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Phenolic Compounds, Antioxidant and Antibacterial Activities of *Rhus flexicaulis* Baker

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Abstract

The genus *Rhus* (sumac), belonging to the family *Anacardiaceae*, is one of the most widespread and recognizable genera in North America and Africa. It is known to be rich in biflavonoids, urushiols and bichalcones. This study describes the identification and quantification of many phenolic compounds, for the first time, in *Rhus flexicaulis*, including seven flavonoids and seventeen phenols, using liquid chromatography-mass spectrometry (LC-MS). The total phenolic content was (30.31 mg/g), the total flavonoid content was (20.93 mg/g) and the total antioxidant capacity was (2005.17 µmol/g) in *Rhus flexicaulis*. The results showed that the plant extract has an antibacterial activity against *Streptococcus pyogenes* and *Escherichia coli*.

Keywords: Phytochemical, Phenolic compounds, Antibacterial, Antioxidants, *Rhus flexicaulis*.

1. Introduction

The genus *Rhus* (sumac), belongs to the family *Anacardiaceae*, including about 250 species, which occur mainly in the tropics, subtropics and temperate areas of the world, especially in North America and Africa (Gallant *et al*. 1998). The sumac name is derived from "sumaga", meaning red in Syriac (Wetherilt and Pala, 1994). In general, *Rhus* species can grow in non-agricultural regions and various species have been used by indigenous people for medicinal and other purposes, suggesting a potential for commercializing the bioactivity of these plants without competing for food production land uses (Van Wyk and Wink 2004). For example, *R. glabra* (smooth sumac) is traditionally used by native people of North America in the treatment of bacterial diseases such as syphilis, gonorrhea, dysentery, and gangrene (Erichsen 1989). *R. coriaria* is commonly used as a spice by grinding the dried fruits with salt, and is also widely used as a medicinal herb in the Mediterranean and Middle East, particularly for wound healing (Sezik *et al*. 1991). The genus *Rhus* is known to be rich in biflavonoids, urushiols and bichalcones (Masesane *et al*. 2000). Sumac extracts have been shown to exhibit a wide range of biological activities, namely antimalarial, antiviral, antimicrobial and antitumorigenic activities (Ahmed *et al*. 2001 and Choi *et al*. 2012).

Rhus flexicaulis is a densely pilose-tomentose, perennial shrub that reaches up to 2-3 m long. Leaves are compound with three suborbicular to ovate-elliptic, sessile leaflets with an apex of entirely rounded margins; the terminal leaflet is larger than the two lateral leaflets. Flowers are arranged in lax terminal panicles. The fruit is a brownish, glossy drupe. The plant is growing in South-east Egypt, Sudan and Arabia regions (Bolous, 2000).

Recently, the essential oil of *Rhus flexicaulis* was analyzed by Gas Chromatography Mass Spectrometry (GC-MS). It was found that the major constituents of essential oil are β-bisabolene, β-farnesene, β-curcumene and caryophyllene oxide. Four classes of compounds have been detected in the *R. flexicaulis* oil including monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. The phytochemical screening showed that *R. flexicaulis* contains moderate amounts of carbohydrates and / or glycosides, sterols, terpenes, flavonoids and tannins, while alkaloids, saponins, coumarines and anthraquinones were absent (Ibrahim *et al*. 2017).

This study is aimed at investigating the chemical constituents of this plant material collected from the field and its antibacterial activity in order to evaluate its potential uses and medicinal properties.

2. Material and Methods

2.1. Plant Collection and Preparation

The fresh aerial parts and of *Rhus flexicaulis* Baker were collected in March 2016 from Gebal Elba (Wadi Ma'arafai; south east of Egypt at about 600 m height, geographic position coordinates N: 27.5°22′12″; E: 20.2°36′20″) and were identified by the experts in Desert Research Center, Cairo, Egypt. A voucher specimen of the plant has been deposited at the Herbarium of Desert Research Center. The plants were stored in plastic bags under dark, chilled conditions during transportation to the

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laboratory. The plant samples were collected as leaves and stems (shoot system), then washed under tap water, and were air-dried at lab-temperature till constant weight. The plant samples were ground to fine powder to be used for chemical analyses. The plant material was brought to the Institute of Botany, Leibniz University Hannover, Germany.

