

Phytochemical and Antibacterial Assessment of *Rhagadiolus Stellatus* Plant in Jerusalem Area - Palestine

Nidal Jaradat*¹, Motasem Al-Masri², Bassam Abu-Shanab³, Fatima Hussein¹,
Mohammad Qneibi¹, Alaa Nasser Eldin¹, Tariq Yassin¹, Momen Khawaja¹

¹Department of Pharmacy, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, Palestine. ²Department of Biology & Biotechnology, Faculty of Science, An-Najah National University, Palestine. ³Faculty of Agriculture & Veterinary Medicine, An-Najah National University, Palestine

* Corresponding author: nidaljaradat@najah.edu

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ABSTRACT

In Palestine, many edible wild plants serve as food and medicine in the folk traditions. Now days, contemporary people, cultures, and traditions are increasingly losing their close connection with nature. Preliminary phytochemical screening including estimation of total phenols and flavonoids contents, as well as antioxidant and antibacterial properties, were evaluated for *Rhagadiolus stellatus* plant using standard phytochemical and biological methods. Evaluation of antibacterial activities of the aqueous extract was performed using the broth micro-dilution method. Antibacterial activities were examined against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* ATCC's and MRSA. The methanolic plant extract contains a mixture of phytochemical compounds such as flavonoids, glycosides, saponins, phenols, alkaloids, phytosterols and volatile oil. Total flavonoids and phenols contents were 18.35±1.11 mg QUE/g and 38.55±0.95 mg GAE/g of the plant extract, respectively. The methanolic extract also had potential antioxidant properties, while the aqueous extract has significant antibacterial properties. This study has provided initial data that justify the importance of *Rh. stellatus* in traditional folkloric food and medicine. The studied species can be used in the prevention and treatment of various infectious diseases. The tested extract not only exhibited antioxidant properties, but also contained bioactive constituents. It can also be utilized as natural food preservatives, nutritional supplements and in pharmaceutical industries.

Keywords: *Rhagadiolus stellatus*; Antioxidant; Antibacterial; Total Phenols; Total Flavonoid.

INTRODUCTION

From ancient times, plants played significant roles in human life due to their distinctive colors, flavors, and odors, and they were considered the most widely used and versatile ingredients in food preparation and processing throughout the world. They also played a critical role in the preservation of human health as they were used for their therapeutic benefits (1, 2).

Many wild plants can be utilized as food or for treatment or prevention of various diseases as well as many of available drugs in pharmaceutical market were originally investigated from wild plant species (3, 4).

Rhagadiolus stellatus (L.) Gaertn is widely known as “Star Edible Hawkbit” in

English and العكيفة in the Arabic. It is a member of Asteraceae (Compositae) family (5, 6).

Its synonyms are *Rh. stellatus* var. *hebelaeus* DC., *Rh. stellatus* var. *edulis* (Gaertn.) and DC., *Rh. edulis* Gaertn (7).

It is an annual herbaceous plant of 20-60 cm height, with dentate leaves and small flowering heads composed of yellow ligulate flowers. The plant is naturally and wildy distributed in the western and southwestern regions of Asia, southern regions of Europe and northern parts of Africa; and it grows wildy in cultivated and abandoned lands, stony grounds, hill slopes, low mountain ranges and dry, sunny places (7, 8).

It was found out that the aerial parts of *Rh. stellatus* contained flavonoids as kaempferol 3-*O*- β -glucoside, quercetin, quercetin 3-*O*- β -glucoside, luteolin and nicotiflorin also contained phenolic acids as 3,5-dicaffeoylquinic and chlorogenic acids and other components such as hexanoic acid, octanedioic acid, nonanedioic acid, myristic acid, nonanedioic acid, 12-methyl tetradecanoic acid, oleyl alcohol, palmitic acid, margaric acid, (9, 12, 15)-octadecatrienoic acid, stearic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid, lignoceric acid, campesterol, stigmasterol, sitosterol and phytol (9-11).

It is an edible wild plant used as a constituent of salads or vegetable pies in Spain, France, Italy, Greece, Croatia, Jordan, Turkey, Palestine, and North Africa (12-17).

In Italian traditional medicine, especially in Sicily, *Rh. stellatus* is used as a therapeutic agent for treatment of psoriasis; the plant is collected at spring time, and it is used to make decoction, which is prepared by crushing and soaking the plant aerial parts in cold water and untreated wine (10-17% alcohol). With this decoction, the psoriasis affected area is rubbed (9).

