Research Article

BIOMEDICAL Journal of Scientific & Technical Research

ISSN: 2574 -1241

DOI: 10.26717/BJSTR.2021.37.005997

Evaluation of the Phenolic Contents, Antioxidant and Antimicrobial Activities of Leaves Extracts of *Ruta Graveolens*

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ARTICLE INFO

Received: July 06, 2021

Published: July 23, 2021

Citation: Aymen Halouani, Mariem Abdelli, Assia Hamdi, Mariem Ben Rhouma and Ichrak Slama, et al., Evaluation of the Phenolic Contents, Antioxidant and Antimicrobial Activities of Leaves Extracts of *Ruta Graveolens*. Biomed J Sci & Tech Res 37(3)-2021. BJSTR. MS.ID.005997.

Keywords: Ruta Graveolens; Organic and Water Extracts; Polyphenols; Flavonoids and Tannins Contents; Antioxidant and Antimicrobial Activities

Abbreviations: RT: Room Temperature; BHT: Butylated Hydroxytoluene; MHA: Muller Hinton Agar; MFC: Minimum Fungicidal Concentration; MHB: Muller Hinton Broth; MBC: Minimum Bactericidal Concentration; CFU: Colony Forming Units; TPC: Total Phenolic Compounds

ABSTRACT

Ruta graveolens, better known as rue, is a polyvalent herb rich in secondary metabolites that has demonstrated different biological properties and its various medicinal properties. Ruta belongs to the Rutaceae family and is native to the Mediterranean region. We choose same species plant, from two origin, North Africa (Tunisia) and South Europe (France). Different extracts were obtained from dried leaves by using five solvents with increasing polarity, petroleum ether, chloroform, ethyl acetate, ethanol, methanol, and water. Different extracts from both plants, demonstrated in most of tests the same profile. Starting with yield extraction evaluation, a high yields were obtained with ethanol, methanol and aqueous plant extracts. Phenols content assessment reveals an important amount in ethanolic and methanolic extracts of polyphenols, flavonoids and in petroleum ether, chloroform and ethyl acetate of tannins. The antioxidant power was evaluated by using four methods, mainly DPPH, ABTS, β-Carotene and FRAP. Results are expressed as Inhibitory Concentration 50 and the best performance was noted with ethanolic and methanolic extracts.

Effect of five organic and aqueous extracts of both plants on growth of two Gram-negative and two Gram-positive bacteria and *Candida albicans* was evaluated by MIC and MBC determination. Ethanol and methanol extracts of both Ruta exerts a bactericidal effect on different bacterial strain. While, a selective antifungal activity was noted only with methanolic extracts. Results were confirmed with MBC/MIC ratio (less than or equal to 4). Phenols content, especially polyphenols and flavonoids, showed good correlation with antioxidant and antimicrobial activities, which confirm its significant contribution to those activities of Ruta graveolens extracts.

Introduction

Plants were largely known as a biological source of active components with potential activity, namely antioxidant, antibacterial and antifungal activity. The discovery of new active compounds is very important in order to develop new therapeutic strategies. The search for new antioxidant and antimicrobial molecules in plants is advantageous and promising because of their accessibility and their diversity, but also because of their use in traditional medicine [1]. Among the species potentially of interest

in the pharmaceutical field, because of its richness in secondary metabolites, and which have demonstrated different biological activities (antimicrobial, antioxidant, antiviral, anti-inflammatory, etc.), we choose *Ruta graveolens* (*R. graveolens*). It is a wild or cultivated plant widespread in the Mediterranea and the Middle East region and commonly used in ethanobotany because of its multiple virtues [2]. *R. graveolens*, (garden rue or common rue), belonging to family Rutaceae.

The plant has been studied for its major components, mainly coumarins, terpenoids, alkaloids, flavonoids, aliphatic acids, ketones, rutarin, isorutarin, rutin, isorhamnetin-3-O-rutinoside, cnidioside A, methylcnidioside A, cnidioside B, methyl ester [3], caffeic acid, chlorogenic acid, cinnamic acid, p-coumaric acid and protocatechuic acid [4], ɣ-fagarine and kokusaginine [5], limonene, thymol and carvacrol [6]. The richness of Ruta in active molecules may explain its great effectiveness in therapeutic treatments and its use in folk medicine, mainly as an antispasmodic, anticancer, treatment of menstrual disorder, an abortifacient, a sedative, anti-inflammatory, antipyretic, anti-microbial, etc. [7]. Free radicals and reactive species are liberated from inflammatory cells, which lead to exaggerated oxidative stress and known to play an important role in the pathogenesis of severe inflammation of many organs and several chronic diseases, such as diabetes, central nervous system injury, and cancer [8]. Plants are potential sources of biological antioxidants, and a much interest has been devoted to natural antioxidant [9].

One of the major world health problem was the emergence of clinical bacterial strains resistant to one or many antibiotics. The use of extracts from medicinal plants is an interesting alternative to deal with this problem [10]. Thus, in the ultimate perspective of finding biologically active natural compounds, as an alternative to the antimicrobial drugs currently used, we proposed to study the antimicrobial activities against the most frequent bacteria and fungi strains, which known with serious consequences on health and economy. To the best of our knowledge, previous study conducted in Tunisia were rather interested on essential oil analysis of *R. graveolens* [11-13]. However, as far as we know, no previous study was interested on the assessment of antioxidant and antimicrobial activities of *R. graveolens* extracts from France. We choose to work in our current study with organic and aqueous extracts from leaves of Tunisian or France *R. graveolens*. The aims of this work were to study the phenolic content of five organic and water extracts of *R. graveolens* leaves, antioxidant and antimicrobial activity assessment, and to correlate phenolic content with the antioxidant and antimicrobial activity of different extracts. In addition, the results will be compared to data from a systematic review of the literature.

Materials and Methods

Plant Material

Areal parts of *R. graveolens* were collected from the region of Beni Khalled, Nabeul, northeastern Tunisia (Ruta T), in November 2016 and from Le Beausset, Marseille, Southern France (Ruta M) in January 2018. Leaves were air-dried for one month at room temperature (RT) under-shade.

Maceration

Dried leaves were crushed with electronic blender and kept in glass bottle until extraction. Powder was submitted to extraction by maceration, using different solvents with increasing polarity [14]. Briefly, 100 g of powder was treated with 100 mL of each solvent, petroleum ether, chloroform, ethyl acetate, ethanol, methanol (all from Honeywell Fluka), and water, serially at RT in the dark with continuous agitation. After 12 h, the supernatant was filtered using Whatman Nº1 paper. The filtrate obtained was then placed in the ventilated oven set at 37 °C until the solvent and water have evaporated completely.

Estimation of Plant Extraction Yield

The extracts are weighed to calculate the yield and then stored at 4 °C.

The extraction yield is calculated according to the following formula [15]:

$$Yield\ (\%) = \left(W_{ext} / W_{samp}\right) \times 100$$

Where W ext and W samp are the weight of the extract after evaporation of solvent (in g) and the dry weight of the organ sample (ing) respectively.

