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Effect of extraction solvent on total phenol content, total flavonoids content, antioxidant and antimicrobial activities against phytopathogenic and foodborne pathogens bacteria of *Ruta montana* extracts

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Abstract In this study, water and various concentrations of methanol and ethanol in water were used as solvent in the extraction of aerial part of *Ruta montana*. The aims were to investigate the effects of solvents on the extraction of polyphenol from *Ruta montana*, from Taza region, and evaluate *in vitro* the antioxidant and antimicrobial activities against phytopathogenic and foodborne pathogens bacteria of the extracts. The results showed that the *Ruta montana* extract obtained by 80% methanol exhibited the highest phenolic (117.70 ± 2.82 mg GAE/g Ext) and flavonoid (77 ± 6.55 mg ER/g Ext) contents. The antioxidant potential was examined by Diphenyl picryl hydrazinyl radical (DPPH) and ferric reducing power assays (FRAP). The 80% methanol extract presented the greatest capacity to reduce the DPPH (IC_{50}=10.66 ± 1.52 µg/mL), and the highest ferric reducing power (66.66 ± 5.67 mg AAE/g of extract). The antimicrobial activity of extracts from *Ruta montana* against phytopathogenic and foodborne pathogens bacteria was qualitatively and quantitatively tested by the development of inhibition zones (ZID) and MTT-assay. The ZID ranged between 10.66 mm and 19.33 mm. *Proteus mirabilis*, *Clavibacter michiganensis* subsp. *michiganensis 1616-3*, *Bacillus subtilis DSM 6633*, *Listeria innocua CECT 4030* were the most sensitive bacteria with MIC varied from 1.56 to 12.5 mg/mL. These finding suggest that *Ruta montana* may be considered as a potential sources of phenol contents with antioxidant and antibacterial potentials against phytopathogenic and foodborne pathogens bacteria.

Keywords: *Ruta Montana;* Antioxidant and antimicrobial activities; Phytopathogenic; Foodborne pathogens.

1. Introduction

The protection of agricultural crops and the preservation of the organoleptic and health qualities of food products represent a major challenge for the agricultural and agro-food industries [1-2]. The strategy to inhibit the growth of undesirable microorganisms is the use of pesticides against phytophatogenic bacteria and synthetic compounds to preserve the food products. However, these chemical molecules present a risk on environment and with potential effects on the consumer [3-6]. For these reasons, a consumer demand for natural preservatives has increased. Essential oils and extracts obtained from many plants have recently gained a great popularity and scientific interest for their biological proprieties, which attributed as secondary metabolites. Among these molecules, the phenolic compounds exhibit a wide range of biological properties, such as antioxidant [7-8], antimicrobial [9-10], anticancer [11-13].

Many methods of extraction have been used to explore the phenolic compounds in plants. However, extraction yield of phenolic compounds not only depend on the used method but also on the extraction solvent. The commonly used solvents for extracting polyphenols were water and organic solvents singly or in combination with water (ethanol, methanol, acetone, diethyl ether) [14-17]. Additionally, the solvent polarity played a key role in increasing phenolic solubility [18]. Morocco is fortunate to have such a diversity of climatic conditions and biotopes that almost any plant can grow and be cultivated economically. It well known for its diverse flora: more than 4500 taxa of vascular plants and between 800 and 951 taxa were endemic [19-22]. *Ruta montana* specie, a member of *Rutaceae* family, locally known as "Fidjl/Aourmi" is a commonly used plant in Moroccan traditional medicine to treat diabetes mellitus, in abscesses treatment, emetic in pediatric and to treat psychic sicknesses [23-25]. In literature, only a few reports about this species are described. For these reasons, the aims of this work were to investigate the effects of solvents on the extraction of polyphenol from *Ruta montana* from Taza region, and to evaluate *in vitro* the antioxidant, antimicrobial activities against phytopathogenic and foodborne pathogens bacteria of their extracts.

2. Materials and methods

2.1. Plant Materials

The specie *Ruta montana* was collected, based on ethnopharmacological information and traditional uses, from Taza region (North-East of Morocco), and was authenticated in the Laboratory of Natural Resources and Environment by the botanist Dr Khabbach Abdlemjid.

