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Antibacterial and Radical Scavenging Activities of *Ruta buxbaumii* Poir. (Rutaceae) Growing in Raman Mountain-Batman

Alevcan KAPLAN*[1]

Abstract

The members of the Rutaceae genus have found a wide application area in traditional medicine in many countries from ancient times to today; they have been used for many purposes in Turkey. *Ruta* species have many pharmacological properties such as inducing abortion, antirheumatic, hypoglycemic, anthelmintic, antipyretic, antiepileptic, antidiabetic, against epilepsy, vertigo, headache and in eye diseases, anthelmintic and against poisoning. In this paper, it was aimed to determine the antimicrobial and radical scavenging activities of methanol (MeOH), petroleum ether (PE), ethyl acetate (EtOAc) and ethanol (EtOH) extracts of *Ruta buxbaumii* Poir. growing naturally in Raman mountain, Batman. Antimicrobial activities of extracts were evaluated using disc diffusion method. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615) and two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) were used to determine the antibacterial activity. Results demonstrated that although *R. buxbaumii* extracts showed close antibacterial effects, EtOAc extract showed the highest effect against *S. pyogenes* with 23.5±1.2 inhibition zones, while EtOH extract showed the lowest effect against *P. aeuroginosa* with 11.8±0.2 inhibition zones. All the extracts showed no clearance of zone inhibition for against *E. coli*. Antioxidant properties and activities were evaluated by using total phenolic content (TPC), total flavonoid content (TFC), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging activity. The results showed that while activity of the EtOH extract of *R. buxbaumii* was the highest (54.10±0.13 mg GAE/g extract) for total phenolic substance content, MeOH and EtOH extracts were found to be higher (47.52±0.19 mg QE/g extract and 46.86±0.16 mg QE/g extract) for total flavonoid content, respectively. Also, radical scavenging activities such as (DPPH) of extracts were investigated, and it was revealed that EtOH extracts (73.6 %), MeOH (60 %), EtOAc (40 %) and PE extract (25 %), respectively. From these results indicates that this species can be used in the pharmaceutical application as a valuable bioproduct with new functional properties in foods.

Keywords: Antibacterial activity, DPPH, total flavonoid content, total phenolic content, *R. buxbaumii*.

1. INTRODUCTION

The use of herbs in treatment began with the history of humanity. Thousands of years ago, people realized the therapeutic power of herbs and started benefiting them to sustain a healthy life. In Anatolia, where folk medicine practices are common, folk remedy practices have survived to the present day after long experiences. Many drugs used in modern medicine are also derived

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from plants [1]. The therapeutic use of herbs varies according to the development level of the countries. In developing countries, 80% of the population benefit from herbal products for therapeutic purposes [2]. In some Asian, African, and Middle Eastern countries, this rate rises to 95%. In developed countries, this rate is less; 40-50% in Germany, 42% in the USA, 48% in Australia, and 49% in France [3]. The World Health Organization predicts that the treatment with medicinal plants will increase within the next years all over the world. Plants can produce secondary metabolites to protect them from natural enemies such as bacteria, viruses, fungi, and insects [4]. Medicinal plants are rich in chemical bioactive components such as terpenoids, phenolics, alkaloids, flavonoids, amino acids, saponins, glycosides, diterpenes and triterpenes [5].