Estimation of Total Polyphenol Content

Total phenols were determined by Folin-Ciocalteu reagent [16]. Briefly, 25 µL of Folin-Ciocalteu reagent was added to 10 µL of extract dissolved in distilled water at 1 mg/mL. The mixture was incubated at RT for 5 min after which 25 µL of 20% sodium bicarbonate ($NaCO_3$) and up to 200 µl distilled water were added. The mixture were then incubated at RT for 30 min, then the absorbance were measured at 760 nm with a plate reader (Thermo Scientific). The standard curve was prepared using different aliquots of Gallic acid (0-250 µg/mL) prepared under the same conditions as extracts. Total phenol values are expressed in terms of Gallic Acid Equivalent (mg GAE/g) of extract.

Estimation of Total Flavonoid Content

The quantification of flavonoids in each extract is estimated by the colorimetric method by using aluminum trichloride ($AlCl_3$) [17]. In practice, 100 µL of each extract (10 mg/mL) are added to 100 µL of distilled water. Then, 100 µL of 2% $AlCl_3$ solution in methanol are added to the diluted extracts. The solution was mixed well and incubated in the dark at RT for 10 min. The absorbance is then measured at 367nm with a plate reader. A calibration curve produced using a standard, which is quercetin, at different concentrations (0-500 mg/L) and prepared following the same experimental conditions as those of the extracts. Total flavonoids amounts were expressed as mg of quercetin equivalent per g of extract (mg QE/g).

Estimation of Total Tannins Content

Condensed proanthocyanidins (tannins) were determined according to the method of Sun et al. (1998) [18]. Briefly, 50 µL of extract was diluted with 300 µL of vanillin solution in methanol (4%) and 150 µL of concentrated HCl (36%). The mixture was incubated 15 min at RT in the dark, and absorbance was measured at 500 nm. The quantification of tannins is determined from a calibration range established with catechin (flavonoid) (0-400 µg/mL). The amount of total condensed tannins is expressed as mg of catechin equivalent per g of extract (mg CE/g).

Evaluation of Antioxidant Activity of Plant Extracts

Free Radical-Scavenging Ability by the use of a stable DPPH Radical: The ability of the corresponding extracts to donate hydrogen atoms or electrons was measured from the bleaching of purple coloured methanol solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) [19]. One hundred µL of various concentrations (0,007 to 1 mg/mL) of different extract was added to 100 µL of DPPH radical solution in methanol (0.2 mmol/L). The absorbance of the resulting solution was measured at 517 nm after 30 min of incubation at RT. DPPH scavenging effect was calculated according to the following formula:

$$Inhibition\ percentage = \left[\left(A0 - A1\right) / A0\right] \times 100$$

where A0 and A1 are the absorbance at 30 min of the control and the sample, respectively. The antiradical activity was expressed as Inhibitory Concentration 50 (IC50 expressed in mg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC50 value corresponds to a higher antioxidant activity.

ABTS: ABTS (2-2'-Azino-di-[3-ethylbenzothiazoline sulfonate]) radical scavenging activity of extracts was determined according to Re et al. (1999) [20]. The $ABTS^{+}$ cation radical was produced by the reaction between 7 mM $ABTS^{+}$ solution and 2.45 mM potassium persulfate (K2S2O8) solution, stored in the dark at RT for 12-16 h. This solution was diluted with methanol to get an absorbance of 0.7 ± 0.02 at 734 nm. In a final volume of 200 µL, the reaction mixture comprised 150 µL of $ABTS^{+}$ solution and 50 µL of each extract at various concentration. The ascorbic acid was used as positive control. The absorbance was measured at 734 nm. The IP of $ABTS^{+}$ radical was calculated using the same formula as for DPPH. The inhibition curves were prepared and IC50 values were obtained.

β-Carotene: The antioxidant capacity is determined by measuring the inhibition of volatile organic compounds and conjugated diene hydro-peroxides resulting from the oxidation of linoleic acid [21]. Briefly, 2 mg of β-carotene are dissolved in 1 mL of chloroform, then 25 µL of linoleic acid and 200 mg of Tween 40 were added, and the mix is emulsified. Chloroform was evaporated under vacuum and 100 mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. From this new solution, 2.5 mL are transferred into test tubes, and 350 µL of each extract (1mg/mL) and of the Butylated Hydroxy Toluene (BHT) control are added. The absorbance is immediately measured for BHT and after 2 h of incubation, in the dark, for the extracts at 490 nm [19]. The inhibition percentage of β-carotene was calculated using the following formula:

$$\beta - carotene\ \ bleaching\ \ inhibition\ \ (\%) = [S - C_{120} / C_0 - C_{120}] \times 100$$

where C_0 and C_{120} were the absorbance values of the control at 0 and 120 min, respectively, and S is the sample absorbance at 120 min. The results were expressed as IC50 values (mg / mL).

Ferric Reducing Antioxidant Power (FRAP): The ferric reducing capacity of different extracts was determined following the method described by El Jemli and colleagues (2016) [22]. The method consist of mixing 200 µL of each extract at different concentrations, 500 µL of phosphate buffer (0.2 M, pH 6.6), and 500 µL of potassium ferricyanide $K_3Fe(CN)_6$ (1 %). After 20 min of incubation at 50 °C (to reduce ferricyanide into ferrocyanide), the reaction was stopped by adding 500 µL of 1% trichloroacetic acid. The working solution was submitted to a centrifugation at 200 xg for 10 min and then 500 µL of the supernatant was mixed with 500 µL of distilled water and 100 µL of $FeCl_3$ (0.1 %). The absorbance was measured at 700 nm with a plate reader. The extract concentration providing 0.5 of absorbance (IC50) was determined by Graph-Pad Prism software.

Antimicrobial Activities

The Gram-negative bacterial strains used to assess the antibacterial properties of the test samples were *Salmonella enterica* (NCTC 6017) and *Escherichia coli* (ATCC 8739). The Gram-positive strains were *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538). To evaluate antifungal activity we have used *Candida albicans* (ATCC 2071). The minimum inhibitory concentration (MIC) was the lowest extract concentration that produces a 90% reduction in the growth (populations) of microbial colonies at which bacteria failed to grow in Muller Hinton Broth (MHB), but bacterial growth was observed after transferring 25 µL to Muller Hinton Agar (MHA). Similarly, the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) were the lowest concentration at which bacteria or yeast failed to grow in MHB and after the subsequent transfer to MHA. MIC and MBC values of the different samples were determined by micro-dilution method using resazurin colorimetric assay as previously described [23].

Briefly, the samples were dissolved in 5 % dimethyl-sulfoxide (DMSO)/MHB and serially diluted two fold (in a 96-well microplate). Then, 100 µL of inoculum (1.5 × 106 Colony Forming Units (CFU) per mL) prepared in MHB was added in each well. Gentamicin or Amphotericin B were used as reference drug and the well containing the vehicle (DMSO 2.5%) as control. After 18-24 h of incubation at 37 °C, the MIC and MBC (or MFC) value were determined. The MBC/MIC or MFC/MIC ratio makes it possible to characterize a bacteriostatic or bactericidal and / or fungicidal nature of an extract. When this ratio is less than or equal to 4 [24], the extract is considered to be bactericidal or fungicidal.

