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Antioxidant activity of extracts formulated from *Citrus aurantium* and *Artemisia herba alba*

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ABSTRACT: Plants still present a large source of novel active biological compounds with different activities. The antioxidant activity of three extracts was evaluated by DPPH and ferric reducing antioxidant power methods. The formulated extracts were analyzed by thin layer chromatography (TLC) and then confirmed by High performance liquid chromatography coupled with DAD detector (HPLC-DAD). The results showed the richness of these extracts in phenolic compounds. Three major compounds, resveratrol (17.98%), kaempferol-glucoside (7.23%) and vanillic acid (10.64%) were detected in methanolic extract of Citrus aurantium peel, aqueous extract of *Citrus aurantium* L. leaves and ethanolic extract of *Artemisia herba alba* Asso respectively by HPLC-DAD. However, the ethanolic extract of *A. herba alba* achieved 50% of the anti-radical activity at a concentration equal to 0.8 mg/ml. A higher antioxidant activity measured by ferric reducing antioxidant power was marked in the same extract with an absorbance equal to 0.824. The ethanolic extract of the aerial part of *A. herba alba*, the methanolic extract of *C. aurantium* peel and the aqueous extract of *C. aurantium* leaves were considered as powerful scavengers of free radicals and can be incorporated into the pharmaceuticals preparations to treat many diseases.

Keywords: *Artemisia herba alba* Asso; *Citrus aurantium* L.; TLC; HPLC-DAD; Antioxidant activity.

1. INTRODUCTION

Phenols are one of the largest groups of secondary plant constituents. They are defined as compounds that bear at least one hydroxyl group attached to an aromatic or benzene ring system. In addition the ring system may bear other substitutes especially methyl groups [1]. *Artemisia herba alba* Asso is a perennial, 30-40 cm, with a characteristic smell of thymol, very leafy and with tomentose young branches. The leaves are hairy, silvery, small deeply bi-pennated, with linear strips. The flowers are all hermaphrodite, packed together in very small capitula, sessile and in bunch. Flavonoids, coumarins, pentacyclic triterpens, anthracenosids and tannins were found in this plant [2]. *A. herba alba* was found therapeutic applications due to their antihepatoxic, choleretic, spasmolytic, anthelmintic, antiphlogistic, antibiotic or antimicrobial activity [3]. *Citrus* is one of larges species among plant; it consists of 40 species which are distributed in all continents [4]. The genus *Citrus* belongs to the Rutaceae family. *Citrus* is classified into two botanical species: *C. medica* L. and *C. aurantium* L. to which belong several varieties. *Citrus aurantium L.* var amara, commonly named Bigarade or bitter (sour) orange, is one of the earliest flowering *Citrus* trees [5]. Organic and water extracts of

fruit peels of *Citrus aurantium* was effective against all the tested human pathogenic organisms [6]. The use of traditional medicine is widespread and plants still present a large source of novel active biological compounds with different activities, including anti-inflammatory, anticancer, antiviral and antibacterial activities, the antioxidants may play a role in health-promoting activity as nutraceuticals. However, antioxidants are substances that delay the oxidation process by inhibiting polymerization chains initiated by free radicals and other subsequent oxidizing reactions [7]. The aim of the present study is to evaluate the antioxidant activities of three organic and aqueous extracts, contained *Artemisia herba alba* and *Citrus aurantium* collected in North West of Algeria (Mascara province).

2. MATERIALS AND METHODS

2.1. Plant material

Citrus aurantium and *Artemisia herba alba* were harvested in March in Tighennif circle and October 2015 in Oued El Abtal region respectively, because these periods represent the flowering stage of each plant in order to collect all the organs including flowers and fruits. The plants were chosen according to their high frequency of use in Mascara region to treat several skin diseases. After harvest, the medicinal plants were identified by comparing their forms to those mentioned in the literature and by botanists from the faculty of SNV at the University of Mascara and stored as voucher specimens under these codes: AS00006 for *Artemisia* and RU00002 for *Citrus*.