To the best of our knowledge, other traditional uses and evidence based *in-vivo* or *in-vitro* studies or toxicological studies for *Rh. stellatus* are not documented in the literature.

MATERIALS AND METHODS

Chemical reagents

The reagents that were utilized for the estimations of the antioxidant potential included: Methanol (Lobachemie, India), n-hexane (Frutarom LTD, Israel), Trolox ((s)-(-)-6 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, Denmark), and (DPPH) 2,2-Diphenyl-1-picrylhydrazyl (Sigma Aldrich, Germany). For phytochemical screening, the utilized reagents included: Millon's reagent (Gadot, Israel), Ninhydrin solution (Alfa Agar, England), Benedict's

reagent (Gadot, Israel), Molish's reagent, H₂SO₄ and Iodine solution (Alfa-Aesar, England), NaOH (Gadot, Israel), Chloroform, HCl (Sigma-Aldrich, Germany), Magnesium ribbon, Acetic acid (Frutarom LTD, Israel), FeCl₃ (Riedeldehan, Germany).

Folin-Ciocalteu's reagent (Sigma-Aldrich, Germany) was used for the evaluation of the total phenol contents. The reagents, utilized for evaluation of total flavonoid content, included: quercetin, (Sigma-Aldrich, Denmark), AlCl₃ and Potassium Acetate (Sigma Aldrich, Germany).

Nutrient broth (Himedia, India) and Dimethyl sulfoxide (DMSO) (Riedeldehan, Germany) were used for antimicrobial screening experiments.

Instrumentation

Rotary evaporator (Heidolph OB2000, VV2000, Germany), freeze dryer (Mill rock technology, model BT85, Danfoss, China), grinder (Moulinex model, Uno, China), balance (Radwag, AS 220/c/2, Poland), filter papers (Macherey-Nagel, MN 617 and Whatman no.1, USA), micropipettes (Finnpipette, Finland), incubator (Nüve, Turkey), syringe filter 0.45 μ m pore size (Microlab, China) and micro broth plate (Greiner Bio-one, North America).

Collection and preparation of plant materials

The aerial parts of *Rh. Stellatus* plant was collected in April 2015 from Jerusalem Mountains in Palestine. Voucher specimen was identified by pharmacognosist Dr Nidal Jaradat and deposited in the Pharmacognosy Laboratory, Faculty of Medicine and Health Sciences at An-Najah National University (voucher codes: Pharm-PCT-2773).

The aerial plant parts were collected, washed with distilled water and later completely dried in the shade at room temperature; the dried aerial parts were grounded into a fine powder using a mechanical blender.

The finely powdered plant was stored in tightly sealed special containers until use.

Preparation of plant extracts for biological screening

A total of 25 g of the powdered plant was weighed and then exhaustively extracted by adding 50 ml of n-hexane and 125 ml of 50% ethanol in triple distilled water. The mixture was then shaken for 72 hours at room temperature using a shaker that was set at 200 rpm. Afterward, the mixture was filtered using suction flask and Buchner funnel filtration. A separatory funnel into 2 phases - a lower aqueous phase representing the first aqueous extract and the upper organic phase representing the organic extract separated the obtained filtrate individually. The remaining solid materials were re-extracted separately by 125 ml of 50% ethanol in triple distilled water, and the re-extraction process was carried out as described above. The extract containing 50% ethanol was evaporated by using rotary evaporator at 35 °C for 6 hours, then the aqueous residue was dried using a freeze dryer for 12 hours. Meanwhile, organic extracts were placed in a rotary evaporator for 1 hour at 40°C to evaporate leftover organic solvents, and completely dried organic extracts were then stored at 2-8°C until use (18).

Preparation of plant extracts for phytochemical analysis and antioxidant assessment

About 10 g, of the grounded plant, were soaked in 1 liter of methanol (99%) and put in a shaker device at 100 rpm for 72 hours at room temperature and then stored in a refrigerator for four days. The extracts were then filtered using filter papers and concentrated under vacuum on a rotator evaporator. The crude extract was stored at 4°C for further use (19).

Antioxidant activity

A stock solution of a concentration of 1 mg/ml in methanol was initially prepared for plant extract and Trolox standard. Stock solu-

tions were used to prepare working solutions with the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) by using serial dilution in methanol.