The Rutaceae family is part of the order Sapindales, part of the Eurosid II group, which is part of the rosids in eudicots in cladistic studies based on DNA sequence analysis in angiosperms [6-7]. The Rutaceae family is a large family consisting of trees, shrubs, and woody species, containing 1600 species and ~155 genera, mostly tropical and subtropical [8-9]. The genus *Ruta* is represented by nine species all over the world; four species in the Mediterranean phytogeographic region, two species in Corsica, three species in the Canary Islands, and 55% of them are endemic [10]. The Rutaceae family includes species that are particularly appreciated in folk medicine for their numerous medicinal properties and benefits to human health. *Ruta* is one of the most well-known genera of the Rutaceae family, native to the Mediterranean region and known for its use in traditional medicine [11]. Today, this genus is cultivated in many parts of the world for its medicinal properties [12]. The most studied species of the genus *Ruta* in the world are *R. chalepensis* L., *R. graveolens* L., *R. montana* L. Extracts and essential oils of these species have a wide variety of medical uses such as gastric, diuretic, inflammatory, rheumatic disorders, anti-inflammatory, antioxidant, hypoglycemic, emmenagogue, spasmolytic, menstrual problems, as a sedative, antipyretic, antiplatelet and anticholinesterase, an antibacterial, antifungal and as an antihelminthic agent and also as a food flavoring agent [13-14-15-16-17-18-19-20]. In their study on the genus *Ruta*, Coimbra et al. (2020) confirmed that different parts of the plant are used in folk medicine to treat a wide variety of diseases. Although its main use is in the gynecological field, it has also been described in the treatment of pain, fever, nausea, inflammation, infections, and nervous disorders. *Ruta* species contain many bioactive substances. The main classes of compounds are coumarins, alkaloids, terpenes, and flavonoids. Due to their wide range of biological abilities, the components of *Ruta* species are of great interest in medicinal chemistry, and many are used in medicine [22-23].

According to Townsend [24], the genus *Ruta* is represented by two species, *R. chalepensis*, and *R. montana*, in Turkey. However, in later studies, the genus *Ruta* is represented with six species such as *Ruta buxbaumii* (sin. *Haplophyllum buxbaumii*. Recently, several taxa from the genus *Haplophyllum* were transferred to *Ruta* and *R. buxbaumii* is one of them.), *R. chalepensis*, *R. montana*, *R. suaveolens*, *R. thesioides* and *R. villosa.* [25-26-27-28]. A literature review on *R. buxbaumii*, which grows on Mount Raman in Batman, one of the *Ruta* species traditionally known for its sedative and gas-digesting effects [29], reveals that there is no information about its biological properties. In addition, the region where medicinal and aromatic plants grow, climatic conditions, and soil properties directly affect plant components. The fact that Mount Raman is a unique region and there are not many studies in this sense reveals the necessity of this study. In line with this background, in the present study, the effect of the antibacterial and antioxidant activities of different extracts of the *R. buxbaumii* plant was investigated for the first time in this study.

2. MATERIALS AND METHOD

2.1. Source of Plant Material

The aerial parts of plant material was collected at flowering time in May 2020, from natural habitat

of Raman mountain (Batı Raman campus of Batman University). The taxonomical identity of the plant was confirmed by Dr. Alevcan Kaplan. Voucher specimens have been deposited at Batman University (voucher no. 2020/013).

2.2. Preparation of Plant Extract

The aerial parts of the plant were washed and dried in the shade at room temperature to remove contamination. The plants were dried at room temperature for two weeks. The samples were then ground with a blade-carbide grinding. The ground sample was macerated at room temperature using 1: 20 MeOH, EtOH, PE, EtOAc respectively to prepare various extracts. The beaker was covered with aluminum foil and shaken continuously using a rotary shaker at 100 rpm. The extraction process was performed in the dark for 3 days. New solvent volumes were changed until the color of the extract became colorless. The extracts were filtered through filter paper. The total volume of the extract was recorded. Subsequently, the extract solution was concentrated to dryness under vacuum and reduced pressure using a rotary evaporator at 60 ° C to obtain concentrated extracts. These extracts were stored at 4ºC for further bioassays [30-31].

2.3. Biological Evaluations

2.3.1.Antibacterial Screening

Disc diffusion method was performed according to Shryock et al. [32] with some changes. Four microbial strains were tested, including two Gram-positive bacteria (*S. aureus* ATCC 25923, *S. pyogenes* ATCC 19615) and two Gram-negative bacteria (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922). The blank discs were filled with concentration of 5.0 mg/disc of extracts. Then, the petri dishes were incubated at 37 °C or 24 hours. The diameter of the inhibition zone around each disc was then measured. Antibacterial activity was determined by the diameter of the inhibition zones around the disc on the agar surface. All tests were performed in triplicate.

