BIOLOGICALLY ACTIVE POLYPHENOLIC COMPOUNDS FROM *Acacia ehrenbergiana*

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ABSTRACT

A new acylated flavonol diglycoside, myricetin 3-*O*-(3'''-*O*-galloyl)-β-D-rutinoside (**1**) was isolated from the aerial parts of *Acacia ehrenbergiana* Hayne. Additionally, ten known compounds, namely gallic acid, methyl gallate, rutin 2''-*O*-α-L-rhamnopyranosyl, myricetin 3-*O*-β-D-rutinoside, rutin, myricetin 3- *O*-β-D-glucoside, quercetin 3-*O*-β-D-glucoside (isoquercitrin), myricetin, quercetin and catechin were isolated. The structure elucidation of the isolated compounds was established using chemical and spectroscopic methods of analysis including UV, MS, ^{1}H-, and ^{13}C-NMR. The *in vitro* antimicrobial evaluation of some isolated compounds showed that they have significant antibacterial activity against selected strains of Gram positive bacteria.

Key words: *Acacia ehrenbergiana*; Fabaceae; flavonoids; antimicrobial activity.

RESUMEN

Un nuevo flavonol diglicosido acilado, la mircetina 3-*O*-(3'''-*O*-galoil)-β-D-rutinosido (**1**), fue aislado de las partes aéreas de *Acacia ehrenbergiana* Hayne. Adicionalmente, fueron aislados diez compuestos conocidos: ácido galico, galato de metilo, rutina 2''-*O*-α-L-ramnopiranosil, miricetina 3-*O*-β-D-rutinosido, rutina, miricetina 3- *O*-β-D-glucosido, quercetina 3-*O*-β-D-glucosido (isoquercitrina), miricetina, quercetina y catequina. La elucidación estructural de los compuestos aislados fue establecida usando métodos de análisis químicos y espectroscópicos: UV, MS, ^{1}H-, y ^{13}C-NMR. La evaluación antimicrobiana *in vitro* de algunos de los compuestos aislados muestra que tiene una significativa actividad frente a cepas seleccionadas de bacterias Gram positivas.

Palabras clave: *Acacia ehrenbergiana*; Fabaceae; flavonoides; actividad antimicrobiana.

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INTRODUCTION

Acacia (Family Fabaceae) is a cosmopolitan genus containing in excess 1350 species (Seigler, 2003). *Acacia* species are used in folk medicine as antidiarrhoeic, antiamoebic, hypotensive, antidiabetic and anti-inflammatory (Boulos, 1983). Some *Acacia* species exhibit antimicrobial activity (Almagboul *et al.*, 1988). Little information is available in the literature about *Acacia ehrenbergiana* Hayne, which is native to Egypt. The antioxidant activity and the total phenolic contents of two different types of Yemeni honey (*Acacia ehrenbergiana* and *Acacia edgeworhi*) have been studied (Mohamed Al- Mamary *et al.*, 2002). Recently, the antimicrobial activity and the nitrogen fixation ability of *Acacia ehrenbergiana* have been studied (Younis *et al.*, 2003). While many antimicrobial agents from phytochemical studies have been introduced into therapy (Yan *et al.*, 2000; Fang *et al.*, 2000), however the field of pharmacology still needs extensive efforts for the development of new antimicrobial agents of superior activity and less toxic side effects as well as to overcome the highly resistant strains of microorganisms. The present study deals with the isolation and identification of polyphenolic constituents of *Acacia ehrenbergiana* as well as the antimicrobial activity of isolated compounds.

RESULTS AND DISCUSSION

The aerial parts of *Acacia ehrenbergiana* were exhaustively extracted with 70% ethanol. The resulted extract was then fractionated with ethyl acetate. The ethyl acetate extract was subjected to column chromatography on polyamide to give seven collective fractions, from which five compounds were isolated using successive CC on cellulose and/or sephadex LH-20. These fractionation processes led to the isolation of a new acylated flavonol diglycoside, myricetin 3-O-(3''''-O-galloyl)-β-D-rutinoside (**1**) along with ten seconda-ry metabolites for the first time from this plant. The UV spectral data, ^{1}H- and ^{13}C-NMR spectroscopic analysis confirmed the structures of the known compounds as gallic acid (**2**), methyl gallate (**3**) (Kia-Jin *et al.*, 2007), Quercetin 3-O-(2``,6``-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**4**) (Yinrong et *al.*, 2000), Myricetin 3-*O*-(6``-*O*-α-L-rhamnopyransyl)-β-D-glucopyranoside or myricetin 3- rutinoside (**5**), (Anna *et al.*, 1996, Yinrong *et al.*, 2000,Yinrong *et al.*, 2003), rutin (**6**), (Anna *et al.*, 1996), myricetin 3-*O*-β-D-glucoside (**7**), quercetin 3-*O*-β-D-glucoside (**8**), (Anna *et al.*, 1996, Yinrong *et al.*, 2003), myricetin (**9**), quercetin (**10**) (Mabry, *et al.*, 1970) and (+)- catechin (**11**) (Jeannette *et al.*, 1995).

