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Chemical investigation of polar extracts from *Ruta chalpensis L.* growing in Tunisia: Correlation with their antioxidant activities.

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Abstract – *Ruta chalpensis* has been used as a spice and as natural additive for more than 1000 years. Ruta is a genus of Rutaceae family and features primarily shrubby plants that are native to the Mediterranean region. The aim of this study was to investigate the antioxidant activities of extracts obtained from aerial part of *Ruta chalepensis* using the non-enzymatic DPPH and ABTS assays systems. Methanol and aqueous extracts exhibited high antioxidant with IC50 values 54.1±1.5 and 73.6±2.6 µg/ml respectively for DPPH assay. Moreover, the results revealed that methanol and aqueous extracts present antioxidant efficiency against the $ABTS^{\cdot+}$ radical with respectively 92.4 % and 87.5% at the highest concentration of 3 mg/mL.
The present study has demonstrated that *Ruta chalepensis* extract possess potent antioxidant activities which could be derived from compounds such as flavonoids and polyphenols.

Keywords: *Ruta chalpensis*, free radical-scavenging activity, polar extracts, ploy phenol content.

1. Introduction

Living organisms are constantly exposed to oxidation. Endogenous by-products (peroxides, transition metals) and exogenous exposure, such as UV rays and other high-energy and heat radiation, lead to the formation of reactive oxygen and nitrogen species, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\cdot}$), singlet oxygen ($^1O_2$) hydroxyl ($OH^{\cdot}$), peroxy ($ROO^{\cdot}$) and alkoxy ($RO^{\cdot}$) radicals (Brewer, 2011). Living organisms synthesize various substances with a strong antioxidant effect in order to reduce the amount of reactive oxygen species and other free radicals. The idea of applying natural antioxidants as stabilizers instead of synthetic compounds first emerged in the food industry to hinder the disintegration of food and beverages (Embuscad, 2015).
However, many degenerative human diseases including cancer, diabetes mellitus, cardio- and cerebrovascular diseases have been recognized being a possible consequence of free radical damage to lipids, proteins and nucleic acids (Babbar et al. 2011). Natural antioxidants protect the living system from oxidative stress and other chronic diseases; therefore, they can play an important role in health care system (Ben sghaier et al. 2016).
Spices are also rich sources of phytochemicals and, particularly, *Ruta chalpensis* has been used as a spice and as natural additive for more than 1000 years. Ruta is a genus of Rutaceae family and features primarily shrubby plants that are native to the Mediterranean region, but it is widely growing in many parts of the world in temperate and tropical countries (khlifi el al. 2013). The genus Ruta (Rutaceae) encompasses more than 1800 species, mainly found in tropical and temperate regions with major centres of diversity in Southern Africa and Australia. The three most diffused species are *Ruta chalepensis L.*, *Ruta graveolens L.*, and *Ruta montana L.* It is used for culinary purposes to flavor foods and as an aperitif (Pollio et al. 2008).
R. chalepensis L. is used in the traditional medicine for treatment of variable diseases. This plant is described for its emmenagogue, abortifacient, antihelmintic and spasmolitic effects and for its depressant activity on the central nervous system (Pollio et al. 2008).
With regard to the phytochemical composition of Ruta chalepensis extracts, many authors have reported that its leaves and young stems contain alkaloids, flavonoids, coumarins, tannins, volatile oil, glycosides, sterols, triterpenes and saponins as possible active constituents (El Guiche et al. 2015). The infusions/decoctions of the two species (*R. montana* and *R. chalepensis*) are widely used in alternative medicine. They are used as a tonic, a febrifuge and a treatment of malaria as well as inflammatory, antioxidant and microbial processes (Khlifi et al. 2013; Kacem et al. 2014). In some countries of the

Mediterranean area, like Algeria, Cyprus or Israel, infusions of *R. chalepensis* leaves are taken to treat mental disorders. Furthermore, *R. chalepensis* is used in many countries of that area as a digestive and it is known that inhibitors of AChE are also used to increase gastrointestinal motility (Jarvie et al. 2008). According to previous literature reports illustrating the importance of Ruta as a medicinal plant, and because of its availability in Tunisia, in this work we aimed to evaluate the phenolic composition and the antioxidant polar extracts of Tunisian *R. chalepensis*. The results of this work highlighted the possibility of using *R. chalepensis* as a potential source of natural antioxidants agent in the food industry.

