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Research Article

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Chemical composition and biological activities of various alimentary preparations of *Citrus aurantium* leaves

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ABSTRACT

Tunisian Sour oranges leaves were studied. Alimentary preparations of C. aurantium were obtained by maceration, infusion, decoctions (15, 30 and 60 minutes). Different yields were obtained for different extractions. Their chemical composition was evaluated revealing few amounts of tannins and anthocyanins whereas some of them presented considerable ones of total phenolics (gallic acid equivalent 0,062 to 0,792 g/Kg Dry matter) and flavonoids (quercitin equivalent 0,039 to 0,694 g/Kg Dry matter). A screening of antioxidant activities was applied to all samples using DPPH and ABTS assays; their antimicrobial activities also were studied by 7 bacteria (3 Gram-positive and 4 Gram-negative), 2 yeasts and 3 fungi. The alimentary mode of extraction influenced extracts chemical composition and their antioxidant activity. Correlations between chemical composition and antioxidant activity were studied.

Key words: antimicrobial activity, antioxidant activity, *Citrus aurantium*, extraction method, alimentary preparations.

INTRODUCTION

The oxygen paradox is determined by how essential oxygen is for eukaryotic organisms and at the same time how fatal can it be to them. The oxygen is vital for respiration, energy recuperation mechanisms as ATP [1]. Plant and animal tissues contain unsaturated fatty acids primarily in the phospholipid fraction of cell membranes. These lipids are especially susceptible to oxidation because of their deficient double-bonds [2]. In physiological conditions, oxygen leads to the formation of oxygen reactive species like free radicals that are highly toxics to cells integrity. These reactive species may interact with a variety of biological components such as lipids, proteins, DNA,… which may lead to partial degradation causing cancer and many other serious diseases [3] [4]. Lipids are important components in food staff, therefore preventing their oxidation may improve food preservation. This protection is widely accomplished by synthetic antioxidants such as butylated hydroxyltoluene (BHT) and butylated hydroxyanisole (BHA). However, their intensive use may be hazardous. This is why a great interest was accorded to natural antioxidants within last years [5]. Microbial activity is another mode of deterioration of many foods and is often responsible of the loss of quality and safety. The increase in outbreaks of food-borne diseases leads to a great concern over pathogenic and spoilage microorganisms in foods. Many strains developed a great resistance to antibiotics. Therefore, antimicrobial properties have been reported more frequently in a wide-range of natural products: essential oils and plant extracts in an attempt to discover new chemical classes of drugs that would resolve these problems [6]. A variety of herbs and plants are known to be sources of bioactive compounds, studies aiming to isolate the active molecules and to evaluate their antioxidant and antimicrobial activities are needed to be carried out [7]. Besides the plant cultivar, other factors have been revealed to influence the quality of plant extracts among them the solvent, the extraction method,… [8]. Thus, studying the solvent effects is a critical point for screening solvent,

selection of the extraction, fractionation and purification steps in plant processing. When various points such as solvent properties, plant matrix component properties, solvent-solutee interaction are understood many further steps can be achieved namely rapid extractions and fractionation, isolation of desired components,… [9]. Tunisia has a multitude of aromatic and medicinal plants among which *Citrus aurantium* is ethno-pharmacologically considered as an important one. It has been reported that different vegetable parts of sour orange had several biological activities: adrenergic stimulation of cardiovascular system, LDL protection, prevention of cardiovascular diseases, antiproliferative activity against cancer cells,… [10] [11] [12]. Antioxidant activity has been reported for sour oranges peels by [13]. The aim of this study was to investigate the effect of different extracting alimentary preparations of Tunisian *C. aurantium* leaves on their chemical composition, antioxidant and antimicrobial capacities.

EXPERIMENTAL SECTION

2.1 Plant material

Leaves *Citrus aurantium* were gathered from a standard orchard supervised by Commissariat Régional du Développement Agricole in Nabel (Longitude 36°45'00'' North, Latitude 10°45'00'' East, Altitude 0 m), Tunisia in January 2009. The leaves were kept to dry at shadow until constant mass.
When dried the leaves were ground with a Kenwood blender.