A solution of DPPH was freshly prepared at a concentration of 0.002% w/v. Then, it was mixed with methanol along with each of the working concentrations in a ratio of 1:1:1. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentrations was DPPH with methanol only. The solutions were incubated at room temperature in a dark cabinet for about 30 minutes. Then, their optical densities were determined by using the spectrophotometer at a wavelength 517 nm.

The percentage of antioxidant activity of plants extract and the Trolox standard were calculated using the following formula: DPPH inhibition activity (%) = $(B-S)/B \times 100\%$.

Where B is the optical density of the blank and S is the optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC_{50}) of the plant extract, as well as the standard deviation was both calculated using BioDataFit edition 1.02 (data fit for biologist).

The antioxidant activity was reported as the percentage of inhibition. The inhibition of the plant extract and Trolox standard, at different concentrations, were plotted and tabulated. Then, the IC_{50} was calculated using the BioDataFit program.

Qualitative phytochemical analysis

Preliminary qualitative phytochemical screening of primary and secondary metabolic compounds such as proteins, starch, phenols, glycosides, saponin glycosides, flavonoids, steroids, volatile oils, and tannins was carried out according to the standard common phytochemical methods described by Evans, 1983 (20) and Harborne, 1998 (21).

Determination of total phenol content in the methanolic extract

Total phenols content in *Rh. stellatus* methanolic extract was determined using the spectrophotometric method with some modifications (22). 1 mg/ml aqueous solution for methanolic *Rh. stellatus* extract was prepared to determine the total phenol content. The reaction mixture was prepared by mixing 0.5 ml of plant extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO₃ aqueous solution. The samples were later incubated in a thermostat at 45 °C for 45 min. The absorbance was determined at wavelength of 765 nm using a spectrophotometer. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Gallic acid, and the calibration line was construed. Based on the measured absorbance, the concentration of Gallic acid equivalent was expressed regarding of (mg of GAE/g of extract).

Determination of total flavonoid content in the methanolic extract

Total flavonoid content was determined in regards of the calibration curve of Quercetin, and it was expressed as milligram of Quercetin Equivalent per gram of extract (mg QUE/g extract). The total flavonoid content was determined according to the procedure of Chang *et al.*, which was validated by Nugroho with some modifications (23, 24). Quercetin (10 mg) was dissolved in 10 ml of distilled water and was diluted to 100 ml. Subsequently, the stock solution was diluted to provide a series of concentrations (10, 30, 40, 50, 70, 100 µg/ml); from each solution, (1 ml) was mixed with 0.3 ml of 10% AlCl₃, 0.3 ml of sodium nitrite, 1 ml of 2M NaOH and 1 ml of distilled water. The samples were incubated at room temperature for 30 minutes. The absorbance was determined at wavelength of 510 nm using a spectrophotometer. Total flavonoid in extracts was expressed

regarding of Quercetin Equivalents (mg of QUE/g of *Rh. stellatus* extract).

Statistical analyses

Determination of antioxidant activity, total flavonoids, and total phenols contents of the *Rh. stellatus* was carried out in triplicate for each sample. The obtained results were presented as means ± standard deviation (SD) and then were compared using unpaired *t*-test (25).

Antimicrobial tests

The aqueous extract of *Rh. stellatus* was screened for antibacterial activity by using well diffusion method. The bacterial suspension was prepared by picking some colony of overnight agar culture of the test organism. Then, adding it to a test tube containing 5 ml of nutrient broth, then the turbidity was compared with that of McFarland nephelometer tube No. 0.5 (1.5*10⁸ CFU/ml). Then taking 1000 µl of suspension diluted it, and it was added to 2 ml of nutrient broth (0.5*10⁸ CFU/ml).