2. Materiel and Methods

2.1. Plant material

The aerial part of *R. chalepensis* was collected in March 2016 from the "Amdoune mountainous" region of Beja in North West of Tunisia. A voucher specimen (RC.03.16) was deposited at the Herbarium for future reference. The leaves were shade-dried, powdered, and stored in a tightly closed container for further use.

2.2. Preparation of plant extracts

The fresh leaves of *R. chalepensis* were dried at room temperature and reduced to coarse powder. One hundred grams of the powdered leaves were extracted with boiling water (1 L) for 15–20 min. After filtration, the crude extract obtained was frozen and lyophilized, leading to the aqueous extract which was dissolved in water.

The methanol extract (MeOH) was obtained by Soxhlet extraction (4 h) using 100 g of the powdered leaves and 1 L of solvent. This extract was also concentrated to dryness and the residue was kept at 4°C.

2.3. Determination of total polyphenol and flavonoid contents

Determination of total polyphenol content (TPC) of *R. chalepensis* extracts was quantified by the Folin-Ciocalteau's reagent assay and was expressed as gallic acid equivalents (Yuan et al. 2005). Aliquots of test samples (100 µL) were mixed with 2 mL of 2% Na_2CO_3 and incubated at room temperature for 2 min. After the addition of 100 µL of 50% Folin-Ciocalteau's phenol reagent, the reaction tube was further incubated for 30 min at room temperature, and finally, absorbance was read at 720 nm. Gallic acid (0–500 mg/L) was used for calibration of a standard curve. The results were expressed as µg gallic acid equivalent (GAE)/mg dry weight of plant material. Triplicate measurements were taken and mean values calculated. The flavonoid content was estimated according to the method of Zhishen et al. (1999), a known volume of each extract was placed in a 10 mL volumetric flask, distilled water was added to make the volume 5 mL, then 0.3 mL $NaNO_2$(1:20 w/v) was added to this dilution. Three milliliters of $AlCl_3$ (1:10w/v) were added 5 min later. After 6 min, 2 mL of NaOH 1 N were added and the total absorbance was measured at 510 nm (Kumar and Chattopadhyay, 2007). The flavonoid content was expressed as µg of quercetin equivalent (QE) /mg of each extract. Triplicate measurements were taken and mean values calculated.

2.4. Determination of tannins

According to Nwabueze (2007), extraction of tannins in the samples was achieved by dissolving 5 g of extract in 50 mL of distilled water in a conical flask, allowing the mixture to stand 30 min with the shaking of the flask at 10 min intervals and then centrifuging it at 5000 g to obtain a supernatant (tannin extract). The extract was diluted to 100 mL in a standard flask using distilled water. Five milliliters of the diluted extract and 5 ml of standard tannic acid (0.01 g/L) were measured into different 50 ml volumetric flasks. One milliliter of Folin–Denis reagent was added to each flask, followed by 2.5 ml of saturated sodium carbonate solution. The solutions were made up to the 50 mL mark with distilled water and incubated at room temperature (20–30 °C) for 90 min. The absorption of these solutions were measured against that of the reagent blank (containing 5 mL of distilled water in place of extract or standard tannic acid solution) in a Spectronic Genesys 10 s, Thermo Electron Corp (Madison, WI, USA) spectrophotometer at a 760 nm wavelength.

Tannin content (tannic acid equivalents) was calculated in triplicate, using the following formula:

$$\% \text{ Tannins} = [(DO_{extract}/\varepsilon x1)/\text{extract concentration}] \times 100$$

where ε is the molar extinction coefficient (l g^{-1} cm^{-1}) of tannic acid (=3.27 l g^{-1} cm^{-1}) and l = 1 cm.