2.2 Chemicals

All chemicals used were of analytical grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France): Sigma (sodium acetate [≥99%], potassium chloride [≥99%], NaH_2PO_4 [≥98%]), NaH_2PO_4 [≥99%], NaCl [≥98%], gallic acid [≥99%], Hydrochloric acid [36.5% to 38%], catechin [≥98%], quercitin [≥98%], cyaniding-3-glucoside [≥95%], nystatin [≥99%], ascorbic acid [≥99%], ampicilline [96 to 100.5 %]), Aldrich (aluminium trichloride [99.99%], 1,1-diphenyl-2-picrylhydrazyl free radical [99%]) Sigma-Aldrich (methanol [≥99%], ethanol [≥99.5%], acetone [≥98%], hexane [≥99%], dimethylsulfoxide [≥98%], Folin-Ciocalteu reagent 2 N, acetic acid [≥99.7%], nalidixic acid [≥98%]) and Fluka (potassium persulfate [≥99%], vanillin [≥98%], H_2SO_4 [≥95%], 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonate [≥99%]).

2.3 Preparation of the extracts

Different water preparations were prepared with different plant materials: maceration at ambient temperature for 16 hours, infusion, 15 minutes decoction, 30 minutes decoction and 60 minutes decoction. All the extracts were cooled, filtered, and concentrated under vacuum to dryness.

The obtained extracts were stocked at 4°C until further analysis.

2.4 Total amount of phenolics in extracts

Total phenolics in the extracts were determined by the Folin-Ciocalteu method [14] using gallic acid as a standard.
The diluted solution of each extract (0.5 ml) was mixed with Folin-Ciocalteu solution (2.5 ml, 0.2N). After 5 minutes, at room temperature, a solution of sodium carbonate solution was added (2 ml, 75g/l in water) and the resulting solution was incubated 1 hour. The absorbances were measured against the adequate solvent as blank at 765 nm with a spectrophotometer (Hélios, Unicam, Cambridge UK). The results were expressed as gram of gallic acid equivalent (GAE)/kg of dry mass.

2.5 Total flavonoids content

Total flavonoids were analyzed by Dowd method as adapted by [15]. Each extract was diluted in the appropriate solvent. Four ml of this solution was mixed to 4 ml of aluminium trichloride in methanol ($AlCl_3$ (2%)). The blank was prepared by 4 ml of each extract mixed to 4 ml of methanol. The samples were incubated during 15 minutes at room temperature, and then absorbance was read at 415 nm. The standard calibration was a methanolic solution of quercetin and results were expressed as gram of quercetin equivalents (QE)/kg of dry mass.

2.6 Condensed tannins amount

The vanillin method as described by [16] was used to evaluate amounts of catechins and proanthocyanidines reactive to vanillin. From the diluted extracts 1 ml was taken and allowed to stay with 2 ml of vanillin (1% in 7M H_2SO_4) for 15 minutes. At the end of incubation time the absorbance was read at 500 nm. A standard calibration curve was plotted with catechin in order to express results as gram catechin equivalents (CE)/kg dry mass.

2.7 Total anthocyanins in extracts

Total anthocyanins were determined by pH differential absorbance method as described by [17]. The absorbance of the extracts was measured at 510 nm and 700 nm in buffers at pH 1 (Hydrochloric acid-potassium chloride, 0.2M)

and 4.5 (acetic acid-sodium acetate, 1M). The incubation duration was 15 minutes. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29600 (cyaniding-3-glucoside) and absorbance of $A = [(A_{510}-A_{700})_{pH=1}-(A_{510}-A_{700})_{pH=4.5}]$. Results were expressed as milligram cyaniding-3-glucoside equivalent (C3GE)/kg dry mass.

2.8 Antioxidant activity

2.8.1 DPPH radical-scavenging activity

The hydrogen atoms or electrons donation ability of the corresponding extracts were measured from the bleaching of purple colored methanol solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) as described by [18] with slight modifications: 1.5 ml of the material to test (plant extracts or pure antioxidant) were mixed to 1.5 ml of a 0.2mM methanolic DPPH solution, the resulted solution was kept to react 30 minutes at 25°C. Absorbances were read at 520 nm and recorded as $A_{(sample)}$, A control experiment was carried out in the same way containing all reagents except the test compound and recorded as $A_{(control)}$. The free radical-scanvenging activity of each solution was calculated in the following way:

$$\%inhibition = 100(A_{(control)}-A_{(sample)})/A_{(control)}$$

Antioxidant activity of sour oranges extracts was expressed as IC_{50}, defined as the concentration of the test material needed to cause a 50% decrease in initial DPPH concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

2.8.2 ABTS radical-scavenging activity

Antioxidant scavenging activities was studied using 2,2-AzinoBis-3ethylThiazoline-6-Sulphonate (ABTS) radical cation as described by [19]. The ABTS solution was prepared by mixing a 7mM of ABTS at pH 7.4 (5mM NaH_2PO_4, 5mM Na_2HPO_4 and 154 mM NaCl) with 2.5 mM potassium persulfate (final concentration) and storing the resulting mixture at dark for 16 hours before use. The mixture was diluted with distillated water to generate an absorbance of 0.7±0.02 at 734 nm using spectrophotometer. From the prepared sample dilutions, 100µl was extracted and allowed to react with 900µl of a fresh ABTS solution, after an incubation of 6 minutes the absorbances were read at 734nm. The used standard was ascorbic acid, the capacity of free radical scavenging was expressed by IC_{50} (mg/l) defined as the required concentration of tested material to scavenge 50% of ABTS radicals. IC_{50} of ABTS was calculated with the same equation presented for DPPH scavenging. All measurements were applied in triplicate.