2.3.2. Determination of Total Phenolic Content (TPC)

The total phenolic content of the extracts were made according to the method of Singleton et al. [33]. 0.2 mL of sample solutions (2 mg/mL) prepared for the study were taken and after adding 9 ml of distilled water, 0.2 mL of Folin Ciocalteu reagent was added and left to stand for 3 minutes. Finally, 0.6 mL of Na_2CO_3 (20%) was added and the total volume was adjusted to 10 ml. After incubating in the dark for 2 hours at room temperature, absorbance was measured at 760 nm. Gallic acid was used to create the standard calibration curve. 0.1 mg/mL was prepared as the master stock and seven different concentrations were obtained by dilution. 0.2 mL of sample solution was added for the control. According to the gallic acid standard, the total phenolic substance in all plant extracts was calculated as mg gallic acid equivalent (GAE)/g extract. The analysis was performed in triplicate.

2.3.3. Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extracts Arvouet-Grand et al. [34] was made according to the method. In the preparation of the experiment, 100 µl of 10% aluminum nitrate and 100 µl of 1 M potassium acetate were taken and the extract was added so that the final concentration of the plant extract was 100 µg/ml. The final volume of the experiment was completed to 5 mL with 99 % ethanol. After incubating in the dark for 40 minutes at room temperature, absorbance was measured at 417 nm. For control, 200 µl of the sample solution was added instead of extract. For the quercetin standard, master stock 0.5 mg/mL was prepared and eight different concentrations were obtained by dilution. The total flavonoid substance content was expressed as mg quercetin equivalent (QE)/g extract. The analysis was performed in triplicate.

2.4. Antioxidant Activity

2.4.1. Determination of DPPH Radical Scavenging Activity

Free radical activities of extracts were determined using DPPH free radical Gezer et al. [35]. For the experiment, the concentration was prepared by dissolving 4 mg DPPH in 100 mL methanol. For each sample, 3.2 mL DPPH radical and 200 µl (500 µg/mL) of extract solutions were added. After 30 minutes of incubation at room temperature in the dark, absorbance was measured at 517 nm. For control, 200 µl extract solvent was added to the test tube. Analysis was performed in triplicate. The following formula was used to determine the % DPPH radical scavenger.

% DPPH scavenging activity = $[(A_{blank}–A_{extract}) / A_{blank}] \times 100$.

A_{blank}: Absorbance of the control.

$A_{extract}$: Absorbance of the reagent with extract.

2.5. Statistical Analysis

The analysis of variance of the data obtained was made according to the ANOVA procedure. The difference between the means was evaluated at the level of $p < 0.05$ according to Duncan comparison test. The data obtained are given as mean ± standard deviation. Data calculations were performed using SPSS for Windows (version 15.0, SPSS®, Chicago, USA).