2.2. Reagents

All the chemical products were purchased from different companies. Butanol (Scharlau), acetic acid (Scharlau), gallic acid (Scharlau), quercetin (Scharlau), catechin (Scharlau), methanol (Hipersolv Chromanorm), ethanol (AnalaR Normapur), formic acid (Emsure), ascorbic acid (Cooper), DPPH (Bockit), phosphate buffer (Chem lab), potassium ferricyanide (Science company), trichloroacetic acid (Ricca chemical).

2.3. Instruments

UV lamp set to 254 nm (Herolab, UV-4 S/L), Agilent 1100/DAD series HPLC system, rotary evaporator (RE302P – Stuart), Lichrochart RP-18 column type (250 x 4 mm, particle size 5 µm), quaternary pump (1260 Infinity II), DAD detector (3000RS), spectrophotometer (SCILOGEX SCI-UV1100), Incubator (IN 30).

2.4. Preparation of organic and aqueous extracts

Five grams of the aerial part of *Artemisia herba alba*, peel and leaves of *Citrus aurantium* reduced to powder, were introduced into 100 ml of 80% methanol, 80% ethanol and distilled water [8]. The operation was repeated three times with renewal of the solvent of each preparation with the same dilution. The combined filtrates were put to rotary evaporation under reduced pressure. Then, the residue was dried, scraped and stored at 5°C in test tubes protected from air and light until ready to use.

2.5. Analysis of extracts by thin layer chromatography (TLC)

The thin layer chromatographic (TLC) analysis of the three selected extracts was carried out by a BAW separation system (butanol/acetic acid/water: 60/15/25) [9]. However, three standards were used for this analysis: gallic acid was prepared at dose of 20 mg/100 ml. Quercetin and catechin were prepared by diluting

3 mg of the powder of each control in 100 ml of methanol. A volume of 5 µl of extracts and standards were deposited on the line of deposits located above the level of the mobile phase using a Pasteur pipette. After migration of the spots, the plate was put to dry then viewed under a UV lamp.

2.6. Analysis of extracts by HPLC coupled with DAD detector

An Agilent 1100/DAD series HPLC system in (reverse phase) was used to analyze the extracts. The quantitative analysis of these samples was achieved using a Lichrochart RP-18 type column (250 x 4 mm, particle size 5 µm). The temperature of the column was maintained at 25°C with a quaternary pump [10]. 10 µl with a flow rate of 1 ml/min were injected. The detector was set to signals between 280 and 370 nm. However two solvents were used as mobile phase. Solvent A: represents distilled water plus 5% formic acid and solvent B: represents methanol only. These were programmed as follows: 0 min (5/95%), 30 min (40/60%), 45 min (65/35%), 55 min (95/5%) and 65 min (100/0%). The total phenolic compounds were determined by comparison of their mass spectra and their retention times with those of the standards produced under the same conditions.

2.7. Evaluation of the antioxidant activity

2.7.1. Free radical scavenging (DPPH)

In the case of phenolic compounds (Φ-OH), the main mechanism of action is the trapping of free radicals by the transfer of the H atom to the DPPH• then transforms into a stable molecule DPPHH [11]. 50 µl of each methanolic solution of the extracts at different concentrations (from 0.312 to 5 mg/ml) were added to 1.95 ml of the methanolic solution of DPPH (0.025 g/l). In parallel, a negative control was prepared by mixing 50 µl of methanol with 1.95 ml of the methanol solution of DPPH. The absorbance reading was made against a blank prepared for each concentration at 515nm after 30 min of incubation in the dark and at room temperature. The positive control was represented by a solution of a standard antioxidant; ascorbic acid was measured under the same conditions as the samples and for each concentration the test was repeated three times. The results were expressed as percent inhibition (I%). IC50 values were determined graphically by regression [12].