Measuring the minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after 18-24hrs. Both organic and aqueous extracts were subjected to serial broth dilution technique to determine their minimum inhibitory concentration for all tested microorganisms. The antibacterial activities of the plant extracts were examined for the growth of four reference bacterial strains. They were obtained from the American Type Culture Collection (ATCC) (*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853)) as well as against the growth of a diagnostically confirmed Methicillin Resistant *Staphylococcus aureus* (MRSA) clinical isolate. The MIC was carried out according to Wikler, 2007. The wells were filled with 100 µl Hinton Broth solution, 100µl of an organic extract of *Rh. stellatus* was added to (C1, D1, G1, H1),

the same amount of aqueous extract of *Rh. stellatus* was added to (A1, B1, E1, F1) by micropipette and they were mixed very well, next 100 µl was transferred to the second column, then to the third and so on; until eleventh column, the 100 µl were discharged (positive control column No. 12). Finally, 1µl from *E. coli* diluted suspension was added to (E, F, G, and H). The same amount of *S. aureus* was added to (A, B, C, and D) rows except for the eleventh column (negative control column No 11). The plate was incubated at 35°C for 24 hours. After that, the last well in each row that appeared clear (not turbid) meant that no detectable growth of the bacteria.

In the same way, the wells were filled with 100 µl Hinton Broth solution, 100µl of an organic extract of *Rh. stellatus* was added to (C1, D1, G1, H1); the same amount of aqueous extract of *Rh. stellatus* was added to (A1, B1, E1, F1) by micropipette and mixed very well; then 100 µl was transferred to the second column, then to the third and so on, up to the eleventh column. The 100 µl was

discharged (positive control column No. 12). Then one µl of MRSA diluted suspension was added to (E, F, G, and H). The same amount of *P. aeruginosa* was added to (A, B, C, and D) row except for column No.11 (negative control column No 11). The plate was incubated at 35° C for 24 hours. After that, the last well in each row that appeared clear (not turbid) meant that no detectable growth of the bacteria (26, 27)

RESULTS

Phytochemical screening

Phytochemical characteristics of *Rh. stellatus* methanolic and n-hexane extracts are summarized in (Table 1). It is shown that flavonoids, glycosides, saponins, phenols, alkaloids, phytosterols and volatile oil were found in the extracts while reducing sugars, proteins, amino acids, starch, carbohydrates, and tannin were not detected in the aqueous extract. Due to the absence of tannin in both plant extracts, the total tannin test was not conducted.

Table (1): Phytochemical screening tests for methanol and n-hexane extract of *Rh. stellatus*.

Phytochemical classes	Test name	Methanolic extract results	n-hexane extract results
Protein and amino acids	Ninhydrin test	-ve	-ve
Carbohydrate & reducing sugars	Fehling's test	-ve	-ve
	Benedicts test	-ve	-ve
	Molisch's test	-ve	-ve
	Iodine test for starch	-ve	-ve
Glycoside	Liebermants test	+ve	-ve
	Keller-kiliani test	+ve	-ve
Flavonoid	Alkaline reagent test	+ve	+ve
Saponins	Frothing test	+ve	-ve
Phytosteroids	Liebermann test	+ve	-ve
Volatile oil	Copper acetate test	+ve	+ve
Phenolic compounds	Folin- ciocalteu test	+ve	-ve
Tannin	Ferric chloride test	-ve	-ve
Alkaloids	Wagner's test	+ve	-ve

Total flavonoid content

Total flavonoid content for *Rh. stellatus* methanol extract presented in (Table 2), as well as absorbance of standard compound Quercetin is shown in this table at different concentrations.

Flavonoid content in the extract was determined. The results of flavonoid content were expressed by mg/ml quercetin using the standard curve equation:

$$Y = 0.0034X - 0.019, R^2 = 0.9937$$

Table (2): Absorbance of standard compound (Quercetin) at $\lambda_{max} = 510$ nm.

Quercetin concentration (mg/ml)	Absorbance (mean value) at $\lambda_{max} = 510$ nm
10	0.01
30	0.08
50	0.17
70	0.22
100	0.32

Where Y is Absorbance at 510 nm and X is flavonoid concentration in the plant extract as illustrated in (Figure1).