2.5. Total anthocyanins content

Total anthocyanins content was determined using two samples containing 0.5 mL of each extract. One sample was treated with 4.4 mL of solvent for methanol extract/distilled water for aqueous extract and the second with 4.4 mL of 13% Sodium bisulfite solution and the mixture was diluted (1:1).
Sample absorbance was determined at 520 nm using a blank solution prepared from 4.9 mL solvent for methanol extract/distilled water for aqueous extract, 0.5 ml of 0.1% HCl in ethanol and 10 ml of 2% HCl in solvent.
The differences (ΔA) between the absorbance value of sample prepared without sodium bisulfite and the absorbance value of the sample prepared with sodium bisulfite were calculated. The anthocyanin content was express in mg/L of extract and was calculated by multiplying the Δ A value by 875 (Ribéreau-Gayon et al. 2006).

2.6. DPPH (2-2-diphenyl-1-picrylhydrazyl) free-radical-scavenging activity

DPPH is a free radical that has a blue-violet color when dissolved in ethanol. The loss of color indicates a radical scavenging activity. In order to measure the antioxidant activity, the DPPH free-radical-scavenging assay was carried out according to the procedure described by Fenglin et al. (2004). An aliquot of each tested compound at various concentrations (250, 125, 62.5, 31.25 and 15.625 µg/mL in ethanol) was mixed with 23.6 µg/ mL of DPPH solution in ethanol. After incubation of the mixture for 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activities were expressed as a percentage of the absorbance of the control DPPH solution (Yagi et al. 2002), and the radical scavenging activity was obtained from the following equation:
Activity (%) = $[(A_{517}\text{control} - A_{517}\text{sample})/A_{517}\text{control}] \times 100$:
The results were expressed as mean of at least three independent experiments. Results were expressed as percentage activity. Mean inhibiting concentrations IC_{50} were calculated by use of the Litchifield and Wilcoxon test (Galati et al. 2001). Data were collected and expressed as mean±standard deviation of three independent experiments.

2.7. Radical-scavenging activity on $ABTS^{+\cdot}$

An improved ABTS [2, 2′-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid) diammonium salt] radical cation decolorization assay was used. It involves the direct production of the blue/ green $ABTS^{+\cdot}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the preformed radical cation reduces it to ABTS, to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant, and the duration of the reaction (Re et al. 1999). ABTS was dissolved in water to a 7 mM concentration. $ABTS^{+\cdot}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS+· solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm. In order to measure the antioxidant activity of extracts, 10 µL of each sample at various concentrations (0.25, 0.5, 1, 1.5, and 3 mg/mL) was added to 990 µL of diluted $ABTS^{+\cdot}$ and the absorbance recorded every 1 min. We stop the kinetic reaction when the absorbance becomes stable. Each concentration was analyzed in triplicate. The percentage decrease of absorbance at 734 nm was calculated for each point, the antioxidant capacity of the test compounds was expressed in percent inhibition (%). The percentage scavenging of $ABTS^{+\cdot}$ was calculated by the following formula:

$$\text{Scavenging activity \%} = [(A_0 - A_x)/A_0] \times 100$$

A_0 and A_x were the absorbance at 734 nm of samples without and with extract, respectively.

IC_{50} value was calculated from regression analysis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard in comparison for the determination of the antioxidant activity of a compound.

2.8. Calculation of Trolox equivalent antioxidant capacity (TEAC)

A calibration curve was prepared with different concentrations (standard range 0–15 µM, final concentration) of Trolox. Trolox equivalent antioxidant capacity (TEAC) can be assigned to all samples able to scavenge the $ABTS^{+\cdot}$ by comparing their scavenging capacity to that of Trolox, a water-soluble vitamin E analog. This index is defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the extract (Antolovich et al. 2002).

2.9. Statistical analysis

The results were expressed as means±S.D. from at least three independent experiments. Statistical analysis was performed using unpaired Student's test. The value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Phytochemical study and metabolite content of *R. chalepensis* extracts

Phenolics are aromatic secondary plant metabolites, and are widely spread throughout the plant kingdom. Phenolics have been associated with color, sensory qualities, nutritional, and antioxidant properties (Robbins, 2003).

Considered to be the most frequent antioxidant compounds in human diets, polyphenols possess multiple biological properties, making it vital to learn about their amounts and varieties in medicinal plants and natural foods (Vlase et al. 2013). Flavonoids are phenolic compounds, which are very effective antioxidants. The Folin–Ciocalteu method is a rapid and widely used assay, to investigate the total phenolic content but it is known that different phenolic compounds have different responses in the Folin–Ciocalteu method (Kahkonen et al. 1999).