2.2. Extraction of Phenolic Compounds

Fifty mg of the dried plant material was used. Extraction was performed three times with 2 mL 80 % Methanol (MeOH) using an overhead shaker and ultrasonic bath. After each extraction step, the samples were centrifuged for twenty minutes at 13,000 rpm, 10000 g and the supernatant was saved. The resulting supernatants were combined and centrifuged again for thirty minutes at 13.000 rpm to remove any suspended particles. The clear supernatant was used for further analysis (Dewanto *et al.* 2002).

2.3. Determination of Total Phenolic Content

Analysis of the total phenol content is based on a colorimetric measurement at 765 nm (Dudonné *et al.* 2009). A standard series of gallic acid (GA) was used for quantification. Each sample was measured as technical triplicate. Results were given as GA equivalents (GAE)/g dry weight. Twenty-five µL of extracted sample (1:10 diluted with H_2O) was incubated with 125 µL of "Folin-Ciocalteau" phenol reagent (1:10 diluted with water) for eight minutes at room temperature (RT). Exactly 125 µL of sodium carbonate (7.5 %) was added to the well and mixed by pipetting. The mixture was incubated for two hours in the dark. Absorbance was measured at 765 nm with a microplate reader (BioTek, Winooski, USA). The total phenol content was calculated by the factor estimated with the GA standard series.

2.4. Determination of Total Flavonoid Content

The extraction follows the procedure as described for the determination of total phenol content (Dewanto *et al.* 2002). Into each well 150 µL of deionized water was filled and 25 µL of a sample or one of the catechin hydrate standard solutions were added. Next 10 µL of a 3.75 % $NaNO_2$ solution was added, and the plate was gently shaken and afterwards incubated at room temperature for six minutes in darkness. After incubation, 10 µL of $AlCl_3$ (10 %) was added, and the plate is again gently shaken and incubated for five minutes at room temperature in the dark. After this step 50 µL of 1 M NaOH were added and the absorbance at 510 nm was measured using a microplate reader (BioTek).

2.5. Determination of Total Antioxidant Capacity (Oxygen Radical Absorbance Capacity) (ORAC)

The measuring system relies on the decrease of fluorescence over time. Fluorescein di-sodium salt was used as fluorophor, being slowly oxidized by 2,2'-azobis (2-methylpropionamidin) dihydrochloride (AAPH) (Huang *et al.* 2002, Gillespie *et al.* 2007). Depending on the amount of anti-oxidants in the sample, this process is decelerated. For quantification a calibration series with 6-hydroxy- 2, 5, 7, 8- tetramethylchroman-2-carbonic acid (Trolox™) was used. Results are given in Trolox™ equivalent (TE) pertaining to 1 g of dry weight.

The extraction follows the following procedure. In each well of a black, pre-cooled microtiter plate 120 µL of 112 nM fluorescein solved in 75 mM phosphate buffer (pH 7.4) was pipetted. Then 20 µL of trolox-standard solution, 20 µL of phosphate buffer (75 mM, pH 7.4) as blank were repeated three times on the plate, and then 20 µL of the samples have been added each in three replicates. The samples had to be diluted 1:50 with phosphate buffer. The plate was then placed into the pre-heated (37°C) microplate reader (BioTek) and incubated for fifteen minutes before fluorescence was measured at 485/520 nm. After the first measurement 80 µL of a 62 mM AAPH-solution in phosphate buffer was added to each well. Then the fluorescence at 485/520 nm was measured over the course of eighty minutes once every minute. The difference between each measurement was calculated and quantified using the standard row.

2.6. Identification of Phenolic Compounds by LC-MS

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed using the HPLC system (Shimadzu; Darmstadt, Germany) consisting of a controller (CBM-20A), two pumps (LC-20AD), a column oven (CTO-20AC) and a photo diode array detector (SPD-M20A). A Vertex Plus column (250 x 4 mm, 55 µm particle size, packing material ProntoSIL 120-5 C18-H) with pre-column (Knauer, Berlin, Germany) was used for sample separation.

