PAPER

COMPARATIVE STUDY ON TOTAL POLYPHENOLS CONTENT OF TUNISIAN WILD *RHUS PENTAPHYLLA* FRUIT EXTRACTS AND THE EVALUATION OF THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT

This study was focused on determining the total polyphenols content and biological activities from wild *Rhus pentaphylla* fruits at two different maturity stages, (stage 1: green-yellow fruits and stage 2: purple-red fruits Highly significant ($p \leq 0.01$) increase was observed in total phenolic contents of fruits ranging from 158.65±0.06 to 177.57±0.06 mg Eq.AG.100 mg^{-1}DW. In contrast, both total flavonoid (from 157.34±0.07 to 152.69±0.14 mg Eq.Cat.100 mg^{-1}DW) and condensed tannins contents (from 200.26±0.30 to 131.23±0.24 mg Eq.Cat.100 mg^{-1}DW) showed dramatic decreases ($p \leq 0.01$). Evaluation of biological activities has provided good effects. Our results show the importance of using this wild fruits in pharmaceutical industries.

Keywords: *Rhus pentaphylla,* ultrasonic extraction, total polyphenol content, antioxidant activity, antimicrobial potential

1. INTRODUCTION

The human body is constantly exposed to a multitude of microbes (bacteria, viruses, parasites, fungi) (TILLE, 2013). Although it has a complex defense system that allows it to meet or host these microbes without allowing them to invade its tissues, it is sometimes necessary to use antimicrobial agents, antivirals etc. (HAMBLIN and HASAN, 2004). But in some cases, these agents are unable to treat certain infectious diseases (ATANASOV *et al*.,2015). Faced with this incapacity, a trend has developed in recent years with the aim of finding new natural sources of plant-based bioactive molecules (CARINA, 2012).
The genus *Rhus pentaphylla* consists flowering species belonging to *Anacardiaceae* family, often grows in non-agricultural areas and widely used in foods (LEE *et al*., 2010). They are recognized in traditional medicine by their therapeutic interests (anticancer, anti-inflammatory, antidiarrhea), and it provides bioproducts that have desirable biological activities: antifungal, anti-inflammatory, antimalarial, antimicrobial, antitumorigenic, antioxidant, antiviral and hypoglycemic (ABED, 2013). The genus has several varieties in Spain, in the region of Malaga, as well, it is divided into northern Africa, which is common in the west of Algeria and Morocco (RACHED, 2009). In Tunisia, the genus *Rhus* is represented by two species: *Rhus tripartita* (Ucria) and *Rhus pentaphylla* which spreads in north and center of the country (ITIDEL *et al*., 2013). *R. pentaphylla* fruits, fresh or dried are slightly acidic (LAHSISSENE *et al*., 2010), and have a pleasant taste (RACHED, 2009), also it has been used in the treatment of diarrhea (LAHSISSENE *et al*., 2010). This genus has also been introduced as a medicinal vascular plant protection and cardiovascular diseases preventive in traditional medicine (SABZGHABAEE *et al*., 2014). Owing to its richness in tannins, flavonoids and coumarin, these natural molecules have anti-substantial activity of butyrylcholinesterase, and therefore can be used in the treatment of Alzheimer's disease (GHOUILA *et al*., 2014). This immense family of polyphenols has an important role in food quality through its antioxidant properties; contribute taste, astringency, flavor, color, long-term stability, and their antimicrobial activities (LARCHER *et al*., 2013). So in order to search for new drugs to contribute the bacteria and viruses, the aim of this study is to investigate the total polyphenols content of wild *R. pentaphylla* fruits collected at different maturity stages and to evaluate their biological activities.

2. MATERIAL AND METHODS

2.1. Fruit sampling

R. pentaphylla were collected from March to June 2015 in Kroumirie Mountains (Ain Draham Municipality, Governorate of Jendouba situated at an altitude of 602 m, at 36°44’57 North latitude and 08°41’12 34’’ East longitude) Tunisia (EL MAKNI and EL AOUNI, 2011). An experienced plant taxonomist presented during collection. At this point, 3 kg of fruits at two ripening maturities stages, green-yellow color fruits (un-ripe stage; S1) and purple-red color fruits (fully-ripe stage; S2) were collected at the same time to ensure subsequent maturity classes would develop on the clone. The collected fruits were transported in the same cooling and light conditions to chemistry department of Faculty of Sciences Bizerte. The different stage of fruits was immediately washed with distilled water and lyophilized at -80°C for 48 hours. Chemical analyses were conducted on the composite lyophilized fruit samples collected over time.