The results of the phytochemical study and metabolite content of *R. chalepensis* extracts are represented in Table 1. Thus, the TPC values were expressed as gallic acid equivalents (µg GAE/mg DW). The calculation of total flavonoid content was carried out using the standard curve of quercetin and presented as quercetin equivalents (µg QE/mg DW).

The methanol extract was more enriched in flavonoid and total polyphenolic compounds than the aqueous extract. In fact, the polyphenolic and flavonoid contents in methanol extract were respectively, equivalent to 71.28 µg of gallic acid and 14.69 µg of quercetin. The tannin content in methanol and aqueous extracts were respectively, 2.25 and 1.2 µg equivalent of tannic acid. Moreover, the methanol extract was more enriched in anthocyanins compounds than the aqueous extract with 465.81 mg EM3G/ L.

Phenolic and flavonoids contents of aqueous extract of *R. chalepensis* leaves have been already reported in Tunisia, with comparable results (Khlifi et al. 2013; Khadhri et al. 2016; Ouerghemmi et al. 2017).

In conclusion, our findings showed the richness of *R. chalepensis* extracts on phenolic contents allowing us to study of their potential biological activities.

Table 1. Quantitative polyphenol, flavonoid, tannins and anthocyanin contents of extracts from *Ruta chalpensis* aerial parts

Extracts	Metabolites			
	Polyphenols (µg GAE/mg DW)[a]	Flavonoids (µg QE/mg DW)[a]	Tannins (µg TAE/mg DW)[a]	Anthocyanins (mg EM3G/ L)[a]
Methanol extract	71.28±3.5	14.69±0.1	2.25±0.3	465.81±4.5
Aqueous extract	26.87±1.5	3.58±0.15	1.2±0.07	245.85±2.5

GAE: gallic acid equivalents; QE: quercetin equivalents; TAE: tannic acid equivalents; EM3G: malvidin-3-glucoside equivalents.
[a]Averages ± standard deviation were obtained from three different experiments.

3.2. DPPH free radical-scavenging activity

Several methods are commonly used to measure the antioxidant capacity of extracts. Each method results in the generation of or uses a different radical that is directly involved in the oxidative process through a variety of mechanisms. No single assay can represent the total antioxidant capacity, and for this reason two different and complementary assays were used to evaluate the extract antioxidant activities (Ouerghemmi et al. 2017). DPPH is a molecule containing a stable free radical; in the presence of an antioxidant that can donate an electron to DPPH, the purple color typical of the free DPPH radical decays, a change that can be followed spectrophotometrically at 517 nm. This simple test can provide information on the ability of a compound to donate an electron, the number of electrons a given molecule can donate, and on the mechanism of antioxidant action (Ben sghaier et al. 2011).

The results from the radical scavenging assays for all extracts are presented in Table 2 as IC_{50} (µg/mL). The best results were obtained with methanol extract (IC_{50}=54.1±1.5 µg/mL) compared to aqueous extract (IC_{50}=73.6±2.6 µg/mL). The obtained values are near to others previous investigations (IC50=70.01 ± 2.11 µg/mL) (Khlifi et al. 2013). Also, the result obtained by Ouerghemmi et al. (2017) are better than the antioxidant activity of our tested extracts with IC_{50} of 30.69 ± 0.041 µg/mL.

These Ruta extracts have higher antioxidant activity than those of *R. graveolans* that showed an IC_{50} of 459 µg /mL.

Table 2. Radical scavenging activity of *Ruta chalpensis* extracts by DPPH method

Extracts	Concentrations (µg/ml)	Antioxidant activity (%)[a]	IC50 (µg/ml)[a]
	15,625	18,4 ± 0.62	
	31,25	33,6 ± 0.81	
Methanol extract	62,5	52,2 ± 1.32	54.1±1.5
	125	73 ± 1.84	
	250	82.4 ± 2,35	
	15,625	11.8 ± 1.68	
	31,25	22.6 ±1.81	
Aqueous extract	62,5	45.2±2.1	73.6±2.6
	125	60,5 ± 2.35	
	250	71.15 ± 3.14	

[a]Averages ± standard deviation were obtained from three different experiments.