2.2. Solvent extracts

Dried powder (10g/100mL) of aerial part of the plant was extracted with water and various dilutions (80% and 100%) of methanol and ethanol in water. The extracts were filtered and the methanol and ethanol solvents were eliminated using a rotary evaporator to obtain a dry extract; for aqueous extract the water was eliminated with the freeze-drying apparatus. All extracts were stored at 4-8°C until analyses.

2.3. Determination of total phenolic and flavonoids content

Total phenolic content (TPC) were determined according to the Folin-Ciocalteu method as described by Lister and Wilson [26]. A 0.5 mL of a sample solution was mixed with 2.5 mL of Folin-Ciocalteu reagent (1/10) with methanol, then 4 mL of sodium carbonate (Na_2CO_3) (7.5 %, w/v) was added. The mixture is then incubated in a water bath at 45°C for 30 min and the absorbance was measured at 765nm using UV-Vis spectrophotometer, against blank sample. The total phenolic content was measured as mg of gallic acid equivalents per g of extract dry weight (mg GAE/g of extract). The total flavonoid contents (TFC) in the extracts were determined using the aluminum chloride method [27]**.** In brief, the distilled water was added to 1 mL of dissolved sample to obtain a total volume of 5 mL and then 0.3 mL

of 5% sodium nitrite ($NaNO_2$) was added. After 5 min, 0.3 mL of 10% aluminium chloride ($AlCl_3$) was added and the mixture was allowed to stand for another 6 min. Then, 2 mL of 1 M sodium hydroxide (NaOH) was added and the total volume was increased to 10 mL with distilled water. The solution was mixed vigorously and allowed to stand for 30 min. The absorbance was recorded against a blank at 510 nm. The flavonoid content was determined as the rutin equivalent from the calibration curve of rutin standard solutions and expressed as rutin equivalent (mg RE/g of extract).

2.4. Antioxidant activity

2.4.1. Diphenyl-2-picrylhydrazyl radical-scavenging activity (DPPH)

The free radical scavenging activity of extract was measured by 2,2'-Diphenyl-1-picrylhydrazyl hydrate (DPPH) [28], with some modifications. A 0.5 mL of DPPH (0.2 mM in methanol) was added to 2.5 mL of extract was allowed to stand at room temperature for 30 min, and then absorbance was measured at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration. Using the following equation:

$$\% RSA = [(AD - AE)/ AD]*100$$

Where AD is the absorbance value of the DPPH blank sample, and AE is the absorbance value of the test solution. The acid ascorbic was used as standard antioxidant.

2.4.2. Ferric reducing antioxidant power (FRAP) Assay

The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) was conducted using the protocol as described by Oyaizu [29] with minor modifications. The samples (extract or ascorbic acid) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v). The mixture was incubated in a water bath at 50°C for 20 min, and then 2.5 mL of trichloroacetic acid (10% w/v) was added to the reaction mixture. Thereafter, it was centrifuged at 3000 rpm for 10 min. A volume of 2.5 mL of upper layer was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL 0.1% w/v). The absorbance was measured at 700 nm at the reaction time of 30 min. The reducing power of the extracts was represented as ascorbic acid equivalent (mg AAE/g of extract).

2.5. Antimicrobial activity

2.5.1. Microorganisms and growth conditions

Bacteria strains tested included phytopathogenic and foodborne pathogens bacteria: Gram positive (*Listeria innocua CECT 4030, Staphylococcus aureus CECT 976, Bacillus subtilis DSM 6633* and *Clavibacter michiganense subsp. Michiganense 1616-3*) and Gram negative (*Proteus mirabilis, Escherichia coli K12, Pseudomonas aeruginosa CECT 118* and *Pseudomonas savastanoi pv. Savastanoi (2636-40)*) were obtained from the Laboratory of Biology and Health, Sciences Faculty of Tetouan. A fungus was also tested: *Candida albicans ATCC 10231* obtained from the Laboratory of Agri-Food and Health of FST-Settat. The pathogens bacterial strains were cultivated in Mueller–Hinton agar (MHA) at 37°C. The fungi and the phytopathogens bacteria were cultured on YPGA medium (5 g yeast extract, 5 g Peptone, 10 g Glucose, 15-18 g Agar, in 1liter) and incubated at 28°C. For the test, final inoculum concentrations of 10^6 CFU/mL for bacteria were used and 10^5 spores/mL for Fungi.