2.2. Chemicals, standards, and reagents

Gallic acid, (±)-catechin hydrate ($C_{15}H_{14}O_6$),and 2,2-diphenyl-1-Picrylhydrazyl (DPPH) were purchased from Univar and Sigma-Aldrich (France).Folin-Ciocalteu's reagent was purchased from Carlo Erba Reagents. 2,4,6-Tri (2-pyridyl)-s-triazine ($C_{18}H_{12}N_6$) was purchased from Acros Organics. Butylated hydroxytoluene (BHT, $C_{15}H_{24}O$), and Iodonitrotetrazolium chloride ($C_{19}H_{13}C_{11}N_5O_2$) were purchased from Fluka (Turkey). Aluminium chloride ($AlCl_3$), Vanillin, all other used chemicals and solvents (methanol, ethanol, acetone, hexane, HCl and DMSO) were purchased from Merck (Darmstadt, Germany).

2.3. Extract preparation

R. pentaphylla fruits at different maturity stages were processed separately. Soxhlet, maceration and ultrasonic extraction techniques were recommended in the literature (MOLLICA *et al*., 2018; GÜNAYDıN *et al*., 2017). Approximately, 6 g of freeze-dried fruits were stirred with 60 mL methanol: water (80:20,V/V) with vortex (IKA MS 3 basic) at 2300 rpm for 1 min. The mixtures were then extracted three times in an ultrasonic bath for 15 minutes (Elma Transsonic DIGITAL, T=25 °C, and F= 150W). The collected extracts were centrifuged at 4000 rpm (Eppendorf Centrifuge and Rotor Packages Model 5810), for 10 minutes, then filtered through a Whatman No. 4 paper and the organic phase (methanol) were evaporated under vacuum evaporator (Heidolph). The resulting aqueous phases were washed with water to dissolve the extracted material from the glassware. Afterwards the aqueous material was lyophilized and stored until use. The obtained lyophilized materials were dissolved in a known volume of methanol and DMSO for the quantification of polyphenols content, and the determination of antioxidant and microbial activities, respectively.

2.4. Determination of total phenolics content

The total phenolic contents of each extract were determined by the modified Folin Ciocalteu method described by Singleton (SINGLETON *et al*., 1999; BLAINSKI *et al*., 2013). Briefly, 1 ml of methanol extracts was added to 1 ml of Folin Ciocalteu reagent diluted with distilled water (1:9). The samples were left in room temperature for 5 minutes to develop color; the reaction was then stopped by adding 10 mL of Na_2CO_3 (7%). After homogenization and vortex, the mixture was heated for 10 minutes at 40°C, and then incubated in dark for 30 min. Measurement of the absorbance was carried out in UV spectrophotometer (Thermo scientific EVOLUTION 201) at 765 nm. Total phenol concentration was calculated from the regression equation of the calibration curve established using different concentrations from stock solution of gallic acid (12-152 $\mu g.mL^{-1}$). The results were expressed as mg of gallic acid equivalent per gram of dry weight (mg Eq. GA. 100 mg^{-1}DW).

2.5. Determination of flavonoids content

Flavonoids content of *R. pentaphylla* fruit extracts were carried out by following colorimetric assay using aluminum trichloride ($AlCl_3$) (ZHISHEN *et al*., 1999). 0.3 mL of $NaNO_2$ (5%) was added to 1 mL of methanol extract of each sample. After 6 min, 0.3 mL of 10% methanol solution of $AlCl_3.6H_2O$ was added to the extract samples. To the mixtures, 2 ml of NaOH (1M) and 10 mL of H_2O were added, followed by keeping them in dark for 2 hours. Absorbance was read at 520 nm after incubation in the dark at room temperature

for 10 min. The results were expressed in mg catechin equivalent 100 mg^{-1} dry weight (mg Eq.Cat.100 mg^{-1}DW).