Radical scavenging activity (I%) = [(Abs control – Abs test) / Abs control] x 100

2.7.2. Ferric Reducing Antioxidant Power (FRAP)

The method was based on the reduction reaction of Fe^{3+} present in ferrocyanide complex to Fe^{2+}. 0.5 ml of the sample at different concentrations (0.312-5 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer solution (pH = 6.6) and 1.25 ml of 1% $K_3Fe(CN)_6$ potassium ferricyanide solution. The whole was incubated at 50°C for 20 min and then cooled at room temperature. 2.5 ml of 10% trichloroacetic acid were added to stop the reaction, then, the tubes were centrifuged at 3000 rpm for 10 minutes. 1.25 ml of the supernatant were added to 1.25 ml of distilled water and 250 µl of a solution of iron chloride ($FeCl_3$, $6H_2O$) at 0.1%. The absorbances were read against a blank at 700 nm using a spectrophotometer. The positive control was represented by a solution of a standard antioxidant; ascorbic acid. And its absorbance was measured under the same conditions as the samples [13].

2.8. Statistical analysis of data

The experimental results were mentioned as mean ± standard deviation (SD) of three tests. Then, the results were treated by ANOVA (one way), followed by Bonferroni's multiple comparison. The graphs were

drawn by Graph pad prism software version 7. The P values below to 0.05 were considered statistically as significant.

3. RESULTS

3.1. Analysis of extracts by TLC method

Three extracts were formulated, namely, the ethanolic extract of the aerial part of *Artemisia herba alba*, the aqueous extract of *Citrus aurantium* leaves and the methanolic extract of *Citrus aurantium* peel. These extracts were chosen for their richness in polyphenols. After migration of methanolic extract of *Citrus aurantium* peel, four spots were appeared ocher yellow with a frontal ratio between 0.08 and 0.89. The distance traveled by the solvent is equal to 5 cm. However, a single ocher-colored spot of ethanolic extract of the aerial part was visualized after migration. Under UV lamp, three spots of colors totally different were found. Chromatographic analysis of the aqueous extract of *Citrus aurantium* leaves revealed the presence of two spots of colors and frontal ratios different from those of gallic acid, quercetin and catechin.

3.2. Analysis of the extracts by HPLC-DAD

Nine pure phenolic compounds were used in the HPLC analysis as controls, (gallic acid, caffeic acid, vanillic acid, catechin, epicatechin, resveratrol, kaempferol, myretin, kaempferol-glucoside).After comparing the retention times and the mass spectrum of the peaks obtained and those of the standards, four compounds were revealed in the methanolic extract of *Citrus aurantium* peel of the nine studied (gallic acid (1): 0.157% and kaempferol-glucoside (2): 5.13%). Visualized by the wavelength of 280 nm as minor compounds and two others (resveratrol (3): 17.98% and myrecetin (4): 12.89%) visualized by the wavelength of 370 nm as the main compounds (Table 1). In parallel, four phenolic compounds were identified in the aqueous extract of *Citrus aurantium* leaves (gallic acid (1): 0.12%, catechin (2): 3.22%, epicatechin (3): 0.50% and kaempferol-glucoside (4): 7.23%), which were visualized by the wavelength of 370 nm (Table 2). Chromatographic analysis of the ethanolic extract of *Artemisia herba alba* revealed the presence of eight polyphenols: gallic acid (1): 0.014%, catechin (2): 1.68%, kaempferol-glucoside (3): 1.35%, kaempferol (4): 0.43%, vanillic acid (5): 10.64%, caffeic acid (6): 1.31%, resveratrol (7): 3.13% and myricetin (8): 1.35% (Table 3).

Table 1. HPLC-DAD chromatochraphic profile of the methanolic extract of *Citrus aurantium* peel.