Statistical Analyses

Independent samples of each item were analyzed in triplicate and data were presented as mean ± SD (standard deviations). IC50 values were calculated by Graph-Pad Prism. P value < 0.05 was considered to represent a statistically significant difference. *P value < 0.05, **P value < 0.01. The Pearson's correlation test was used to analyze the correlation between total phenolic, total flavonoids and tannin content and antioxidant and antimicrobial activities. Correlation coefficient r >0: positive correlation; Correlation coefficient r <0: negative correlation.

Results

Extraction Yield

Low extraction yields, around 1%, were obtained with the petroleum ether (0.87 % and 0.91 % respectively) and chloroform (0.95 % and 1.45 % respectively) extracts for the two plants (Figure 1). Likewise for Ruta T with ethanol (1.04 %) extract and for Ruta M with aqueous extract (1.49 %). For ethyl acetate the extraction yield was lower, 0.57 % for Ruta T and 0.79 % for Ruta M. A medium extraction yield was obtained for the methanolic extract of Ruta M, about 2.76 %. Very high extraction yields were obtained for methanol and aqueous extracts for Ruta T (4.94 % and 5.22 % respectively) and for ethanolic extract for Ruta M (5.06 %).

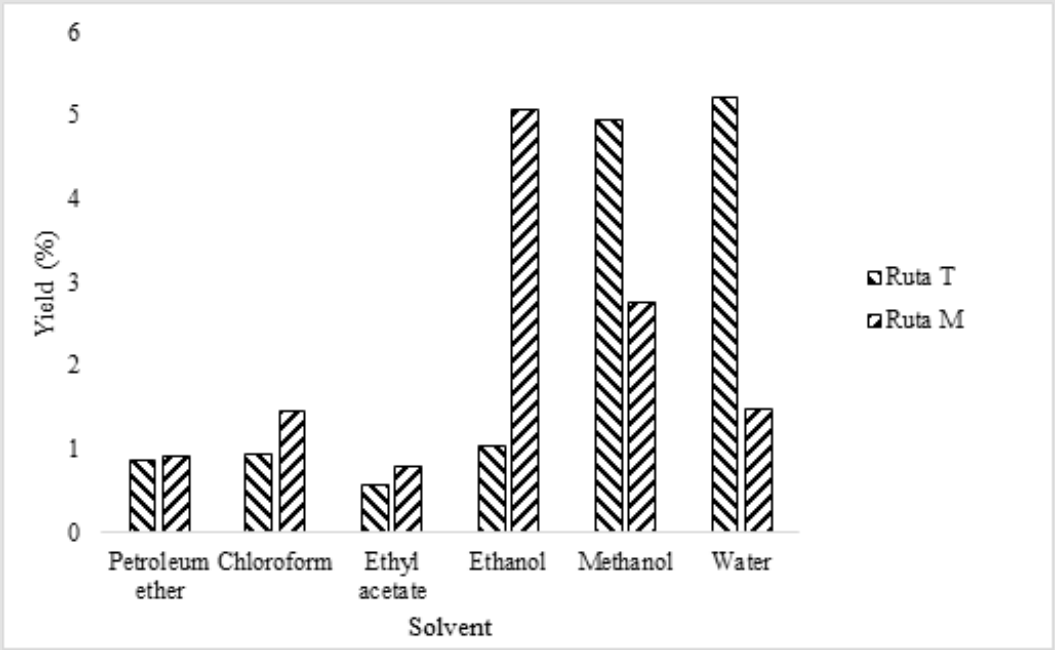

Figure 1: Percentage yield of plant extracts. The leaves powder of each dried Ruta, Tunisia (T) or Marseille (M), were submitted to extraction by maceration by using different organic solvent and water. After that, solvent and water were evaporated and percentage yield of each extract was determined according to the formula described by Falleh, et al. [15].

Amount of Total Phenolic Compounds

Polyphenolic: The lowest amount of polyphenols were determined in petroleum ether of Ruta T and M and for ethyl acetate extract of Ruta M, accounting for 7 ± 2,95, 16 ± 0,98 and 30 ± 0,24 mg GAE/g extract respectively (Figure 2). A moderate amount of total polyphenols were detected in chloroform extract of Ruta T and M, ethyl acetate, ethanol, methanol and aqueous extracts of Ruta T and in aqueous extract of Ruta M with content around 78 ± 0,24, 89 ± 0,73, 80 ± 1,72, 127 ± 0,49, 108, 85 ± 0,73 and 96 ± 0,24 mg GAE/g extract respectively. A highest amount content of polyphenols were determined in ethanol and methanol extracts of Ruta M (214 ± 1,23 and 194 ± 0,98 mg GAE/g extract respectively).

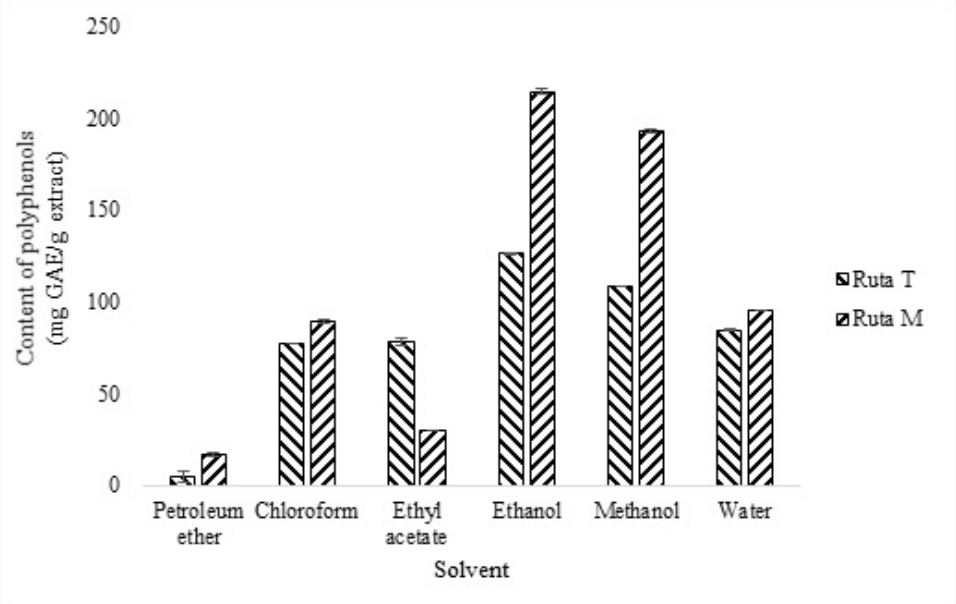

Figure 2: Total polyphenols content of *Ruta graveolens* extracts. The quantification of total phenols in each extract of both Ruta, Tunisia (T) or Marseille (M), were determined by Folin-Ciocalteu method [16] for each extract of both Ruta and results are expressed in terms of Gallic Acid Equivalent (mg GAE/g) of extract. Each bar represents the mean ± SD of triplicate determinations (n=3).