Prior to analysis the samples were diluted 1:2 in 80 % methanol (LC-MS grade) and 10 µL was used. The temperature of the column oven was set to 30ºC. Ammonium acetate (2 mM) was added to eluents of water (A) and methanol (B). Both eluents had a flow rate of 0.8 mL min^{-1}. The gradient was applied in the following manner: starting with 10 % B, then switching linearly to 90 % B in thirty-five minutes, two minutes of 90 %, switching to 10 % B in one minute and the subsequent equilibration at 10 % B for two minutes. UV/Vis spectra from 190-800 nm were recorded. Components were injected into an AB Sciex Triple TOF mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA) following HPLC separation for identification. A temperature of 600°C was used for negative electrospray ionization. Mass spectra with the range of 100-800 Da were measured in the TOF range. MS/MS spectra from 50-800 Da at a collision energy of -30 were additionally recorded.

The phenolic acid and flavonoid standards for the identification and quantification were prepared in the same way. Peaks were compared by examining retention time and fragmentation pattern using the PeakView (SCIEX) software. Quantification of the identified compounds was done using a regression line generated by the software MultiQuant (SCIEX) that compares the area under the curve. Protochatechuic acid (PCS) was used as internal standard. For example, to quantify chlorogenic acid in *Rhus flexicaulis* extract, a quadratic regression line was formed using the following concentrations of the chlorogenic acid standards: 50 nM, 100 nM, 1 µM, 50 µM and 100 µM + 50 µM PCS. For vanillin and catechin, a linear regression line was formed using the following concentrations of the appropriate standard: 50 nM, 100 nM, 500 nM, 1 µM, 50 µM and 100 µM + 50 µM PCS and 10 nM, 100 nM, 1 µM and 10 µM + 50 µM PCS,

respectively. All standards were measured in the same way.

2.7. Antibacterial Assay

Disc diffusion method was used to determine the antibacterial activity of *Rhus flexicaulis* extract against *Streptococcus pyogenes* and *Escherichia coli* using 100 µL of suspension containing 10^8 CFU/mL of bacteria spread on Muller Hinton agar (Selim *et al*. 2013). The sterilized dried ethanol plant extract was loaded on filter paper discs to obtain a final concentration of 7.5 mg/disc. Loaded filter paper discs were placed on the top of Mueller-Hilton agar plates. Plated then incubated at 35°C for twenty-four hours to allow the diffusion of herbal plant extract. The presence of inhibition zones was measured by Vernier caliper, recorded and considered as indication for antibacterial activity. Negative controls were prepared using the same solvent employed to dissolve extract. After incubation for forty-eight hours at 37°C, the diameter of inhibition zones was recorded.

3. Results

3.1. Determination of Total Phenolic Content

Total phenolic content was examined with "Folin-Ciocalteau", a commonly used reagent for the estimation of the content of phenols in a sample. Results were expressed as gallic acid equivalents in (mg GAE g DW^{-1}). The results showed that the total phenolic content of *Rhus flexicaulis* was 30.31± 1.63 mg GAE g DW^{-1}.

3.2. Determination of Total Flavonoid Content

The total flavonoid content was determined following the same procedure described for the total phenol content. Results were expressed as catechine-hydrate equivalents in [mg g DW^{-1}]. The results showed that the total flavonoid content of *Rhus flexicaulis* was 20.93± 1.47 mg CE g DW^{-1}.

3.3. Determination of Total Antioxidant Capacity (ORAC)

The ORAC test is a standard test for total antioxidant capacity, standardized as Trolox™ equivalent (TE) in µmol/g. Data showed that the total antioxidant capacity of *Rhus flexicaulis* was 2005.17± 5.17 µmol TE g DW^{-1}.

3.4. Identification of Phenolic Compounds by LC MS

The LC-MS measurement resulted in data for the mass spectrum (ES^-), the retention time and the UV spectrum. These parameters have been compared to the standards and peaks found in the extract data. If both values accorded to each other, the compound was shown to be found in the plant extract (Table 1). The LC-MS analysis indicated that twenty-four compounds were present in the *Rhus flexicaulis* extract. These compounds include seven flavonoids namely kaempferol (1), quercetin (2), apigenin (3), catechin (4), epicatechin (5), isorhamnetin (6) and taxifolin (7), and seventeen phenolic acids namely cinnamic acid (8), gallic acid (9), ferulic acid (10), benzoic acid (11), gentisic acid (12), chlorogenic acid (13), p-coumaric acid (14), caffeic acid (15), P-Hydroxybenzoic acid (16), vanillic acid (17), vanillin (18), anisic acid (19), rosmarinic acid (20), pyrogallol (21), sinapic acid (22), syringaldehyde (23) and syringic acid (24) in *Rhus flexicaulis* for the first time. The total ion chromatogram is shown in figure 1.