2.5.2. Agar diffusion method

The agar disc diffusion method was used for testing the antimicrobial activity of *Ruta montana* extracts according to Kelen and Tepe [30], with some modifications. First, 0.1 mL of microbial suspension was spread, by using a sterile swab, on the surface of MHA, for foodborne pathogens, or YPGA for phytopathogenic bacteria and fungi. Sterile filter

paper discs (Whatman Grade 1.6 mm diameter) were placed aseptically on the previously inoculated agar plates then were individually loaded with 20 µL of each extract at a concentration of 60 mg/mL dissolved by dimethylsulfoxide (DMSO) 10%. Plates were kept at room temperature for 2 h to allow the diffusion of all products tested, and then incubated as described above. The antimicrobial activity was evaluated by measuring the diameter of circular inhibition zones around the disc that including the diameter of the paper disc (6 mm diameter). *Vancomicym* (30µg), *Streptomycin* (25µg), *Amphotericin* (10µg) antimicrobial agents were used as positive control and DMSO 10% was used as negative control.

2.5.3. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MICs) of the active products only were determined according to Gulluce et al. [31] with minor modifications. Briefly, the test was performed in sterile 96-well microplate with a final volume in each microplate well of 100 µL. For susceptibility testing, 100 µL of Mueller–Hinton broth (MHB) for bacteria and YPG for fungi was distributed from the second to the twelfth test wells. The stock solution of the extract were prepared, in the first well of microplate, by dissolving all extract with 10% DMSO in MHB for bacteria and YPG for fungi (v/v) to reach a final concentration 25 mg/mL. Then 100 µL of scalar dilutions were transferred from the first to the ninth. Thereafter and from each well, 10 µL of the suspension was removed and replaced by the bacterial or fungi suspension to final inoculum concentrations of 10^6 CFU/mL for bacteria and 10^5 spores/mL for fungi. The final concentration adopted to evaluate the antimicrobial activity was 0.097 to 25 mg/mL. The tenth well was considered as sterile medium, since neither extract solution nor microorganism were added, the eleventh was used as a positive control for growth microorganism and twelfth well containing 10% DMSO/MHB or 10% DMSO/YPG, without extract, were used as negative control. The plates were then covered with the sterile plate covers and incubated 24 h at 37°C for bacteria and 72 h at 28°C for fungi. The MIC was defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth after incubation. As an indicator of microorganism growth, 25 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) (0.5 mg/mL) dissolved in sterile water was added to each wells and incubated 30 min at 37°C for bacteria and at 28°C for fungi. Where microbial growth was inhibited, the solution in the well remained clear after incubation with MTT. To determine the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values, 10 µL of broth from the uncolored wells was inoculated in MHA for foodborne pathogens and LPGA for phytopathogenic bacteria and fungi, respectively. The dishes were incubated for 24 h at 37°C and 72h at 28°C, MBCs and MFCs were defined as the lowest recorded extract concentration of the wells in which microbial strains failed to grow in microbial medium.

2.6. Statistical Analysis

All determinations were done in triplicates. Values of each experiment were expressed as mean±standard deviation (SD) and were subjected to analysis of variance (One-way ANOVA). The statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Inc., San Diego, California). Differences (between groups) were considered as statistically significant at $p<0.05$.

3. Results and discussion

3.1. Effects of solvent on extraction yield, Total phenolic and flavonoids contents

In this study, *R.montana* extracts were obtained by using water and organic solvents (methanol and ethanol; 80% and 100%). Extraction yields ranged from 3.87 ± 0.32% to 15.00% ± 2.82% (**Table 1**). Indeed, the yields of extraction by various solvents decreased in the following order 100% ethanol > 100% methanol > aqueous > 80%Ethanol >

80%methanol > 80%ethanol. Phenolic and flavonoid contents are expressed respectively in mg of gallic acid or rutin equivalent per gram of extract (mg GAE/g of extract - mg RE/g of extract) in the same table. Clearly, the highest phenol content was found in 80% methanol extract (117.7 ± 2.82), followed by 80% ethanol extract (97.87 ± 2.22) > 100% methanol > 100% ethanol > aqueous. For flavonoids, they decrease in the following order 80%methanol (77 ± 6.55mg RE/g of extract) > 80% ethanol > 100% ethanol > 100% methanol > aqueous (0.32 ± 0.02 mg RE/g of extract). Our results are similar to those reported in literature indicating that the aqueous solvents gave the best yields of phenol content than the absolute solvents [32-33]. For others, the solvent polarity played a key role in increasing phenolic solubility [18]. In addition, Naczk and Shahidi [34] noticed that many factors influence the extraction of phenolic compounds from plant such the chemical structure, the method of extraction, and the size of the particles forming the sample.