	Retention time (min)	**Percentage (%)**
	280 nm wavelength	
Gallic acid	7.035	0.16
Kaempferol-glucoside	39.095	5.13
	370 nm wavelength	
Resveratrol	36.16	17.98
Myrecetin	38.52	12.89

Table 2. HPLC-DAD chromatochraphic profile of the aqueous extract of *Citrus aurantium* leaves.

	Retention time (min)	**Percentage (%)**
Gallic acid	7.03	0.12
Catechin	17.64	3.22
Epicatechin	23.32	0.5
Kaempferol-glucoside	39.09	7.23

Table 3. HPLC-DAD chromatochraphic profile of the ethanolic extract of *Artemisia herba alba.*

	Retention time (min)	Percentage (%)
Gallic acid	7.035	0.014
Catechin	17.64	1.68
Vanillic acid	20.14	10.64
Caffeic acid	21.08	1.31
Resveratrol	36.163	3.13
Myricetin	38.525	1.35
Kaempferol-glucoside	39.095	1.35
Kaempferol	46.13	0.43

3.3. Free radical trapping (DPPH)

The free radical inhibition percentages DPPH of the three extracts and their IC50 values were mentioned in (Figure 1). In view of the set of percentage inhibition represented above, the percent reduction of 50% free radical DPPH by the aqueous crude extract of *Citrus aurantium* leaves gave a concentration corresponding to 9.77 ± 0.12 mg/ml. This concentration appeared not significant compared to the ascorbic acid ($P = 7.34 * 10^{-13}$). The ethanolic extract of *Artemisia herba alba* achieved 50% of the anti-radical activity at a concentration equal to 0.8 mg/ml which appeared significant compared to the other extract ($P = 1.5 * 10^{-5}$). While the methanolic extract of *Citrus aurantium* peel revealed a reduction activity of 50% of the DPPH radical with a concentration equal to 2.39 mg/ml.

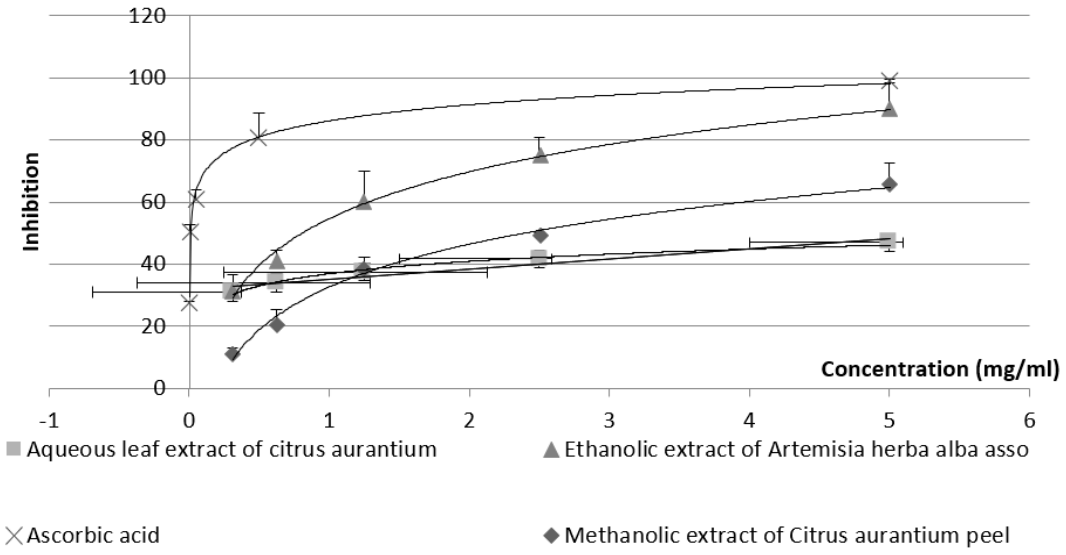

Figure 1. Percentage inhibition of the DPPH radical depends to the ethanolic extract of the concentration of the extracts.