3. RESULTS AND DISCUSSION

Plants have been used for centuries for medicinal purposes in the treatment of various diseases and enteritis [36-37]. The properties of plants that are lethal for microorganisms and important for human health have been investigated in laboratories since 1926. Recently, as well as all over the world, the use of plants found in the natural flora for different purposes such as treatment, food, tea, spice, paint, insecticide, veterinary cure, resin, glue, essential oil, beverage, and cosmetics has become a part of our study area [1]. In this context, plant extracts are examined for their biological activities for the management of complex diseases. For instance, plant-based antioxidant compounds have been claimed to inhibit the oxidation process by reacting with free radicals, chelating catalytic metals, and scavenging oxygen molecules found in biological systems. Thus, in terms of biological activities, due to the pluripotential of plant extracts, including antioxidant, antimicrobial, antidiabetic properties, herbal extracts are paving the way for global recognition and inclusion in pharmaceutical drugs [38]. Antibacterial studies on medicinal plants are a rich source of antimicrobial agents. As synthetic and semi-synthetic antimicrobial drugs are abandoned on the market, there is a continuing need for new research to cope with the increasing evolution of many antimicrobial-resistant strains of organisms [39]. In this study carried out for this purpose, antibacterial and some biological activities of *R. buxbaumii* plant extracts were examined. The antibacterial activity of *R. buxbaumii* extracts was determined on four bacterial strains (Table 1). These selected microorganisms are known for their strong resistance, invasive and toxic powers, and are pathogenic in humans. They are frequently found in many infections that cause clinical and therapeutic problems in Turkey. The results of antibacterial screening of MeOH, PE, EtOAc and EtOH extract of *R. buxbaumii* are presented in Table 1. The antibacterial activity of the extracts was evaluated against streptomycin. All extracts exhibited strong antibacterial activity against Gram (+) strain *S. aureus* and *S. pyogenes*. On the other hand, weak to moderate antibacterial activity was found for the Gram (-) strain *P. aeruginosa*. Also, all the extracts showed no clearance of zone inhibition against the Gram (-) strain *E. coli*. EtOAc extract showed the highest zone of inhibition against *S. pyogenes* strains with 23.5±1.2 mm. EtOH extract showed the lowest zone of inhibition against *P. aeruginosa* strain with 11.8±0.2 mm. This suggests that it may have resulted from both the diversity of the compounds contained in the extracts and their synergistic interactions rather than individual activity.

Table 1 Antibacterial activity of the various solvent extracts from *R. buxbaumii* against human pathogenic bacteria.

Microorganisms	İnhibition zone (mm)				Antibiotic
	MeOH	EtOH	PE	EtOAc	Streptomycin
S. aureus ATCC 25923	21.3±0.5	21.6±0.0	22±0.5	21±0.0	27.0±0.1
S. pyogenes ATCC 19615	18±0.0	16.3±0.6	18.1±0.6	23.5±1.2	27.0±0.1
P. aeruginosa ATCC 27853	13.7±0.6	11.8±0.2	13±1.2	14± 1.1	27.0±0.1
E. coli ATCC 25922	na	na	na	na	na

*na: not active

Antimicrobial activities of some species belonging to the genus *Ruta* have been reported by many researchers [22-23-40-41-42-16-43]. Bekkar et al. [20] reported that methanol extract had a strong antimicrobial effect against *S. enterica* ssp *arizonae*. The results of this study are in agreement with the report of Ivanova et al. [44] who confirmed that the different extracts of *R. graveolens* L., a related plant, have antimicrobial activity. Regarding antimicrobial activity, this study found inhibitory activity against *S. aureus* and *S. pyogenes* among the bacterial strains tested, while found no activity against *E.coli*. For *R. chalepensis* plant, antibacterial activity characteristics in the current experiment were close to the content reported by Ouerghemmi et al. [16]. In their study examining the antibacterial activity of the spontaneous and cultivated *R. chalepensis* plants in Tunisia, Ouerghemmi et al. [16] reported that while it exhibited moderate activity against the *S. aureus* strain, low or no activity against *P. aeruginosa* and *E. coli*. The effectiveness of these extracts against bacteria may be partly due to their phenolic composition. The studies of Nagarjuna and Al-Rajab [45] on *R. graveolens* was found antibacterial activity against gram-positive and gram-negative bacteria, resulting in several common human pathogenic bacteria, including methicillin-resistant *S. aureus* and the yeast *C. albicans*. Several studies have linked the inhibitory effect of plant extracts against bacterial pathogens to their phenolic composition [43-46-47]. The inhibitory effect of phenolic compounds can be explained by adsorption to cell membranes, interaction with enzymes, substrate, and metal ion deprivation [48]. Flavonoids and phenolic acids are very strong free radical scavengers. These compounds, taken through food, largely eliminate the effects of oxygen radicals, which are called "oxidation stress", and adversely affect human health [49]. Table 2 shows the total phenolic (TPC) and total flavonoid content (TFC). The formation of blue density indicates the presence of phenolic compounds. Statistical differences were found between samples. The highest phenolic content of *R. buxbaumii* was 54.10 ± 0.13 mg GAE/g extract in the EtOH extract and the lowest phenolic content of *R. buxbaumii* was 34.12 ± 1.2 mg GAE/g extract in the PE. The phenolic substance content of the EtOAc extract was calculated as 44.25±0.06 mg GAE/g extract and was statistically significant. The highest flavonoid content of *R. buxbaumii* was 47.52 ± 0.19 mg GAE/g extract in the MeOH extract and the lowest phenolic content of *R. buxbaumii* was 18.94 ± 0.24 mg GAE/g extract in the PE. The levels of MeOH and EtOH extracts were high in terms of flavonoid but not significantly different between the extracts. The flavonoid substance content of the EtOAc extract was calculated as 24.02 ± 0.54 mg QE/g extract and was statistically significant. As can be seen from the data, PE extract contains less amount of both phenolic and flavonoid substances than other extracts, and this was statistically significant. Total phenolic content (TPC) in this experiment was close to the content reported by Fakhfakh et al. [50]; they found phenolic content of 54.13 mg GAE/g extract in EtOH extract. Kacem et al. [16] obtained the highest TPC content of EtOH, water, hexane, EtOAc from EtOH extract with 178 mg GAE/g extract. Gali and Bedjou [30] found the highest TFC in butanol extract with 210.00 ± 4.93 µg GAE/mg extract, even reported that EtOAc extract was higher than EtOH (61.61 ± 0.70 µg GAE/mg extract) extract. [51] found that the MeOH extract TFC 1328.8 mg GAE/100 g dry weight. Yaman et al. [52] examined the