Table 1. Extraction yield, phenolic content and Antioxidant activity of *R.montana* extracts.

solvent systems	Yield	TPC[1]	TFC[2]	IC_{50}[3]	FRAP[4]
Aqueous	9.66% ±1.54^{b}	19.45 ± 2.14^{d}	0.32 ± 0.02^{c}	>100^{c}	2.19 ± 0.44^{d}
100% Methanol	11.33% ± 0.57^{b}	82.62 ± 2.43^{b}	1.86 ± 0.17^{c}	82,12 ± 0,03^{b}	8.67 ± 2.89^{cd}
80% Methanol	5.66% ± 0.28^{c}	117.70 ± 2,82^{a}	77.00 ± 6.55^{a}	10.66 ± 1.52^{a}	66.66 ± 5.67^{a}
100% Ethanol	15.00% ± 2.82^{a}	58.46 ± 4,68^{c}	8.29 ± 0.97^{c}	71.45 ± 0.04^{b}	15.29 ± 0.54^{c}
80% Ethanol	3.87 % ± 0.32^{c}	97.87 ± 2,22^{b}	62.66 ± 3.55^{b}	13.00 ± 1.73^{a}	36.33 ± 8.05^{b}

Data are reported to mean (n = 3) ± SD. Values in the same column not sharing a common letter (a to c) differ significantly at p<0.05

[1] mg GAE/g of extract

[2] mg RE/g of extract

[3] IC_{50} expressed in µg/mL needed to decrease the initial DPPH concentration by 50 %

[4] mg AAE/g of extract

3.2. Antioxidant activity

The antioxidant activities of *R.montana* extracts were evaluated by two methods, namely by DPPH and reducing power test. Results, summarized in **Table 1**, showed that the 80% methanol extract has the high capacity to reduce the DPPH (IC_{50}=10.66 µg/mL), following by 80% ethanol extract (IC_{50}=13.00 ± 1.73µg/mL), 100% ethanol extract, 100% methanol and aqueous extracts (IC_{50}=71.45 ± 0.04 µg/mL, IC_{50}=82.12µg/mL, and IC_{50} >100µg/mL, respectively). But they were all less potent than the standards used as positive controls, namely Trolox and Ascorbic acid IC_{50}=1.4±0.04µg/mL and IC_{50}=1.82±0.025µg/mL, respectively, (statistically significant at $p < 0.05$). For the FRAP test, with results expressed in milligram equivalence of ascorbic acid per gram of extract (mg AAE / g EO), the highest reducing power was obtained for 80% methanol extract (66.66 ± 5.67 mg AAE/g of extract), following by 80% ethanol (36.33 mg AAE/g of extract), 100% ethanol (15.29 mg AAE/g of extract), 100% methanol (8.67 ± 2.89 mg AAE/g of extract) and aqueous (2.19 ± 0.44 mg AAE/g of extract). According to Liu et al. [35], the phenolic compounds presented an antioxidant potential that correlated with their total contents. In the present work, there is a close association between total phenolic content and antioxidant activity of *R. montana* extracts. Contrary, several studies have shown no relationship between the content of phenolic compounds and antioxidant activity [36-37]. This could be explained by numerous factors; (i) the presence of non-phenolic products such as sugars, amino acids which reacts with Folin-Ciocalteu giving a high apparent phenolic level [38-39], (ii) the antagonistic interaction between

phenolic and non-phenolic compounds [40], (iii) the antioxidant activity depends on the numbers and position of functional groups [41].

3.2. Antimicrobial activity

Table 2a. Antimicrobial activities of the extracts from R.montana against plant pathogenic and foodborne pathogens bacteria.

	inhibition zone diameter (mm)				
	Solvent systems				
Strains	Aqueous	100%MetOH	80%MetOH	100% EtOH	80% EtOH
S. aureus CECT 976	-	10.66±0,57	-	-	-
B. subtilis DSM 6633	-	17.33±0.57	16.33 ±1.50	-	14.33± 1.50
L. innocua CECT 4030	-	-	16.00 ±1.00	-	18.00 ± 1.00
E. coli K12	-	-	-	-	-
P. aeruginosa CECT 118	-	-	-	-	-
P. mirabilis	-	19.33±0.57	16.00 ±1.00	-	15.00 ± 2.00
C. michiganensis 1616-3	-	18.33±0.57	-	13.66±0.57	-
P. savastanoi (PSS2636-40)	-	13.33±0.57	-	11.33±0.57	-
C. albicans ATCC 10231	-	-	-	-	-

MetOH: Methanol, EtOH: Ethanol, (-): inhibition zone less than 8 mm.