2.9 Antimicrobial activity

All strains were obtained from the Laboratory of Chemical Engineering, Bioprocess Systems Microbiens Department, Ecole Nationale Supérieure Agronomique de Toulouse. The tested strains were 3 Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* CIP7625, *Listeria monocytogenes* Scott A 724), 4 Gram-negative bacteria (*Pseudomonas aeruginosa* CIPA22, *Escherichia coli* ATCC 10536, *Klebsiella pneumonia* CIP8291, *Salmonella enterica* CIP833), 2 yeasts (*Saccharomyces cerevisiae* ATCC 4226 A, *Candida albicans* IPA200) and 3 fungi (*Mucor ramannianus* ATCC 9314, *Aspergillus parasiticus* CBS 100926, *Fusarium culmorum* NRRL 3288). The bacterial and yeast strains were cultured on nutrient agar for 48h at 37°C, while fungi were propagated in PDA(Potato Dextrose Agar) at 30°C for 48h to 3 days before used. All microorganisms were stocked at -6°C in appropriate conditions and regenerated twice before use in the manipulations.

The agar disc diffusion method was employed to determinate the antimicrobial activity of the essential oil [20] with modifications. A suspension of the test microorganisms (0,2 ml of 4.107 cells/mL for bacteria, 0,3 ml of 12,5.105 spores/mL for yeasts and fungi) was spread in worm sterile nutrient media.The resulting agar solutions were immediately poured into petri dish after vortexing and allowed to solidify. The dish was left to cool down and to solidify at room temperature for 30 min. Dilutions of the extracts in the appropriate solvents (15mg/ml) were prepared and 60 µl of these solutions were impregnated into each standard empty disc (9mm in diameter), then the discs wee sterilized by UV at 300 nm for 5 minutes. The sterilized discs were placed on the plates and kept for 2 hours at 4°C in order to allow the material to diffuse before microbial growth. Bacterial Petri dishes were incubated at 30°C for 24 hours while yeasts and moulds remained 36 hours at 30°C. The diameters of inhibition zones were calculated in millimeters. Each assay was performed in duplicate.

2.10 Statistical analysis

All data were expressed as means ± standard deviation of triplicate measurements. The confidence limits were set at $p<0.05$. The statistical analysis of extracts chemical composition was carried out by a one-way ANOVA and T-test by SPSS for Windows 17.0.0, SPSS Inc. 1989-2010. Correlation coefficients (R^2) to determine the relationship

between chemical composition and antioxidant activity were calculated using MS Excel XP software (Correl statistical function).

RESULTS AND DISCUSSION

Alimentary preparations used distillated water to get maceration, infusion and different duration's decoctions (15, 30 and 60 minutes). The yields of different extracts are presented in Table 1. Increasing decoction period led to increase yields 8.5 to 10.6%. Li et *al*. [21] proposed a hypothesis: conjugating the effects of high temperature and water had direct effect on increasing yields as they allow the degradation of cell membranes. This hypothesis can explain the increase noted between maceration and infusion (0.9% and 6.05% respectively). Comparing infusion and decoction 15' yields showed that the combination of boiling temperature and period increased them. This observation is in accordance with what was reported in literature [22] [23] [24].

Table 1: Extraction yields (%) of alimentary extracts of *C. aurantium* leaves

	Yield
Maceration	0.9 ± 0.02^{a}
Infusion	6.05 ± 0.8^{b}
Decoction15'	8.5 ± 0.5^{c}
Decoction 30'	10.4 ± 1.1^{c}
Decoction 60'	10.6 ± 1.3^{c}

Values in the same column with different subscript are statistically different (p<0.05)