The current results showed that phenolic acids were the major chemical constituents present in *Rhus flexicaulis*. Catechin was found to have the highest amount (34.99 µg/g), while Sinapic acid had the lowest amount (0.02 µg/g).

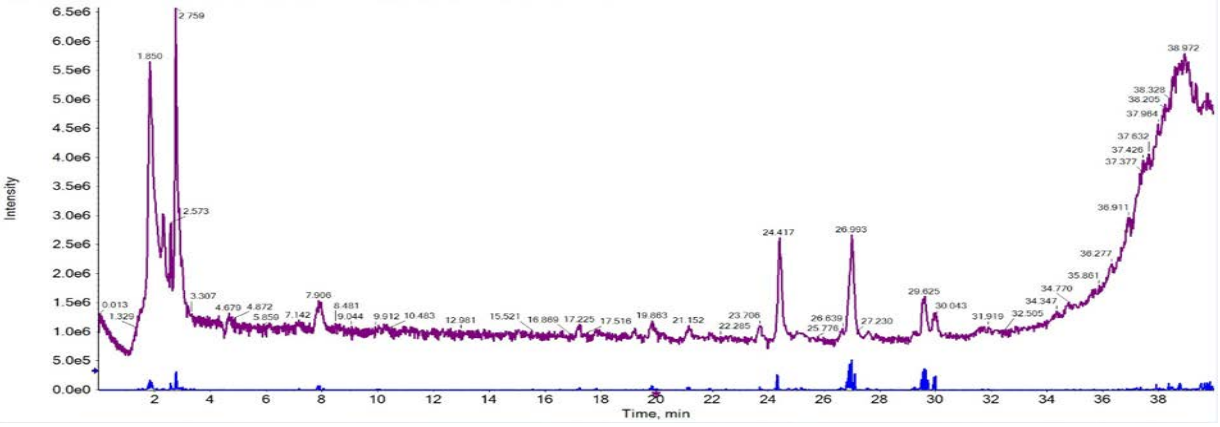

Figure 1. Total chromatogram of phenolic compounds of *Rhus flexicaulis*

Table 1: Phenolic acids and flavonoids detected in the Rhus flexicaulis extract. The identification was based on the m z -1 ratio and retention times compared to standards.

Peak No.	Compound	Mass	Retention Time (min)	$[MH]^-$	Chemical formula	Concentration(µg/g)
1	Catechin	290.27	12.3	289.2	$C_{15}H_{14}O_6$	34.99
2	Ferulic acid	194.1	16.5	193.1	$C_{10}H_{10}O_4$	0.30
3	Gallic acid	170.1	4.4	169.1	$C_7H_6O_5$	5.0
4	Apigenin	270.2	29.5	269.2	$C_{15}H_{10}O_5$	0.36
5	Gentisic acid	154.1	6.6	153.1	$C_7H_6O_4$	0.03
6	Chlorogenic acid	354.3	9.1	353.3	$C_{16}H_{18}O_9$	0.08
7	P-coumaric acid	164.1	15.7	163.1	$C_9H_8O_3$	0.25
8	Caffeic acid	180.1	12.2	179.1	$C_9H_8O_4$	0.33
9	Isorhamnetin	316.2	29.3	315.2	$C_{16}H_{12}O_7$	0.14
10	Quercetin	302.2	26.6	301.2	$C_{15}H_{10}O_7$	0.89
11	Cinnamic acid	148.1	22.2	147.1	$C_9H_8O_2$	0.03
12	Taxifolin	304.2	19.1	303.2	$C_{15}H_{12}O_7$	20.0
13	Epicatechin	290.2	15.4	289.2	$C_{15}H_{14}O_6$	0.50
14	Kaempferol	286.2	29.06	285.2	$C_{15}H_{10}O_6$	0.19
15	P-Hydroxybenzoic acid	138.1	10.08	137.1	$C_7H_6O_3$	0.09
16	Vanillic acid	168.1	11.1	167.1	$C_8H_8O_4$	2.54
17	Vanilin	152.1	16.9	151.1	$C_8H_8O_3$	1.90
18	Anisic acid	152	19.09	151	$C_8H_8O_3$	0.25
19	Rosmarinic acid	360.3	16.4	359.3	$C_{18}H_{16}O_8$	0.18
20	Pyrogallol	126.1	6.7	125.1	$C_6H_6O_3$	0.23
21	Sinapic acid	224.2	16.2	223.2	$C_{11}H_{12}O_5$	0.02
22	Syringaldehyde	182.1	17.4	181.1	$C_9H_{10}O_4$	1.82
23	Syringic acid	198.1	11.6	197.1	$C_9H_{10}O_5$	0.83
24	Benzoic acid	122.1	15.2	121.1	$C_7H_6O_2$	0.46

