules is probably the reason for this behavior. On the other hand, sesquiterpenes hydrocarbons and their oxygenated derivatives have very low antioxidant activity (Ruberto and Baratta, 2000).

CONCLUSION

Citrus aurantium L. flowers have a very strong aromatic odor. The high *in vitro* antimicrobial activity of Neroli oil could be attributed to the presence of a mixture of monoterpenes, sesquiterpenes, alcohols and esters which can be considered as promising for future utilization as a food preservative eliminating the growth of important foodborne pathogens and contributing to enhance food safety and shelf life. Separation of terpenes, responsible for the antimicrobial activity, would be an interesting study to identify the molecules generating the good efficacy.

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