3.4. Reducing power

The antioxidant activity of the EAH extract was marked significant after comparison with the other extracts (Figure 2) with P value equal to $2.98 * 10^{-13}$. Followed by the ascorbic acid with a better absorbance of 0.838 ± 0.2, a high value of 0.824 ± 0.1 was recorded after analyzing the concentration of 5 mg/ml of the

methanolic extract of *Citrus aurantium* peel. Low absorbance values were observed in the aqueous extract of *Citrus aurantium* leaf ranging from 0.448 ± 0.01 to 0.665 ± 0.09.

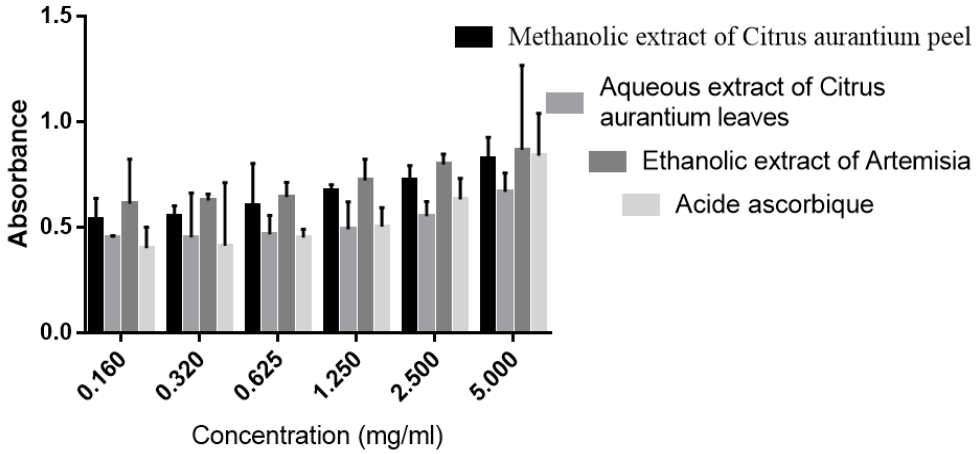

Figure 2. Reducing power of extracts and ascorbic acid.

4. DISCUSSION

In this study, it is clear that the methanolic extract of *Citrus aurantium* peel contained a flavonic compound which is quercetin belonging to the flavonol family, since their frontal ratios were found to be equal. Likewise the light color of the migrated spot is explained by its low concentration compared to the standards which are pure (dark). According to the literature, the chromatographic analysis of the methanolic extract of *Marrubium deserti* leaves, harvested in the Daya-Mogheul region, wilaya of Bechar, revealed the presence of quercetin with an Rf equal to 0.88 [14]. Similarly the Rf values are specific for each phenolic compound and elution system [15]. Regarding the results obtained, the ethanolic extract of the aerial part of *Artemisia herba alba* seemed rich in phenolic acids other than gallic acid, in terms of color, frontal ratio and fluorescence. Because, these constituents give a fluorescent white blue color after viewing under UV lamp, set to 254 nm [16]. Following the observations, we can report that the aqueous extract of *Citrus aurantium* peel contained two phenolic compounds. Indeed, the spot colored in white fluorescent blue corresponds to phenolic acids. The other appeared under UV, dark blue indicated the possibility of the presence of one of the three families of flavonoids, flavonols, flavonones, or aurones [16]. But their chemical composition was different from the standards. It should be noted that, the flavonoids are amphiphilic compounds and that they are entrained by the mobile phase, because of the resulting compatibility between the polar solvents and the hydroxyl part of the flavonoids (polar/polar) [17]. flavanones, flavonols and methoxyflavones have Rf values of 0.5 to 0.7 [18]. Following the results recorded, the methanolic extract of peel and aqueous of bitter orange leaves were found rich in phenolic acids and flavonoids. So these results agree with those revealed by the TLC which lacked specificity, in terms of the exact determination of the nature of the polyphenolic constituents.