Table 2b. Antimicrobial activities of the antibiotics against plant pathogenic and foodborne pathogens bacteria.

	inhibition zone diameter (mm)		
	Antibiotics		
Strains	Vancomicym	Streptomycin	Amphotericin
S. aureus CECT 976	30.66±0.57	nt	Nt
B. subtilis DSM 6633	27.66±0.57	nt	Nt
L. innocua CECT 4030	25.33±0.57	nt	Nt
E. coli K12	8±00	nt	Nt
P. aeruginosa CECT 118	-	nt	Nt
P. mirabilis	24.33±0.57	nt	Nt
C. michiganensis 1616-3	nt	24.66±0.57	Nt
P. Savastanoi (PSS2636-40)	nt	26.33±0.57	Nt
C. albicans ATCC 10231	nt	nt	18.66±1.15

nt: not tested, (-): inhibition zone less than 8 mm.

The antimicrobial activity of extracts from *Ruta montana* against phytopathogenic and foodborne pathogens bacteria was qualitatively and quantitatively tested by the development of inhibition zones and MTT-assay. The results of the antimicrobial activity of different extracts from *Ruta montana* are presented in **Table 2a and 2b**. According to the *in vitro* test, our results showed that the extracts plant exhibited antimicrobial activity against all the tested strains, but in variable degree. The inhibition zones ranged between 10.66 mm and 19.33 mm. The very sensitive bacteria to the 80%, 100% methanol, and 80% ethanol extracts, were *Proteus mirabilis* (15-19mm), *Clavibacter michiganensis*

subsp. *michiganensis 1616-3* (18mm), *Bacillus subtilis DSM 6633* (16-17 mm), *Listeria innocua CECT 4030* (16-18 mm); with MIC varied from 1.56 to 12.5 mg/mL (**Table 3**). Aqueous extract have no antimicrobial effect against the tested microorganisms. The *Escherichia coli K12 and Pseudomonas aeruginosa CECT 118* are not sensitive to all extracts. No antifungal activity was observed on *Candida albicans ATCC 10231*. The results in this work showed that *R.montana* extracts did not have selective antimicrobial activities on the basis of the gram positive or gram negative strains. These results are similar to those reported previously [31, 42]. However, according to others authors, the Gram-positive bacteria are more sensitive to plant extracts than Gram-negative bacteria [43-44].

Table 3. Minimum inhibitory concentration (MIC) of *R.montana* extracts (mg/mL).

	Solvent systems				
Strains	Aqueous	100%MetOH	80%MetOH	100% EtOH	80% EtOH
S. aureus CECT 976	nt	nt	nt	nt	nt
B. subtilis DSM 6633	nt	12.50	3.12	nt	1.56
L. innocua CECT 4030	nt	nt	12.50	nt	6.25
E. coli K12	nt	nt	nt	nt	nt
P. aeruginosa CECT 118	nt	nt	nt	nt	nt
P. mirabilis	nt	12.50	12.50	nt	1.56
C. michiganensis 1616-3	nt	12.50	nt	nt	nt
P. savastanoi (PSS2636-40)	nt	nt	nt	nt	nt
C. albicans ATCC 10231	nt	nt	nt	nt	nt

MetOH: Methanol, EtOH: Ethanol, nt: not tested.

4. Conclusion

The present study investigated the effect of the solvent extraction on phenol and flavonoid contents, the antioxidant and antimicrobial activities against phytopathogenic and foodborne pathogens bacteria. Our results revealed that the hydroalcoholic extracts of *R. montana* has the highest total phenolic and flavonoid contents and the greatest antioxidant activities compared to absolute extracts. These results showed also the interesting antibacterial activity of *R. montana* extracts against phytopathogenic and foodborne pathogens bacteria. Our finding confirmed that *R. montana* extracts may be useful in food preservation and eco-friendly applications thanks to their biological properties.

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