2.6. Determination of condensed tannins content

Determination of condensed tannins is based on phenolic compounds condensation with vanillin under acidic conditions, which will provide a brown compound (PRICE *et al*., 1978). Briefly, 100 mg of lyophilized plant materials were dissolved in 1mL of methanol and added to 2 ml of 1% vanillin in 70% sulfuric acid H_2SO_4 (1 g vanillin in 77.77 mL of H_2SO_4 completed with distilled water to prepare 100 mL). The entire mixture was placed in a water bath for 15 min at 30°C protected from light. Finally the absorbance of the mixture was read at a wavelength λ = 500 nm using a Thermo scientific EVOLUTION 201 UV-Visible Spectrophotometer. The results were expressed in mg catechin equivalent 100 mg^{-1} dry weight (mg Eq.Cat.100 mg^{-1}DW).

2.7. Antioxidant activities

2.7.1 DPPH assay

Among the tests to determine the radical scavenging ability, 2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis is one of the most widely used. Indeed, the DPPH assay is rapid, simple, stable, and economical to measure the antioxidant capacity of foods or plant extracts (JABBARI and JABBARI, 2016). Briefly for each extract, a 50 $mg.mL^{-1}$ stock solution had been prepared with methanol as solvent, and if necessary sequential dilutions were made (SOKMEN *et al*., 2004). Then, for all extracts solution, 50 μL was added to an equal volume of freshly DPPH radical solution (4.10^{-3}mM) and allowed to stand for 30 min in the dark at room temperature. The experiment was repeated for three times. The entire mixture was placed in the dark at room temperature for 30 min, and the absorbance of the mixture was read at λ = 517 nm using a UV-Visible Spectrophotometer. BHT was used as the standard control.

2.7.2 FRAP assay

Ferric reducing antioxidant power (FRAP) was performed according to the methods of BENZIE and STRAIN (1999) with slight modifications (PULIDO *et al*., 2000) were used as an evaluation of the reducing power of samples. The stock solution (50 $mg.mL^{-1}$) of each extract was prepared in methanol. A portion of 50µL solution was mixed with 1.5 mL of FRAP reagent in a test tube and vortexed. Blank samples were prepared with methanol and deionized water. Both samples and blank were incubated in a water bath for 30 minutes at 37°C and the absorbance of the samples was determined against blank at 593 nm. Series of stock solution at 62.5, 125, 250, 500 and 1000 µM were prepared using an aqueous solution of $FeSO_4.7H_2O$ as the standard curve. The values obtained were expressed as μM of ferrous equivalent Fe (II) per gram of freeze-dried sample. Increases in absorbance due to the formation of a colored TPTZ-Fe^{2+} complex were monitored at 593 nm. A Trolox per gram curve (0.1- 0.8 μM) was used as a standard.

2.8. Antimicrobial/Antifungal Tests

The same procedure given above was employed for the extraction. The evaluation of antimicrobial activities of *R. pentaphylla* extracts was carried out using three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcusfaecalis* ATCC 29212, *Listeria*

monocytogenes ATCC 19115) and two Gram-negative bacteria (*Escherichiacoli* ATCC 25922and *Pseudomonasaeruginosa* ATCC 27853) and against one fungal strain (*Candidaalbicans* ATCC 10231).

Broth dilution method

Micro-dilution technique was one of the most basic methods for antimicrobial susceptibility testing (BALOUIRI *el al*., 2016). The used pre-culture contain Muller-Hinton Broth medium (MHB), although a pre-culture McFarland 0.5~10^8 UFC.mL^{-1} (Colony Forming Unit) ~ 0.2 OD (optical density). The ad of 2 mL of sodium chloride (NaCl) or 0.9% phosphate buffered saline (PBS) to vials followed by the addition of bacteria with a cotton rod, and the optical density is measured at λ = 600 nm to obtain an absorbance 0.2 for all used bacterial strains. PBS is used as a blank. Each well of a sterile 96-well microtitre plate-shaped V was aliquoted with an equal volume of Mueller-Hinton broth and fruits extract initially dissolved in DMSO. We conducted a series of dilution, for the first eleven well, we added 100 μL of fruit extracts and we add then 100 μL of Muller Hinton Broth solution (Ca^{2+}, Mg^{2+}). Concentrations (5; 2.5; 1.25mg.mL^{-1}; and 4.88µg.mL^{-1}) are taken into a waterfall and the last well was used for the addition of control's bacteria. After 10 to 15 min, the microplate was incubated in the oven at 37°C for 24 hours. To each well 40μL of colored indicator iodonitrotetrazolium, $C_{19}H_{13}C_{11}N_5O_2$ chloride (200μg.mL^{-1}) was added.