3.5. Antibacterial Activity

It was found that the average diameter of inhibition zones for the ethanolic extract was 19.51 and 37.3 mm on the growth of *Streptococcus pyogenes* and *Escherichia coli*, respectively.

4. Discussion

Data of this study showed that *R. flexicaulis* collected in Egypt contains appreciable amounts of total phenolic and total flavonoid contents, and a high total antioxidant capacity, suggesting the possibility to use this plant for medicinal purposes. The high antioxidant capacity might be attributed to the high amount of phenolics and flavonoids detected in this plant. Flavonoids are hydroxylated phenolic compounds that are synthesized by plants in response to microbial infections, and they have been found to be antimicrobial substances against a wide array of microorganisms *in vitro*. This activity may be ascribed to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Marjorie, 1996). Flavonoids have enhanced great interest recently because of their potential beneficial effects on human health such as antiviral, anti-diabetic, anti-inflammatory, antitumor, anti-carcinogenic and anti-ageing properties (Cook and Samman, 1996; Ren *et al*. 2003 and Zhou *et al*. 2009). It was declared that these compounds contribute to all the former biological activities via their strong antioxidant potential and free radical scavenger ability (Sharififar *et al*. 2009).

Phenolic compounds give the plant an important value as they exhibit a wide range of anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-oxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Middleton *et al*. 2000; Puupponen-Pimia *et al*. 2001 and Manach *et al.* 2005). Also, phenolic compounds could be a major determinant of antioxidant potentials of foods and could, therefore, be a natural source of antioxidants. Some phenolic compounds recorded in the study plant have medical importance such as P-coumaric acid is believed to reduce the risk of stomach cancer (Ferguson *et al.* 2005).

The results of the present study indicate the existence of antimicrobial activity in the crude extracts of *R. flexicaulis*. This may be due to presence of the phytochemical groups as previously mentioned. Several studies indicated that many *Rhus* species have antimicrobial activity. For instance, *R. coriaria*, has been proven to have a broad range of antimicrobial activity by inhibiting the growth of *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Shigella dysentariae, Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, Enterococcus faecalis*, and *Yersinia enterocolitica* (Nimri *et al.* 1999). It was also found that the *Rhus succedanea* leaf gall extracts have antibacterial activity against *Escherichia coli, Salmonella typhi, Micrococcus luteus*, and *Staphylococcus aureus* (Shrestha *et al.* 2013).

5. Conclusion

The results of the present study indicate that *Rhus flexicaulis* contains appreciable amounts of total phenolics and flavonoids, and a high total antioxidant and antibacterial activities, suggesting the possibility to use this plant for medicinal purposes This study provides valuable information for further phytochemical isolation and characterization studies of active compounds, necessary for the development of new drugs. The research efforts on *Rhus* extracts indicate a promising potential for the plant family to provide renewable bio-products with the following desirable bioactivities: antimicrobial, antifungal, antimalarial, antiviral, antifibrogenic, anti-inflammatory, antimutagenic, antioxidant, antithrombin, antitumorigenic, cytotoxic, hypoglycaemic, and leukopenic.

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