Flavonoids: An amount of flavonoids content lowest than 50 mg QE/g extract was obtained in petroleum ether and ethyl acetate extracts of Ruta M accounting for 29.2 ± 2,48 and 42.3 ± 0,87 mg QE/g extract (Figure 3). Higher amount, between 50 and 100 mg QE/g extract, were obtained in petroleum ether extract of Ruta T, chloroform extract of Ruta T and M, ethyl acetate extract of Ruta T and aqueous extract of Ruta M, accounting for 95.7 ± 0,29, 66.2 ± 0,29, 84.8, 80 and 64.7 ± 0,73 mg QE/g extract respectively. A moderate level of flavonoids content (between 100 and 150 mg QE/g extract) were obtained in ethanol extract of Ruta T and M (145.1 ± 1,75 and 122.9 ± 8,74 mg QE/g extract respectively) and in aqueous extract of Ruta T (116.5 1,17 mg QE/g extract). A highest amount of flavonoids content (>150 mg QE/g extract) were determined in methanol extract of Ruta T as well as of Ruta M (with 190.9 ± 5,83 and 174.9 ± 0,73 mg QE/g extract respectively).

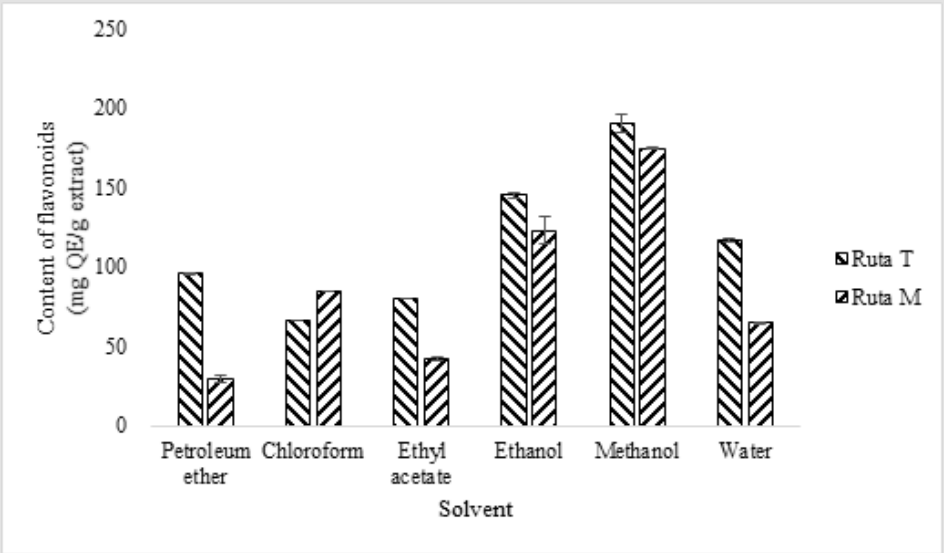

Figure 3: Total flavonoid content of *Ruta graveolens* extracts. The quantification of flavonoids in each extract of both Ruta, Tunisia (T) and Marseille (M), was estimated by Aluminum Trichloride ($AlCl_3$) method [17] and results are expressed as mg of quercetin equivalent per g of extract (mg QE/g). Each bar represents the mean ± SD of triplicate determinations (n=3).

Tannins: As shown in Figure 4, a higher condensed tannin content (> 50 mg CT/g extract) were determined in petroleum and ethyl acetate extracts of Ruta M and in chloroform extract of Ruta T, accounting for 70.66 ± 1,17, 75.66 ± 1,17 and 63.16 ± 2,35 mg CT/g extract respectively. A moderate tannin content (between 25 and 50 mg CT/g extract) were noted in petroleum ether and ethyl acetate extracts of Ruta T and in chloroform extract of Ruta M, accounting for 28.16 ± 2,35, 44 ± 1,17 and 44.83 ± 7,07 mg CT/g extract respectively. A lowest amount of tannin content (< 25 mg CT/g extract) were determined in ethanol and methanol extracts of Ruta T and M (7.33 ± 5,89, 12.33 ± 3,53, 24 ± 1,17 and 7.33 ± 1,17 mg CT/g extract respectively). No traces of tannins were noted in aqueous extract of both plants.

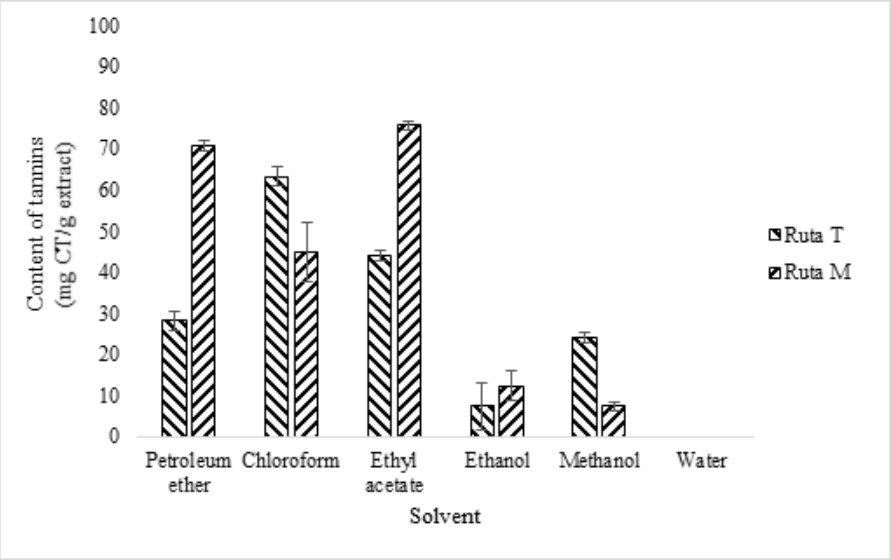

Figure 4: Total tannins content of *Ruta graveolens* extracts. The quantification of condensed tannins in each extract of both Ruta, Tunisia (T) or Marseille (M), were determined according to the method of Sun et al. (1998) [18] and results are expressed as mg of catechin equivalent per g of extract (mg CE/g). Each bar represents the mean ± SD of triplicate determinations (n=3).

Antioxidant Capacity

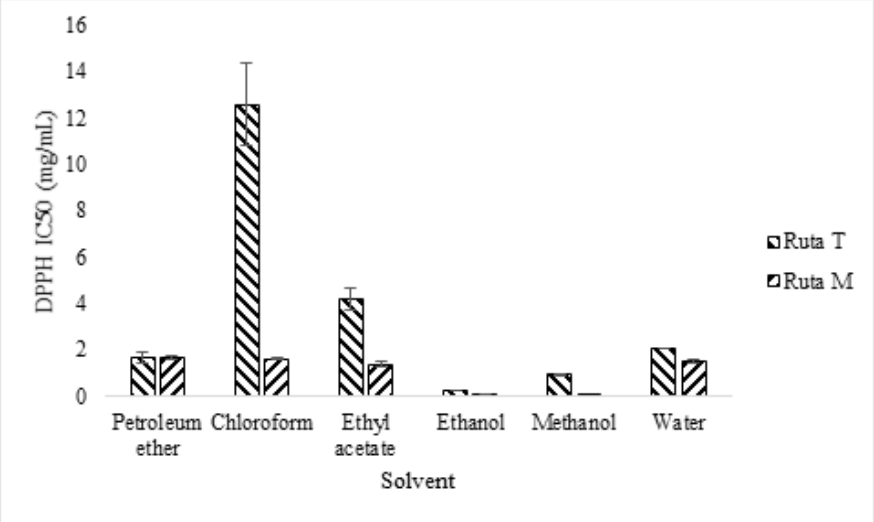

Figure 5: Scavenging of DPPH radical potential of *Ruta graveolens* extracts. The absorbance of the DPPH at 517 nm treated with different concentrations of each extract of both Ruta, Tunisia (T) or Marseille (M), were measured [19] and the Inhibitory Concentration 50 (IC50 expressed in mg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm, were determined. Each bar represents the mean ± SD of triplicate determinations (n=3).