3. Statistical analysis

All results are expressed as (±SD) of total phenolic, flavonoid and tannins contents. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey's HSD post hoc test. Correlation tests were used to determine relationships among the polyphenols content and antioxidant activities during ripening stages of *R. pentaphylla* fruits. Significance overall and within any correlation (confirmed by linear regression test) was accepted at $p<0.05$.

4. RESULTS AND DISCUSSION

4.1. Phenolic contents

R. pentaphylla fruits showed a highly significant ($p\leq0.01$) increase in total phenolic content during ripening stage, The order of total phenolic content (Table 1), of samples during ripening is [(S1)<(S2)].

The concentration increased from 158.65 for the unripe fruits to 177.57 mg Eq.AG.100 mg^{-1}DW for the fully-ripe fruits. From the obtained results, it can be seen that the phenolic contents of fruits could also be affected by experimental conditions and various factors such as variety, growing condition, maturity, season, amount of sunlight received. This result was in agreement with previous work (MARINOVA *et al*., 2005; NAVARRO *et al*., 2006), where phenolic compounds increase during the last stage especially for red colored varieties, and it's increasing with maturation.

Table 1. Total polyphenols content and antioxidant activities at different maturity stages of *R. pentaphylla* fruits extract.

		1st stage: un-ripe	2nd stage: fully-ripe
Total polyphenols content	TPC (mg Eq.AG. 100 mg^{-1}DW)	158.65±0.06**[a]	177.57±0.06**[a]
	TFC (mg Eq.Cat.100mg^{-1}DW)	157.34±0.07**[b]	152.69±0.14**[b]
	CTC (mg Eq.Cat.100mg^{-1}DW)	200.26±0.3**[c]	131.23±0.24**[c]
Antioxidant activities	DPPH (IC_{50} $mg.mL^{-1}$)	0.42	< 0.5
	FRAP (µmol $Fe^{2+}.g^{-1}$DW)	64.36	81.59

Values are expressed as (±SD) (n=2), **highly significant ($p \leq 0.01$), [a]: statistic measurement between un-ripe and fully-ripe fruits for phenolic contents, [b]: statistic measurement between un-ripe and fully-ripe fruits for flavonoid contents, [c]: statistic measurement between un-ripe and fully-ripe fruits for tannins contents.

4.2. Flavonoid contents

Fruit ripening are related to important biochemical changes such as color, texture, taste and other quality traits modify. As seen, ripening stage caused a decrease in total flavonoids content; the results of the distribution of TFC in relation to fruit maturity/ripening stages are presented in Table 1. The concentration of flavonoid varies in the following order: [(S1)> (S2)], so as the fruit maturity progressed, there is a highly significant ($p \leq 0.01$) decrease from 157.34 to 152.69 mg Eq.Cat.100 mg^{-1} DW of FC in *R. pentaphylla* fruits. This decrease in TFC with advanced maturity is in agreement with the study of TLILI *et al*. (2014) who reported that the decrease of flavonoid content during ripening may be due to metabolic production of other phenolic compounds or degradation via enzyme action.

4.3. Condensed tannins content

As seen in Table 1, maturity stages of *R. pentaphylla* fruits had a marked effect on the amounts of condensed tannins. The variations of flavan-3-ol content during ripening follow this order [(S1) > (S2)] of ripening stages (a highly significant ($p \leq 0.01$) decrease). The higher concentrations of condensed tannins are found in S1 un-ripe stage (200.26mg Eq.Cat.100mg^{-1}DW) approximately two times more than those of fully-ripe (S2), Flavan-3-ol present a drastic declined during fruit ripening (KENNEDY *et al*., 2000). This change in tannins content in the later stages of ripening could be explained by the fact that different phenolic acids might have condensed to form complex phenolic compounds such as tannins and lignin (BEN AHMED *et al*., 2009).
A significant difference ($p < 0.05$) was demonstrated by ANOVA with a Tukey's HSD post hoc test (Table 1), which confirm that the variation of this polyphenols content (phenolic, flavonoid, and condensed tannins contents) was influenced by the stage of maturation and/or their interaction according to fruit shape.