3.3. ABTS radical cation scavenging activity

The $ABTS^{+\cdot}$ radical formed as follows $ABTS\text{-}e^{-} \rightarrow ABTS^{+\cdot}$ reacts quickly with electron/hydrogen donors to form colorless ABTS. The reaction is pH-independent. A decrease of the $ABTS^{+\cdot}$ concentration is linearly dependent on the antioxidant sample concentration, including Trolox as a calibrating standard (Van den Berg et al. 2000).

The results revealed that methanol and aqueous extracts present antioxidant efficiency against the $ABTS^{+}$ radical with respectively 92.4 % and 87.5% at the highest concentration of 3 mg/mL (Table 3).

Table 3. Concentration-dependent ABTS free radical scavenging activity of *Ruta chalpensis* extracts

Extracts	Concentrations (mg/mL)	Antioxidant activity (%)[a]	TEAC (mM)[a]
	0.25	29,45 ± 1.62	
	0.5	47,6 ± 1.81	
Methanol extract	1	79.07 ± 2.35	1.84
	1.5	88.9 ± 2.84	
	3	92.4 ± 3.35	
	0.25	22,1 ± 1.68	
	0.5	37,6 ± 1.95	
Aqueous extract	1	70.25±2.28	1.61
	1.5	79,4 ± 3.35	
	3	87.5 ± 3.14	

[a]Averages ± standard deviation were obtained from three different experiments.

The TEAC of different extracts was also calculated. The TEAC values reflect the relative ability of hydrogen or electron donating antioxidants of a sample to scavenge the $ABTS^{\cdot+}$ compared with that of Trolox. When referring to TEAC values, methanol extract seems to be the more potent antioxidant with TEAC value of 1.84 mM. It appears that the highest antiradical activity of methanol and aqueous extracts reflects their high phenolic (71.28±3.5µg and 26.87±1.5µg GAE/mg DW) and flavonoid contents (14.69±0.1µg and 3.58±0.15 QE/mg DW) (Table 1).

These results correlate very well with other investigations by Heim et al. (2002) revealing antiradical and antioxidant activities of small phenolics including flavonoids and phenolic acids. Likewise,

Hagerman et al. (1998) have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\cdot+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups.

3.4. Correlation of the different activities with chemical composition

It is interesting to observe the correlation between the phenolic content and antioxidant activity of plant extracts, since phenolic compounds contribute directly to antioxidant activity. In this study, there was a distinct correlation between studied parameters (total polyphenolic, flavonoid, tannins and anthocyanin content, and antioxidant activities) in selected *Ruta chalpensis* plant extracts. This correlation was demonstrated by linear regression analysis.

With reference to Table 4, the correlation of chemical compostion against the different activities was satisfactory ($r > 0.75$). The high antiradical activity of the different extracts tested could be attributed to their richness of molecules with high antiradical potential such as polyphenols, flavonoids, tannins and anthocyanins. This hypothesis is confirmed by several authors who attribute the antiradical activity of plant extracts to these molecules (Cai et al. 2004; Park et al., 2004, Copland et al., 2003).

Table 4. The correlation between antioxidant assays and total phenol, flavonoid, tannin and Anthocyanin contents.

Antioxidant activity	Extracts	Polyphenols	Flavonoids	Tannins	Anthocyanins
DPPH	Methanol extract	0,86	0,88	0,81	0,80
	Aqueous extract	0,75	0,77	0,79	0,75
ABTS	Methanol extract	0,98	0,97	0,81	0,86
	Aqueous extract	0,95	0,92	0,76	0,85

4. Conclusion

The present study has demonstrated that *Ruta chalpensis* extracts possess potent antioxidant activities, which could be derived from compounds such as flavonoids and polyphenols. These antioxidant activities could have contributed, at least partly, to the therapeutic benefits of certain traditional claims. The results presented here could be an additional argument to support the use of this species in the North African tradition medicine. Furthermore, *Ruta chalpensis* extracts could give rise to antimicrobial, anti-inflammatory and antiulcer agents and could be promising candidates for further studies designed to obtain more evidence on their components with potential chemo-preventive activity. Safety and *in vivo* efficacy studies on this potential plant will be conducted.

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