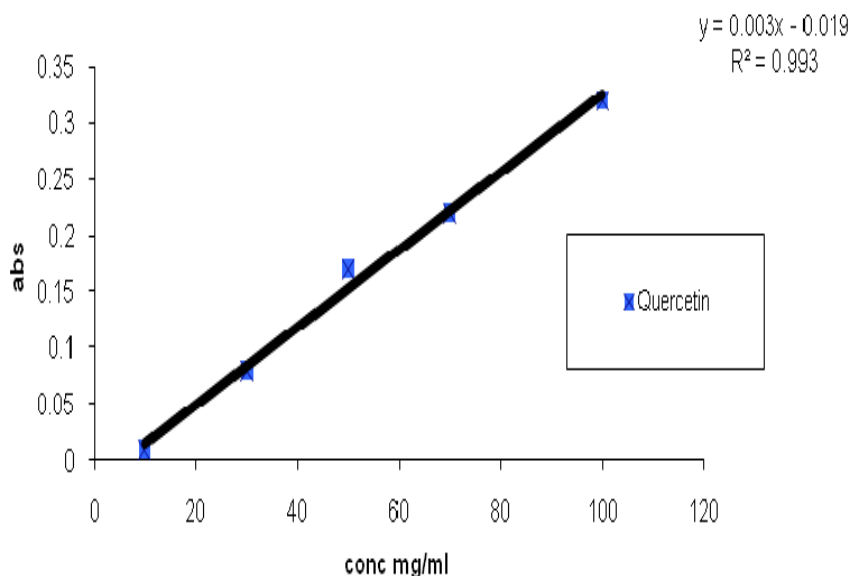


Figure (1): Standard calibration curve of Quercetin.

From standard calibration curve of quercetin, the total flavonoid content in *Rh. stellatus* was 18.35 ± 1.11 mg QUE/g plant extract.

Total phenolic content

The absorbance of standard compound (Gallic acid) at λ_{max} of 765 nm in *Rh. stellatus* is presented in (Table 3) and (Figure 2).

The results of total phenol content were expressed by mg/ml Gallic acid using the standard curve equation.

Table (3): Absorbance of standard compound (Gallic acid).

Gallic acid concentration (mg/ml)	Absorbance (mean value) at $\lambda_{max} = 765$ nm
0.01	0.156
0.02	0.199
0.03	0.256
0.04	0.364
0.05	0.411
0.1	0.764

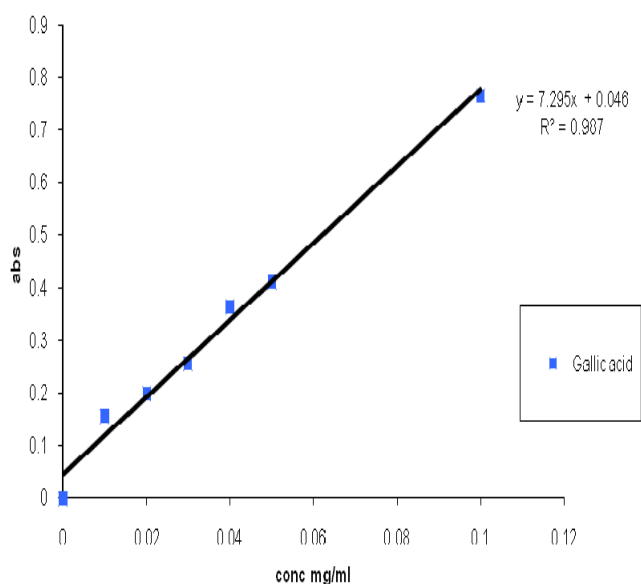


Figure (2): Standard calibration curve of Gallic acid, where Y is Absorbance at 765 nm and X is total phenol in the plant extract.

From standard calibration curve of Gallic acid, total phenol content in *Rh. stellatus* is 38.55 ± 0.95 mg GAE/g plant extract.

Antioxidant capacity

The free radical scavenging activity of the methanolic extract of *Rh. stellatus* has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 $\mu\text{g/ml}$. The zero

inhibition was considered for the solution, which contained only DPPH without any plant extract.

The result revealed a high antioxidant activity with $\text{IC}_{50} = 13.18$ $\mu\text{g/ml}$ which is comparable to Trolox standard at an IC_{50} of 1.85 ± 0.65 $\mu\text{g/ml}$. The detailed results are shown in (Table 4) and (Figure 3).

Table (4): % Inhibition activity for Trolox standard and *Rh. stellatus* aerial parts.