antioxidant activities of MeOH and EtOH extracts of wild *R. chalepensis* L. (above-ground part) and *R. montana* L. (leaf-flower and stem parts). Total flavonoid content in dry weight of MeOH and EtOH extracts of *R. chalepensis* was found higher than other extracts except for MeOH leaves-flowers extract of *R. montana*. In their study on *in vitro*, antioxidant activity and total phenolic content of *R. montana* L., Merghem and Dahamna [53] were found that EtOAc contained high amounts of total polyphenols (257.1 ± 0.703µg gallic acid equivalent/mg of extract), tannins (251 ± 1.41 µg tannic acid equivalent /mg of extract), and flavonoids (117.4 ± 3.451 µg quercetin equivalents/mg of extract), respectively. The differences between the values suggested that the plant species, locality, and extraction methods may cause this.

Table 2 Total phenolic and flavonoid contents of *R. buxbaumii* extracts.

Extracts	**Total Phenolic Content (mg GAE/g extract)**	**Total Flavonoid Content (mg QE/g extract)**
MeOH	49.60 ± 0.17^{b}	47.52 ± 0.19^{a}
EtOH	54.10 ± 0.13^{a}	46.86 ± 0.16^{a}
PE	34.12 ± 1.2^{d}	18.94 ± 0.24^{c}
EtOAc	44.25 ± 0.06^{c}	24.02 ± 0.54^{b}

* Statistically, each column was evaluated separately and the differences were shown in lower case according to the $p < 0.05$ level.

Various methods are widely used to measure the antioxidant capacity of extracts. Each method results in the generation or use of a different radical that is directly involved in the oxidative process through various mechanisms. Among the free radical scavenging methods, the DPPH method is fast, simple, and inexpensive compared to other test models. When the DPPH radical is scavenged with an antioxidant compound via hydrogen donation to form a stable DPPH-H molecule, the color of the solution changes from purple to yellow [54].