Table 2: Chemical composition of *C. aurantium* leaves extracts

	Polyphenols (GAE)	Flavonoids (CE)	Tanins (QE)	Anthocyanins (C3GE)
Maceration	0.062 ± 0.000^{a}	0.039 ± 0.001^{a}	tr	$0.34\ 10^{-10}$
Infusion	0.402 ± 0.005^{b}	0.377 ± 0.017^{b}	tr	$0.15\ 10^{-10}$
Decoction 15'	0.605 ± 0.008^{b}	0.561 ± 0.014^{b}	tr	$3.3\ 10^{-10}$
Decoction 30'	0.695 ± 0.0013^{b}	0.694 ± 0.032^{b}	tr	$0.26\ 10^{-10}$
Decoction 60'	0.792 ± 0.007^{c}	0.578 ± 0.025^{c}	tr	-

Values in the same column with different subscripts are significantly different (p<0,001), tr: trace

Table 3: Antioxidant activities of *C. aurantium* leaves extracts

	DPPH IC_{50} (mg/l)	ABTS IC_{50} (mg/l)
Maceration	$167.06\pm3.17^{a,b}$	65.45 ± 1.90^{e}
Infusion	215.85 ± 7.15^{c}	76.99 ± 1.16^{f}
Decoction 15'	147.07 ± 5.55^{a}	78.94 ± 4.18^{f}
Decoction 30'	176.14 ± 8.14^{b}	113.85 ± 6.65^{g}
Decoction 60'	207.12 ± 11.95^{c}	105.94 ± 1.62^{g}

Values in the same column with different subscripts are significantly different (p<0,001)

Chemical composition of the different extracts is presented in Table 2. Extraction method seemed to be influent on polyphenol amounts: maceration and infusion led to different phenol amounts. Longer decoction time induced higher polyphenolic compound as these compounds are important part of vegetable cell, it is probable that boiling water and long extraction duration made this extraction easier by increasing solubilization as mentioned by [23] [24] [25]. Leaves aqueous extracts were divided into 3 groups: one for maceration, the second contained infusion, decoction 15' and decoction 30' and the last presented only decoction 60'. An important decrease of flavonoids amounts was clear for decoction 60', this reduction may be due to their degradation by high temperature especially in aqueous medium or to hydrolysis [9] [26] [27]. Furthermore, tannins and anthocyanins presented very low content for all extraction procedures. Antioxidant activities for *C. aurantium* leaves extracts has been determined by 2 different test systems: DPPH and ABTS assays. These results are presented in Table 3.

Antioxidant activity varied with extraction procedure for alimentary preparations ($p<0.001$). Especially for decoctions, different IC_{50} values were registered in aqueous preparation, temperature rising allowed higher material transfer rate and solute desorption within active sites in vegetal matrix. On the other hand, many degradation processes could have taken place by hydrolysis (presence of water as solvent) or oxidation of antioxidant molecules by peroxyl and hydroxyl formed radicals (high temperature) [28] [29]. However, antioxidative activity of a given compound may increase, decrease or remain unchanged as a function of temperature [2]. The values of antioxidant activities IC_{50} obtained by DPPH and ABTS were different. These differences were caused by the specificity of each radical. Indeed, DPPH has always been considered as standard analysis for polar and medium polar components whereas ABTS radical is more sensitive especially for plant extracts [28][29][30]. For this reason, the correlation between antioxidant activity and the content of phenolics and flavonoids were studied only for ABTS data. The low correlation coefficient between ABTS assay and total phenolic compounds highlighted that other chemical families than polyphenols were responsible of the antioxidant activity noticed for *C. aurantium* leaves. Positive correlations

expressed by high R^2 value indicated the relation between antioxidant activity and aqueous leaf extracts ($R^2 = 0.72$) indicating the presence of hydrosoluble polyphenolics in *C. aurantium* leaves similarly to [31]. Furthermore, antioxidant activity doesn't depend only on concentration of active components; it is also influenced by various interactions, or different synergisms (positive or negative) that could take place in such a rich matrix that is plant extract [7] [32] [35]. Extracting antioxidant components from a complex matrix such as plant material depends on the solubility of the component, the solvent and the presence of other components that may compete with them in the extraction procedure [2].

The antimicrobial activities of the different *C. aurantium* extracts were evaluated using a paper disc diffusion method against 3 Gram+, 4 Gram- bacteria, 2 yeasts and 3 fungi. No antimicrobial activity was observed against all studied strains.

CONCLUSION

In recent decades, the plants have drawn great interest as sources of natural products. Plant extracts with antimicrobial and antioxidant characteristics as well as several biological activities can replace synthetic antibiotics and antioxidants. In our study, no antimicrobial activity was noticed for *C. aurantium* leaf extracts. While these extracts exhibited moderate antioxidant activity by DPPH and ABTS. A relationship between structure, chemical composition and antioxidant activity has been established for some of them. Alimentary mode of preparation influenced antioxidant activity scavenged by DPPH and ABTS.

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