Concentrations ($\mu\text{g/ml}$)	% Inhibition by Trolox $\pm\text{SD}$	% Inhibition by <i>Rh. stellatus</i> $\pm\text{SD}$
1	36.96 \pm 0.79	21.57 \pm 0.91
2	68.12 \pm 1.43	22.3 \pm 0.45
3	69.88 \pm 1.65	30.9 \pm 0.76
5	80.88 \pm 1.4	31.4 \pm 1.13
7	80.88 \pm 1.33	34 \pm 1.43
10	81.22 \pm 0.98	52 \pm 1.43
20	86.23 \pm 1.11	65.2 \pm 1.32
30	86.23 \pm 1.32	65.2 \pm 0.65
40	86.23 \pm 1.43	70.1 \pm 0.65
50	94.5 \pm 1.12	70.43 \pm 0.98
80	95.31 \pm 1.65	70.4 \pm 0.94
100	95.6 \pm 1.54	73 \pm 1.22

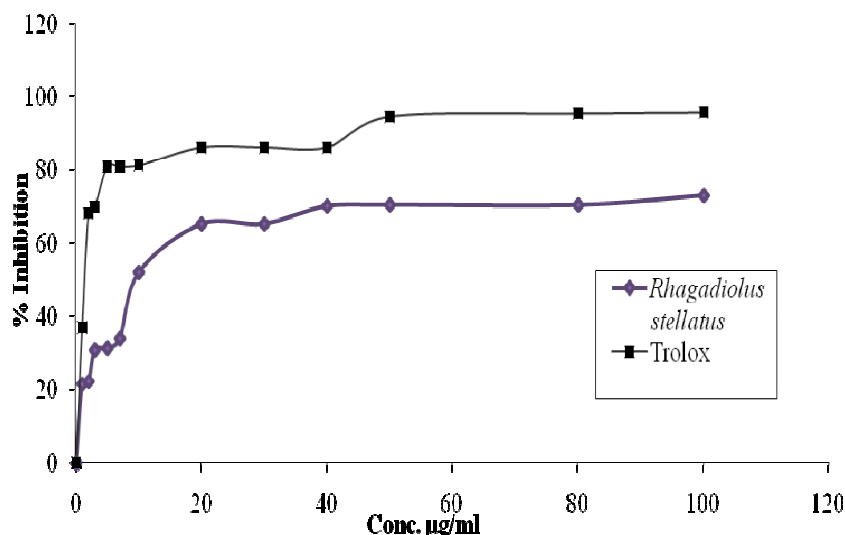


Figure (3): Inhibition activity of Trolox standard and *Rh. stellatus* aerial plant including *E. coli*, *P. arginosa*, *S. aureus* and MRSA pathogens (31-33).

Antimicrobial activity

The aqueous plant extract showed potential antibacterial activity under 50 mg/ml while the organic extract did not show any inhibition at the same concentration as shown in (Table 5).

Table (5): Minimum inhibitory concentrations (MICs) of *Rh. stellatus* aqueous extract.

Microorganisms	MIC (mg/ml)
<i>Staphylococcus aureus</i> (ATCC 25923)	6.25 mg/ml
MRSA	6.25 mg/ml
<i>Escherichia coli</i> (ATCC 25922)	12.5 mg/ml
<i>Pseudomonas arginosa</i> (ATCC 27853)	12.5 mg/ml

DISCUSSION

Wild plants extracts have been used for thousands of years in natural therapies, food preservation, pharmaceuticals, alternative medicine and food as well (28-30). It is essential to scientifically investigate those plants especially ones that have been used in folk medicine for the purpose of improving the quality of healthcare offered to human beings. Plants secondary metabolites are potential sources of novel antimicrobial compounds especially against bacterial pathogens; thousands of studies have been published on the antimicrobial activities of plant extracts against different types of microbes,

In-vitro studies in this work have shown that the plant crude extract had inhibited bacterial growth but at different degrees of effectiveness. It was found out that the aqueous extract of *Rh. stellatus* inhibited the growth of *E. coli* and *P. arginosa* at concentrations of 12.5 mg/ml. At the same time, this extract inhibited the growth of *S. aureus* (ATCC) and MRSA at concentrations of 6.25 mg/ml.

In summary, this study has shown that *Rh. Stellatus* aerial parts have powerful antibacterial and antioxidant activities. Further investigations are required to isolate and identified the biological active compounds in the *Rh. Stellatus* aerial parts.

CONCLUSIONS

The aerial parts of edible wild plant *Rh. Stellatus* in this study has exhibited potential antibacterial activity making them good candidates for manufacturing of novel drugs. Also, this plant species have shown potential antioxidant properties. Accordingly, it is concluded that this plant species can be used in food supplements and pharmaceutical industries; moreover, further investigations are required to prove its efficacy *in-vivo* studies.

CONFLICT OF INTERESTS

The authors report no conflicts of interest in this manuscript.

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