DPPH: The ethanol and methanol extracts from Ruta T and M had the strongest free radical-scavenging activity with IC50 value equal to 0.25 ± 0,01, 0.12 ± 0,04, 0.93 and 0.05 ± 0,01 mg/mL respectively (Figure 5). A medium scavenging activity was obtained in petroleum ether and water extracts of Ruta T (IC50 = 1.67 ± 0,26 and 1.55 ± 0,02 mg/mL respectively) and Ruta M (IC50 = 2.06 ± 0,09 and 1.66 ± 0,07 mg/mL respectively) and in chloroform and ethyl acetate extracts of Ruta M (IC50 = 1.62 ± 0,03 and 1.37 ± 0.11 mg/mL respectively). The lowest capacity to reduce DPPH was observed in chloroform and ethyl acetate extracts of Ruta T with IC50 value equal to 12.58 ± 1,76 and 4.18 ± 0,48 mg/mL respectively.

ABTS: Chloroform, ethyl acetate, ethanol and methanol extracts of Ruta T exhibited the best performance in ABTS assay (IC50 = 0.41 ± 0.06, 0.27 ± 0.01, 0.22 and 0.12 ± 0.01 mg/mL respectively) and for ethanol and methanol extracts of Ruta M (IC50 = 0.91 ± 0.35 and 0.27 ± 0.05 mg/mL respectively) (Figure 6). A medium activity was noted in petroleum ether and aqueous extracts of Ruta T, with IC50 values equal to 1.08 ± 0.06, 1.58 ± 0.42, and Ruta M, with IC50 values equal to 2.57 ± 0.16 and 2.08 ± 0.42 mg/mL respectively, and in ethyl acetate extract of Ruta M with IC50 value equal to 1.97 ± 0.62 mg/mL. While a lower activity was noted only in chloroform extract of Ruta M (IC50 = 6.64 ± 1.96 mg/mL).

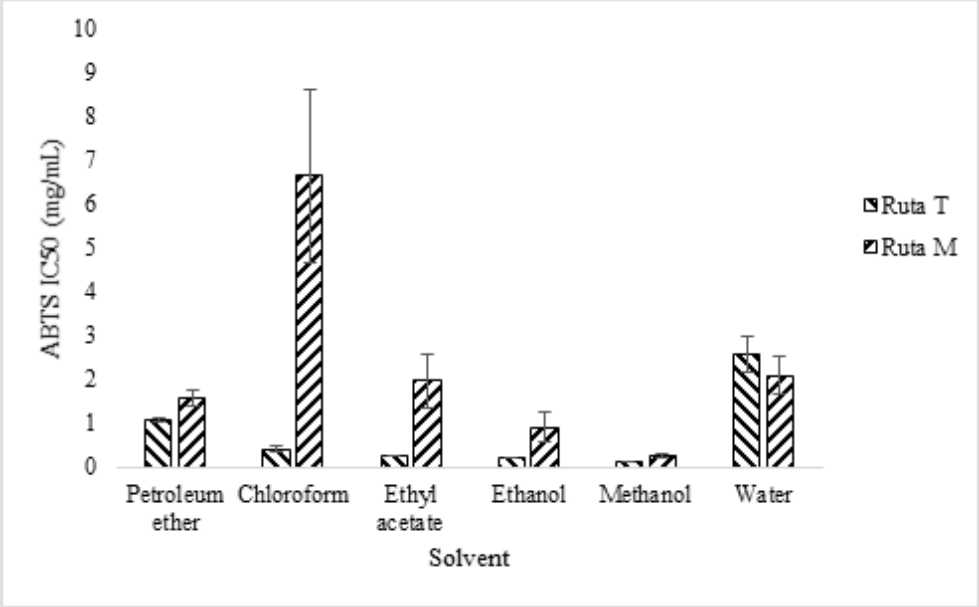

Figure 6: Scavenging of ABTS radical potential of *Ruta graveolens* extracts. ABTS radical scavenging activity of each extract of both Ruta, Tunisia (T) or Marseille (M), was determined according to Re et al. (1990) [20] and the absorbance was measured at 734 nm. The IC50 of ABTS+ radical was calculated using the same formula as for DPPH and results were expressed as IC50 values (mg/mL). Each bar represents the mean ± SD of triplicate determinations (n=3).

β-Carotene: As shown in Figure 7, all the extracts inhibit the bleaching of β-carotene with a low IC50 values (< 0.5 mg/mL). The highest activities were found in methanol extract of Ruta T and M (IC50 = 0.07 ± 0.008 and 0.026 ± 0.001 mg/mL respectively), in peroleum ether and ethanol extracts of Ruta M (IC50 = 0.097 ± 0.019 and 0.101 ± 0.003 mg/mL respectively) and in aqueous extract of Ruta T (IC50 = 0.037 ± 0.001 mg/mL). A medium activity was noted in chloroform and ethyl acetate extracts of Ruta T (IC50 = 0.172 ± 0.020, 0.188 ± 0.016) and Ruta M (IC50 = 0.259 ± 0.012 and 0.207 ± 0.007 mg/mL), in petroleum ether extract of Ruta M (IC50 = 0.299 ± 0,007 mg/mL) and in water extract of Ruta T (IC50 = 0.298 ± 0.032 mg/mL). The lower activity of scavenging effect on the β-carotene was noted in ethanol extract of Ruta M with IC50 value equal to 0.474 ± 0.004 mg/mL. The antioxidant capacity of each extract of both Ruta, Tunisia (T) and Marseille (M), by using the β-Carotene assay was determined according to the method described by Tepe et al., (2006) [21]. The absorbance was measured, at 490 nm, at 0 min and after two hours of incubation at RT. The results were expressed as IC50 values (mg/mL). Each bar represents the mean ± SD of triplicate determinations (n=3).

FRAP: In the presence of antioxidants in the extract, would result in the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by donating an electron. A higher activity was determined in ethanol and methanol extracts of Ruta T (IC50 = 1.297 ± 0.07 and 2.052 ± 0004 mg/mL respectively) and Ruta M (IC50 = 1.571 ± 0.01 and 0.96 ± 0.02 mg/mL respectively) (Figure 8). Petroleum ether and ethyl acetate extracts of Ruta T (IC50 = 4.961 ± 0.4 and 2.335 ± 0.06 mg/mL respectively) and Ruta M (IC = 4.85 ± 0.64 and 3.323 ± 0.53 mg/mL respectively) and in chloroform extract of Ruta T with IC50 value of 2.884 ± 0.16 mg/mL. A lower activity was detected in chloroform extract of Ruta M (IC50 = 6.3 ± 0.48 mg/mL) and in water extract of Ruta T and M (IC50 = 6.214 ± 1.76 and 8.14 ± 1.37 mg/mL respectively).