4.4. DPPH assay

Methanol extracts of *R. pentaphylla* fruits crude were investigated through in vitro models radical scavenging activity using DPPH method. Inhibitory concentration IC_{50} value is the most critical value that reflects the antioxidant action of the tested species. If this value is low, the tested sample has a high antioxidant activity. The IC_{50} value of standard synthetic antioxidant BHT was ranging 0.17 $mg.mL^{-1}$. Table 1 shows that the 1st stage (un-ripe) of *R. pentaphylla* exhibited better antioxidant action about 0.42 $mg.mL^{-1}$, then the 2nd stage (fully-

ripe). The Antioxidant power of *R. pentaphylla* fruits is due to a wealth of flavonoid and tannins, which have been reported to have multiple biological effects, including antioxidant activity due to the redox properties of their phenolic hydroxy groups and the structural relationships between the different parts of the chemical structures (RICE-EVANS and MILLER, 1996).

4.5. FRAP assay

The ferric reducing antioxidant power (FRAP) was measured for each extract obtained at stages 1 and 2. Results were given in Table 1. The obtained values had been expressed as μM of ferrous equivalent Fe (II) per gram dry weight. From the results given in Table 1, the 2nd stage of *R. pentaphylla* exhibited better antioxidant principles then 1st stage (81.59 and 64.36 μmol $Fe^{2+}.g^{-1}$DWrespectively). The order of total activity is the same as total phenol content. A further investigation by PALAFOX-CARLOS *et al.* (2012), who suggest that the physiological and ripening process in fruit may affect directly the presence of phenolic compounds content and their antioxidant activity which increased during ripening.

4.6. Correlation between total polyphenols content and antioxidant activity

As result for this study, there was a positive correlation between the polyphenols content ($r = 0.876$), the two test (DPPH and FRAP) and the maturity stages, but this correlation is not significant (linear regression test $p = 0.0513$); the results of the regression is the same in the case of multiple regression (case of this test) and that when we made the regression one by one.

4.7. Antimicrobial potentials

Antimicrobial potential of *R. pentaphylla* extracts obtained from the two stages was tested separately. The results obtained from the first and the second stages using micro-dilution technique were given in Table 2.

Table 2. Antimicrobial activity of different crude extracts of *R. pentaphylla.*

	MIC($mg.mL^{-1}$) Methanol/Water	
Bacterial strains	**First stage**	**Second stage**
Escherichia coli	1.25	1.25
Staphylococcus aureus	-	-
Enterococcus faecalis	-	-
Pseudomonas aeruginosa	-	1.25
Listeria monocytogenes	-	-

The evaluation of in vitro antibacterial activity of methanol/water extract of *R. pentaphylla* fruits at different maturity stages against the in-use bacteria, showed that the two stages of fruits extract exhibited antibacterial activity against three positive Grams (*Enterococcusfaecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus*) and two negative Grams (*Pseudomonas aeruginosa* and *Escherichia coli)* bacteria at different concentrations. MIC values of the two stages are ranged 1.25 $mg.mL^{-1}$ for the tested microorganisms. Methanol/water extracts of both stages of *R. pentaphylla* exhibited important antibacterial effects against *Escherichia coli* and *Pseudomonasaeruginosa*. From the obtained results

Escherichia coli was found to be the most sensitive organisms for the two stages. The results prove that there might be synergism between polyphenols content and antimicrobial (CUSHNI and LAMB, 2005). However, *R. pentaphylla* showed no antifungal effect against C*andida albicans* at its different maturity stages.

4. CONCLUSIONS

Quantitative analysis of widely *R. pentaphylla* fruit extracts revealed richness in secondary metabolites such as phenolic, flavonoids and condensed tannins. A highly significant difference was demonstrated which confirm that the variation of this polyphenols content was influenced by the stage of maturation and/or their interaction according to fruit shape. DPPH test showed that the first stage has the higher antioxidant activity, which is not the case for FRAP test. A good correlation between the polyphenols content, the two test (DPPH and FRAP) and the maturity stages. As a result of this study, the validation of bioactivity of this fruit as agro alimentary in operating pharmacy was confirmed. In fact, fruit extracts at different maturity stages present a good potential as antibacterial compounds against the tested microorganisms comparing with the commercial antibiotics. So we could suggest that this wild fruits could be used in the treatment of infectious diseases caused by resistant microbes.

ACKNOWLEDGEMENTS

This study was financially supported by the Tunisian Ministry of higher education. The authors wish to acknowledge the University of Carthage for providing financial support for the research work.

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Paper Received June 13, 2018 Accepted September 21, 2018