The results of the antioxidant properties of the crude extracts assessed by the DPPH• free radical scavenging method are shown in Figure 1. In general, the strongest activity was 73.6 % in EtOH extract, followed by 60 % in MeOH, 40 % in EtOAc, and 25 % in PE extract, respectively. Kacem et al. [16] examined the radical scavenging activity of *R. chalepensis* extracts at different concentrations and found that the 0.125 mg / mL sample of ethanol extract had inhibition of approximately 80-70 %. This value was found to be consistent with this study. More recently, Bekkar et al. [20] have reported an important antioxidant activity of *R. chalepensis*. Yaman et al. [52] examined the radical scavenging activities of the extracts such as DPPH and ABTS. It was observed that *R. chalepensis* extracts exhibited higher activity when evaluated on a solvent basis. The studies of Merghem and Dahamna [53] on *R. montana* was found that EtOAc extract showed the highest scavenging capacity followed by MeOH, aqueous and chloroform extract. Ouerghemmi et al. [43] reported that the highest DPPH activity of *R. chalepensis* was in flower, leaf, and stem MeOH extracts, respectively. Kacem et al. [16] reported that the ethanol extract of the leaf-stem sample of *R. chalepensis* species had more radical scavenging activity than water, EtOAc and hexane extract, and even less aqueous extract activity than EtOAc. Antioxidant activities of extracts; It is due to reduction of hydroperoxides, inactivation of free radicals, complexation with metal ions or a combination of these. It is thought that some of the antioxidant activity provided by these mechanisms is due to flavonoids. Additionally, the antioxidant activities observed in plants may result from the synergistic interaction of two or more compounds in the plant. It has been reported that many natural antioxidant compounds generally act synergistically with each other, thus providing an effective defense against free radicals [55].

Due to the structural differences of herbal samples, it is not possible to talk about the use of a single solvent system for each sample in extraction methods. As the results clearly show, the analyzes by working with different solvents,

the most suitable solvent can be selected, so that accurate and high results can be obtained about the antioxidant capacity of the plants. According to the results, it was clearly seen that the differences in the phenolic content of the different extracts of the studied plant affected their antioxidant properties. Based on this, it can be said that the different antioxidant activities of the extracts are due to the amount and chemical structure of phenols that can pass into the solvent during extraction. Lastly, the present study has found parallel results to other studies. It is clear that there is a positive correlation between total phenolic and flavonoid content and antioxidant activity.

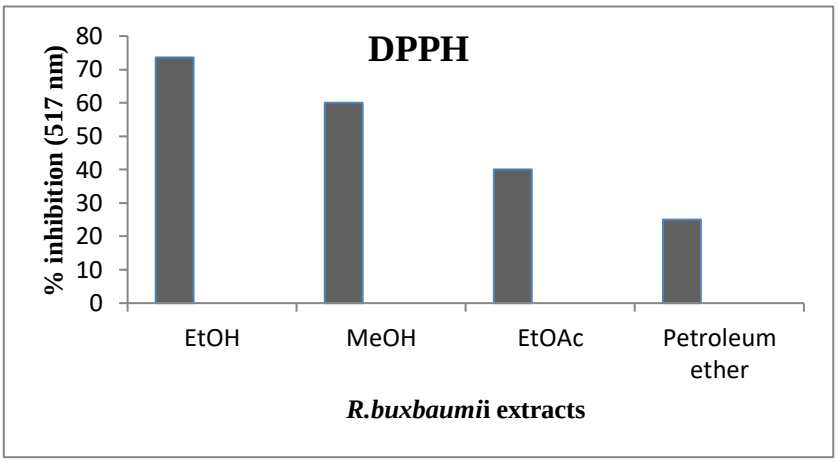

Figure 1 The DPPH free radical activities of the various solvent extracts from *R. buxbaumii*.

4.CONCLUSION

This study showed that *R. buxbaumii* Poir. several extracts have significant antibacterial, antiradical activities and rich phenolic and flavonoid content. Results from experiments support biological activities. It was suggested that the variation of *R. buxbaumii* in this study may be due to differences affecting biochemical and physiological structures such as species, organ, physiological age, harvest time, and locality. Preliminary findings of our study suggest that *R. buxbaumii* Poir. could be used as a natural source of medicinal application for antimicrobial and antioxidant activities. However, further investigations on other species of the family Rutaceae are encouraged to determine their potential anti-agent (antimalarial, antifungal etc.) activities. In addition, the search for natural antioxidants that can replace synthetic antioxidants for the future continues rapidly. It is recommended to determine plant extracts with high antioxidant activity with such studies, to examine their antioxidant effects in food and health systems, and to ensure the continuity of studies to apply alternative ways to treat diseases.

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