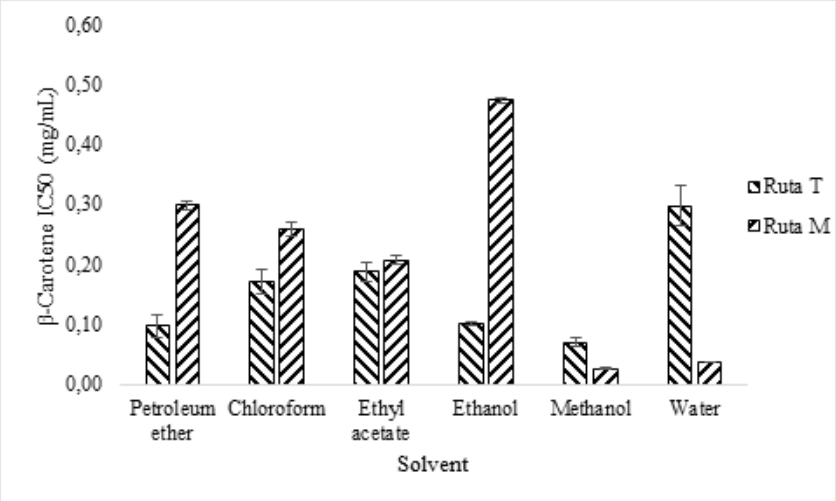

Figure 7: β-Carotene bleaching assay of *Ruta graveolens* extracts.

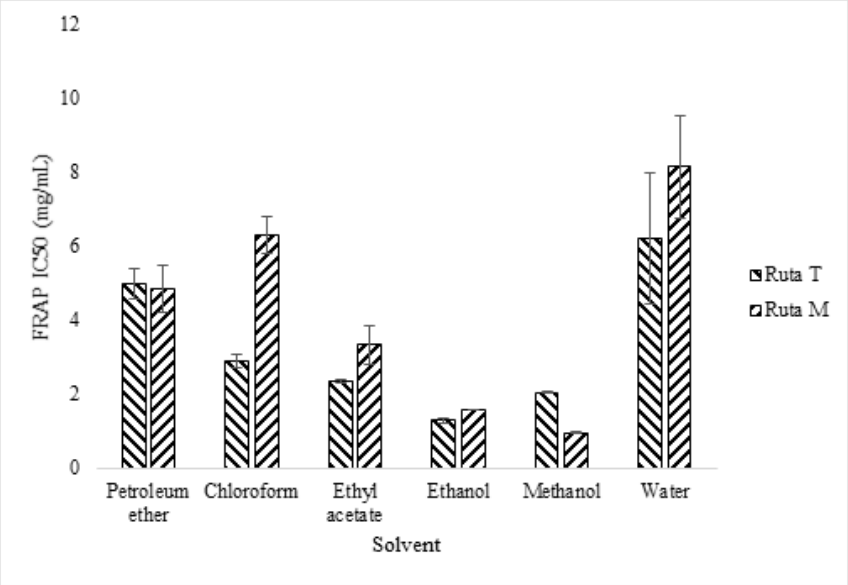

Figure 8: Ferric reducing anti-oxidant power assay of *Ruta graveolens* extracts. The ferric reducing capacity of each extract of both Ruta, Tunisia (T) or Marseille (M), was determined following the method described by El Jemli, et al. [22]. The absorbance was measured at 700 nm. The extract concentration providing 0.5 of absorbance (IC50) was determined. Each bar represents the mean ± SD of triplicate determinations (n=3).

Antimicrobial Activity

Antibacterial and antifungal activity of *R. graveolens* extracts were represented in Table 1. For petroleum ether, chloroform, ethyl acetate and aqueous extracts for 2 plants, no prominent antimicrobial activity (IC50 > 5 mg/mL) was noted against the 4 different strains of bacteria and *Candida albicans* (data not presented to allay the Table). Ethanol extract of Ruta T and Ruta M has an inhibitory effect against both Gram-negative bacteria (*S. enterica* and *E. coli*) with a selective activity against Gram-positive bacteria (Ruta T extract against *B. subtilis* and Ruta M extract against *S. aureus*). Methanol extract of Ruta T show a potential effect selectively against S. enterica and *B. subtilis*. However, for methanol extract of Ruta M, an important inhibition activity was noted against all tested bacteria. Unlike ethanol extract of Ruta T and Ruta M, methanol extract of both plant represent a potential effect against *C. albicans.* For different extracts described above and seems active against different tested strains, MBC/MIC ratio are between 1 and 3.33 (≤4). Ethanol and methanol extracts of Ruta seems to exert a bactericidal (for Ruta T and M), sometimes selectively, and fungicidal (for Ruta M) action against different tested bacteria and the yeast tested strains.

Table 1: MIC and MBC (mg/mL) for organic and aqueous extracts from leaves of *Ruta graveolens*.

		S. enterica			E. coli			B. subtilis			S. aureus			C. albicans		
Solvent		**MIC**	**MBC**	**MBC/ MIC**	**MIC**	**MBC**	**MBC/ MIC**	**MIC**	**MBC**	**MBC/ MIC**	**MIC**	**MBC**	**MBC/ MIC**	**MIC**	**MFC**	**MFC/ MIC**
Ethanol	Ruta T	2.5	5	2	5	5	1	2.5	5	2	-	-	-	-	-	-
	Ruta M	2.5	5	2	2.5	5	2	-	-	-	2.5	5	2	-	-	-
Methanol	Ruta T	2.5	5	2	-	-	-	1.25	2.5	2	-	-	-	0.75	2.5	3.33
	Ruta M	2.5	5	2	2.5	5	2	1.25	2.5	2	2.5	5	2	0.75	2.5	3.33

Note: > 5 mg/mL ; Ruta T : *R. graveolens* from Tunisia; Ruta M: *R. graveolens* from Marseille; MIC : Minimum Inhibitory Concentration; MBC : Minimum Bactericidal Concentration.

Correlation Analysis

The most important antioxidant activity was noted with DPPH test, especially for ethanol, methanol and aqueous extract for both plant. Those extracts present the higher contents of polyphenols and flavonoids content. Therefore, this activity may be due to the high content of these compounds. To better assess the interrelationship between total polyphenols, total flavonoids and condensed tannins content and antioxidant and antimicrobial activity, an eventual correlation between both parameters was evaluated using the Person's correlation test. Pearson's correlation coefficient r was presented in Table 2 (only significant data) for Ruta T (values presented with asterisk *) and Ruta M. Correlation analysis reveal that Ruta M represent a negative correlation between polyphenols and flavonoids content and IC50 value of DPPH test (r= -0.9033; P = 0.0136 and r = -0.8813; P = 0.0203 respectively). For Ruta T, a significant correlation was noted between only tannins content and IC50 value of DPPH test (r = 0.8362; P = 0.038). No correlation (P<0.05) was noted between different compounds and other antioxidant tests (ABTS, β-carotene and FRAP) for both plant.