The HPLC chromatography study of peel and bitter orange leaves has not yet been reported in the literature. While, the other parts of this plant have been studied and which have the same chemical characteristics. The gallic acid is one of the hydroxybenzoic acids identified after chromatographic analysis of the phenolic extract of the flowers of *Citrus aurantium* in the same flowering stage with an amount of 212.4 ± 0.02 µg/g DW [19]. Chromatographic analyzes of the ethanolic extract of *Citrus aurantium* carried out by

contained eight flavonoids, isonaringin, naringin, hesperidin, neohesperidin, narsteritin, ingenine and tangeritine [20]. According to the results observed, it appeared clearly that vanillic acid is a majority compound of the three phenolic acids identified in the ethanolic extract of the aerial part of *Artemisia herba alba*. While flavonoids are present with very small percentages. This extract seemed rich in flavonoids, kaempferol and simple phenols [21]. It can be deduced that the variations in results obtained depend on the availability of the standards used for the chromatographic analysis of the extracts, on the nature of the polyphenols to be sought and on the nature of the mobile phase used for the development.

In view of the set of percentage inhibition represented above, 1 mg/ml of the organic extract of *Citrus aurantium* leaves gave a percentage equal to 92.55% of scavenging activity against free radicals (DPPH) [22]. The ethanolic extract of *Artemisia herba alba* showed the lowest antioxidant activity compared to the results obtained with a concentration of 20.64 ± 0.84 mg/l [23]. While the methanolic extract of *Citrus aurantium* revealed a higher rate of reduction activity of the DPPH radical than that carried out previously (0.3 mg/ml) [19]. As well as, a rate of 1.9 mg/ml of IC 50 has been found [24]. The ethanolic extract of *Artemisia herba alba* has a high content of polyphenols and flavonoids. This reflects their important antioxidant activity compared to the other extracts (aqueous *Citrus aurantium* leaves and methanolic *Citrus aurantium* peel). Depending to the percentage of reduction of the free radical, the antiradical activity of the free radical DPPH is proportional to the levels of polyphenols and flavonoids endowed with their properties to yield a hydrogen atom. So the effect of these compounds contributes to the saturation of the unpaired electron (single) causing many diseases (inflammation, cancer, degenerative, etc.).

Our results were found lowest than that reported previously (0.654 for 1000 µg/ml), which they have noted a high reducing power caused by the same concentration of the ethanolic extract of *Artemisia herba alba asso* with absorbance equal to 0.813 ± 0.018 [25]. For the methanolic peel extract of *Citrus aurantium*, it had the moderate antiradical activity with absorbance of 0.597. However a value of 0.481 was obtained in case of *Citrus aurantium* leaves. The absorbances values were found equal to 0.250 and 0.251 for peels and leaves respectively [22]. According to our results, the absorbance of the extracts was marked proportionally to the concentrations. And by consequence, proportional to the hydroxyl groups of phenolic compounds which can serve as electron donors [12]. The reducing power of a compound can serve as a significant indicator of its potent antioxidant activity [13].

5. CONCLUSION

Regarding to the results observed, the formulated extracts revealed their richness in phenolic compounds, having detected by TLC method. Then, HPLC-DAD confirmed the presence of phenolic acids and flavonoids. The content of these extracts in phenolic compounds gave them a powerful antioxidant activity against free radicals. Therefore, the ethanolic extract of the aerial part of *Artemisia herba alba*, the methanolic extract of *Citrus aurantium* peel and the aqueous extract of *Citrus aurantium* leaves were considered as powerful scavengers of free radicals and can be incorporated into the pharmaceuticals preparations to treat or manage many cancer, inflammatory and even antimicrobial diseases.

Conflict of Interest Disclosures: None

Authors' Contributions: AS: Conceived of the presented idea. AS and SB: Developed the theory and the methodology and acquiesced data, verified the analytical methods. BM and ATTM: Supervised the findings of this work. The final manuscript has been read and approved by all authors.

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