Table 2: Correlation coefficient r between polyphenols, flavonoids and tannins contents and antioxidant and antimicrobial activity.

	Polyphenols content	**Flavonoids content**	**Tannins content**
DPPH (IC_{50})	r= -0.9033 (P = 0.0136)	r = -0.8813 (P = 0.0203)	*r = 0.8362 (P = 0.038)
S. enterica (MIC)	r = -0.9203 (P = 0.0093)	*r = -0.8774 (P = 0.0216) r = -0.8873 (P = 0.0184)	NS
E. coli (MIC)	r = -0.9203 (P = 0.0093)	r = -0.8873 (P = 0.0184)	NS
B. subtilis (MIC)	NS	*r = -0.9269 (P = 0.0078)	NS
S. aureus (MIC)	r = -0.9203 (P = 0.0093)	r = -0.8873 (P = 0.0184)	NS

Note: *Ruta T; NS: not significant.

A negative correlation was noted between polyphenols content of Ruta M extracts and antibacterial activity (MIC) against S. enterica (r = -0.9203; P = 0.0093), *E. coli* (r = -0.9203; P = 0.0093) and *S. aureus* (r = -0.9203; P = 0.0093). Similarly, a negative correlation was also noted between flavonoids content of Ruta M extracts and antibacterial activity (MIC) against same strains (S. enterica with r = -0.8873; P = 0.0184, *E. coli* with r = -0.8873; P = 0.0184 and *S. aureus* with r = -0.8873; P = 0.0184). However, a negative correlation was noted between flavonoids content of Ruta T extracts and antibacterial activity (MIC) against S. enterica (r = -0.8774; P = 0.0216) and *B. subtilis* (r = -0.9269; P = 0.0078).

Discussion

As part of the valuation and exploration of medicinal plants as a source of active biomolecules with potent biological activities, we have chosen *R. graveolens*, which is known for its virtues and its large use in ethnobotany regarding its richness with secondary metabolites [2]. Based on a study conducted by Mohammadi-Motamed, et al. [25], which demonstrate that increasing the temperature during extraction, significantly decreased the ability to scavenge DPPH radicals, and may be also with other activities or chemical contents, we choose to exhibit extraction by maceration for different organic and water extracts at RT. Results show that polar solvents give better yields than nonpolar solvents, since polar solvents have the ability to diffuse inside the plant powder, reach the plant matrix and recover as much metabolites as possible. Previous study shows a relatively similar extraction yields. Indeed, Eldalawy [26] noted an amount of ethanol extract around 7 % but Bañuelos-Valenzuela, et al. [6] noted a two-fold yield of this extract (10.5 %).

Phytochemicals analysis noted a high content of polyphenols in methanolic and ethanolic extracts which may explained by the high power of these polar solvents to dissolve a large number of compounds such as flavones, phenols and polyphenols [27].

According to Wojdyło, et al. [28], the content of phenolic compounds also varies depending on the extraction method. According to Boizot, et al. [29] and Gomez-Caravaca, et al. [30], the results of the determination of total phenolic compounds (TPC) can not exactly indicate the contents of the extracts in these compounds. Indeed, despite the sensitivity of the Folin-Ciocalteu method, this reagent can also react with the aromatic amino acids, protein (especially with tryptophan), reducing carbohydrates, such as glucose and fructose, as well as vitamin C. Qualitative assessment reveals the presence of polyphenols in *R. graveolens* extracts, with different amount from one study to another as determined by quantitative analysis [31,32].

Indeed, TPC of methanol extract of *R. graveolens* leaf/stem was found equal to 28.4 mg GAE/g dry weight [33]. The TPC in the ethanol extract of *R. graveolens*' leaves was found to be 13 µg/mL of Catechol equivalent [34]. Cho et al., (2005) [35] reveals that TPC in the ethanol extract was around 17.07 mg/g and around 16.39 mg/g in water extract and by Maslennikov et al. [36] with TPC equal to 14.9 ± 1.4 mg/g in leaves extracts. Also, the amount of TPC was around 15.28 % in chloroform, 15.62 % ± in ethanol, 16.25 % in methanol, and 10.05 % in water extracts [31]. Much lesser TPC (around 4.3 mg GAE/g dry sample) was detected in *R. graveolens* aqueous methanol extract of Leaves [37]. According to Giresha et al. [38], phenolics are higher than flavonoids, saponins and alkaloids contents in ethanol, water and methanol extracts of *R. graveolens*. Phenolic acids in *R. graveolens* extracts are mostly represented by the gentisic acid (2.4 mg/100 g dry sample), caffeic acid (2.1 mg/100 g dry sample), ferulic acid (4.9 mg/100 g dry sample) and p-Hydroxybenzoic acid (1.2 mg/100 g dry sample) [37].

Each plant was collected in different year; nevertheless, chemical analysis reveals that the year of collection did not interfere with the chemical profile of *R. graveolens* extracts (Bañuelos-Valenzuela, et al.) [6]. Like polyphenols, flavonoid content therefore varies depending on the solvent used. Indeed, Stankovic [39] reported that the flavonoid heterosides are soluble in polar solvents such as methanol-water and acetonitrile-water mixtures, whereas for genins (aglycone part of flavonoids), they are rather soluble in nonpolar solvents. In this assay, it appeared that most of the Ruta flavonoids are glycosylated because they are better solubilized in polar than nonpolar solvents. In a study conducted by Proestos, et al. [37], flavonoid content in *R. graveolens* extracts are represented by Quercetin (3.1 mg/100 g dry sample), Rutin (3.1 mg/100 g dry sample) and (+)-Catechin (10.5 mg/100 g dry sample). The variability between these condensed tannin contents may be due to the effect of several factors, such as the sensitivity of the tannins to several degradation pathways (oxidation, light, etc.), the stage of maturity of the sample plant, growing conditions, solvents used during extraction and many other factors [40].

Qualitative assessment reveals the presence of tannins in aqueous, ethyl acetate and methanol leaves extracts of *R. graveolens* [32]. Estimation of tannins was around 3.10 % in chloroform, 15.15 % in ethanol, 18.25 % in methanol, and 1.04 % in water leaves of *R. graveolens* [31]. Antioxidant activity was evaluated by four different assay, mainly, DPPH, ABTS, β-carotene and FRAP. Results of DPPH method suggests that the presence of phenolic compounds in ethanolic, methanolic and aqueous extracts may be the main cause of their considerable ability to remove radicals from DPPH. Indeed, DPPH radical scavenging activity of *R. graveolens* water extract was showed at 50 µmoL Trolox equivalent per g dry basis [41]. Also, DPPH radical scavenging activity of Leaf methanol extract at 250 mg/mL showed an IC50 value equal to 90.21 % [42]. An important, dose dependant, DPPH radical scavenging activity was showed with ethanol extract, with 08.48, 10.45, 11.15 and 13.01, 19.37% of inhibition at a respective concentration of 10, 50, 100, 250 and 400 µg/mL [34]. An IC50 value for the methanol extract (at 200.5 µg/mL) was much higher than BHT (at 41.8 µg/mL) used as a positive control [25].

Similar to our results, in a study conducted by Giresha, et al. [38], the ethanol extract had the highest antioxidant activity (followed by the aqueous and methanol extracts) with IC50 equal to 3.27 µg/µL for DPPH free radical scavenging, 3.58 µg/µL for ferric reducing, 3.87 µg/µL for superoxide scavenging and 4.77 µg/µL for anti-lipid peroxidation activity. Also, ethanol extract showed a positive DPPH antioxidant activity (IC50 = 540 µg/mL) as compared with standard quercetin [26]. DPPH analysis of ethanol extract reveals an IC50 value equal to 281.02 µg/mL [43]. The methanolic leaves extracts of R. graveolens, and the major polyphenols (Quercetin, chlorogenic acid, and p-coumaric acid) showed higher antioxidant activity than those of *Artemisia abrotanum*. Recently, an IC50 values of DPPH was 21.3 µg/mL, β-carotene bleaching was 26.7 µg/mL, and FRAP assays was 32.8 mM TEAC/g Extract [44]. A potential antioxidant activity, tested by ferrylmyoglobin/ABTS+ method (3.7 mM TEAC/g dry weigh), was noted with methanol extract of *R. graveolens* leaf/stem [33].

Recently, ABTS analysis of ethanol extract reveals an IC50 value equal to 587.98 µg/mL [43]. An important antioxidant activity of Ruta T extracts (petroleum ether, ethanol and methanol) may be due to the complexity of these extracts in polyphenolic substances and the synergy between them for better antioxidant activity [45]. In fact, polyphenols having high stoichiometry have a significant capacity to trap free radicals by multiple transfers of H atoms or electrons from the starting phenol and some of its oxidation products, as in the case of quercetin and rutin [46,47]. The reducing power of a compound can serve as a significant indicator of its antioxidant activity [48] because it is related to its property as an electron donor of polar extracts and, therefore, their

ability to neutralize free radicals and reduce them to a more stable, non-reactive form [49]. Antioxidant activities of *R. graveolens* water extract based on their abilities to reduce the ferric ion-TPTZ complex was showed at 100 mol Trolox equivalent per g dry basis [41].

Ferric oxide radical scavenging activity was showed with ethanol extract, with 0.54 % of inhibition at 500 µg/mL compared to 1.59% of inhibition of the standard drug BHA [34]. Total antioxidant content in *R. graveolens* extracts was 0.47 TAC mg/g [36]. A selective antimicrobial activity was noted with some extracts of both plants. Petroleum ether, chloroform, ethyl acetate and aqueous extracts of both Ruta, very likely, does not contain secondary metabolites with antimicrobial power, like phenolic oligomers, alkaloids (especially quinolines and acridones) [50-52], furoquinolines [48], sterols/tri-terpenes, rutin, and the low content of coumarins compared to methanolic and ethanolic extracts. Indeed, most of the active antimicrobial compounds identified are apolar and insoluble in water [53]. These results can be explained by the high contents of ethanolic and methanolic extracts of phenolic compounds. Indeed, phenols are the compounds with the greatest antibacterial effectiveness. In particular, they cause irreversible lesions on the membranes and are useful in bacterial, viral and parasitic infections, whatever their location [54].

Flavonoids are also antimicrobial agents that work by binding to extracellular or soluble proteins, or by attaching to the cell wall by destabilizing it [55]. In a study conducted in Finland by Ojala, et al. [56] and in Bulgaria by Ivanova et al. (2005) [3], an important antibacterial activity, *B. subtilis* and *S. aureus*, was noted in methanol extract from leaves or aerial part of *R. graveolens*. Anti-microbial activity by formation of clearance of zone was shown with the methanolic extracts from stem (8 mm) against *E. coli*, *Pseudomonas sp*., and *Staphylococcus sp*. and water extract (4 mm) against *Staphylococcus sp*. [13]. Ethanol (16 mm) and water (6.5 mm), but also petroleum ether (8 mm) leaves extract of *R. graveolens* at 1 mg/mL shows an antibacterial activity against *E. coli* Sivaraj, et al. [57]. Kumar, et al. [58] noticed an important antibacterial activity against *Pseudomonas aeruginosa* (*P. aeruginosa*) only with the methanolic extract from *R. graveolens*' leaves (from India). Ethyl acetate Root extract of *R. graveolens* gave a good antibacterial activity against *P. aeruginosa* (7 mm) and *E. coli* (8 mm) [59].

Chloroform, ethanol and methanol extracts of *R. graveolens* showed antimicrobial activity on *E coli, P. aeruginosa*, *B. subtilis* and *S. aureus*. Nevertheless, aqueous extract was not against any of four strains [60]. Sensitivity of *B. subtillis* to methanolic (18 mm) and ethanolic extracts (16 mm) at 100 mg/mL was noted, and *C. albicans* sensitivity to aqueous extract (7 mm) [31]. Antibacterial Activities of the methanolic leaves extract and identified polyphenols showed antibacterial activities against a range of bacteria, inter alia, against *E. coli* (with MIC = 0.75 and MBC = 0.98) and *S. aureus* (with MIC = 0.99 and MBC = 0.84) [44]. This antibacterial activity of *R. graveolens* is attributed to the high contents of specific polyphenols such as isochlorogenic acid and p-coumaric acid and quercetin [44]. Methanolic leaves extracts of *R. graveolens* was also evaluated against C. albicans, with MIC equal to 0.84 and MFC equal to 1.02. This antifungal activity of *R. graveolens* is attributed to p-coumaric acid, quercitrin, rosmarinic acid, but particularly to quercetin and isochlorogenic acid [44].

Correlation test was applied to more explain the relation between phytochemical assessment and different tested activities. A significant correlation was noted only between phenols, flavonoids, and tannin contents, and DPPH assay. Previous significant correlation (R2 = 0.937) was noted between trolox equivalent antioxidant capacity and TPC in methanolic extract of *R. graveolens* [33]. Surprisingly, no previous study was interested on correlation analysis between phytochemical content and antimicrobial activity, which give additional originality to our study. Negligible or absence of correlation between total phenolic content and antioxidant and/or antimicrobial activity in our plant extracts demonstrate that important activity could possibly be due to the presence of some other phytochemicals components (coumarins, terpenoids, alkaloids etc.), also to the synergistic effects among them [61]. Phenols are most wide and important secondary metabolites plant and may be responsible for antioxidant activity due to their redox properties, hydrogen donors and singlet oxygen quenchers [34].

Conclusion

To the best of our knowledge, this is the first report on the phenolic content assessment, antioxidant and antimicrobial activity study of organic and aqueous extracts from leaves of Tunisian or France *R. graveolens*. Comparison between a wild (France) and cultivated (Tunisia) *R. graveolens* reveals an important extraction yield, antioxidant, antibacterial and antifungal activity of ethanolic and methanolic extracts. Correlation analysis explain in part the relationship between phenol contents and different tested activity. Our results are discussed and compared to data from a systematic review of the literature, which are interested on same study conditions, namely organic and aqueous extracts.

Acknowledgement

This work was supported by Ministère de L'enseignement Supérieur et de la Recherche Scientifique, LR99ES27, Tunisia.

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2021.37.005997

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Submission Link: https://biomedres.us/submit-manuscript.php