Compound **1** exhibited chromatographic properties of a myricetin 3-*O*-glycoside structure (Mabry *et al.*, 1970) (R_f-values, dark purple spot on paper chromatography (PC), turned to dark yellow with ammonia vapors) and with the natural product spray reagent (Hierman, 1982). The UV methanol spectrum of **1** exhibited two maxima of the flavonol nucleus (band I at 370 and band II 265 nm) and extra absorption at 272, which could be diagnostic for the presence of galloyl function (Barakat *et al.*, 1999). On addition of NaOMe, a bathochromic shift with increase in the intensity of band I indicated the presence of free 4'-OH group. The bathochromic shift in band II with NaOAc was indicative of free 7-OH group. On the other hand, the strong bathochromic shift in band I (>60 nm) remaining after addition of HCl to $AlCl_3$ suggested the presence of free 5-OH and absence of free 3-OH group. The other shifts observed on addition of the different diagnostic shift reagents were in good agreement with the presence of free hydroxyl groups at C-5, 7, 3', 4' and 5' in the structure of **1** (Mabry *et al.*, 1970). On complete acid hydrolysis, gave gallic acid and myricetin in the organic hydrolysate, while rhamnose and glucose were detected

in the aqueous fraction (Co-PC, aniline hydrogen phthalate reagent). Accordingly, its structure was tentatively identified as myricetin 3-*O*-(galloyl)-rhamnosylglucoside or glucosylrhamnoside. In the aromatic region of the ^{1}H NMR spectrum, two singlets, each one integrated for two equivalent protons at δ 6.91 and 7.16, assigned to H-2'/6' and 2""/6"" of the B-ring and galloyl, respectively. Also the appearance of two *meta* doublets, at 6.35 and 6.17 (*J* =1.8), were characteristic to H-8 and H-6, respectively. A doublet at δ 5.38 (*J* =7.2) was assigned to a β-anomeric proton of a glucose moiety (H-1"), while an α-anomeric proton of a rhamonosyl moiety was detected at δ 4.4 as a broad singlet (H -1'"). The attachment of the galloyl ester at C-3'" of the rhamnosyl moiety was deduced from the down field shft of H-3'" at δ 4.13 (Δ + 0.54), assignable to the the galloylation of OH-3'". on comparison with that of compound (5). The configuration and conformation of the sugar moiety were identified as β-D-$^{4}C_{1}$-glucopyranosyl and α-L-$^{1}C_{4}$-rhamnopyranosyl according to the δ- and J-values of all other signals of H-1", H-1'", H-3'" as well as with the other signals of the sugar moiety (see experimental part or Table 1). These evidences were supported from ^{13}C-NMR, whereby the characteristic five signals of the galloyl moiety were assigned to C-7"",C-3""/C-5"", C-4"", C-1"" and C-2""/C-6"" at δ 166.0, 145.8, 138.8, 119.8 and 109.0, respectively. The galloyl group at OH-3'"was confirmed by downfield shift of C-3'" (72.3) and upfield of both C-4'" (71.0) and C-2'" (70.8) in comparison with the corresponding ones of the myricetin 3-rutinoside (**5)**, (Anna *et al.*, 1996, Yinrong *et al.*, 2000,Yinrong *et al.*, 2003). Also, the location of the rhamnopyranosyl moiety on C-6" of glucose was deduced from the characteristic downfield shift of C-6" signal at 68.6 and upfield of C-5" signal at 76.5 (Harborne and Mabry, 1982). The remaining C-signals (Table 1) were assigned according to a comparison study with the published data of structurally related compounds and of compound **5** (Agrawal and Banzal, 1989; Harborne and Mabry 1982). Thus, **1** was established as myricetin 3-*O*-(3'"-*O*-galloyl)-β-D-rutinoside.

Table 1.^{13}C-NMR spectral data for compounds 1 and 5

C-No.	1	5
glycone		
2	156.8	157.15
3	132.8	133.74
4	177.8	177.52
5	161.7	161.56
6	99.1	98.97
7	164.4	164.81
8	93.4	93.84
9	156.8	157.15
10	104.4	104.23
1'	120.5	120.39
2'	108.9	108.91
3'	146.0	145.72
4'	137.0	137.08
5'	146.0	145.72
6'	108.9	108.91
glucose		
1"	101.4	101.41
2"	74.4	74.29
3"	77.0	76.90
4"	70.5	70.94
5"	76.5	76.44
6"	68.6	67.45
rhamnose		
1'"	101.1	101.12
2'"	70.8	70.67
3'"	72.3	70.41
4'"	71.0	72.16
5'"	67.0	68.57
6'"	18.1	18.03
C-7"" (gallic Carbonyl)	166.0	
1""	119.8	
2"",6""	109.0	
3"",5""	145.8	
4""	138.8	

BIOASSAY

Potent antibacterial activity was observed against tested gram positive bacteria including *Bacillus subtilis, Micrococcus luteus, Bacillus megaterium, Staphylococcus aureus, Streptomyces sp.* and *Bacillus*

Table 2. Antimicrobial activities of the tested compounds expressed as size (mm) of inhibition zone.

		Compound						
	Name	1	2	3	5	10	Ampicillin	Nystatin
Gram positive bacteriana	1- *Bacillus subtilis* NRS-744	+	+ +	+++	+	+	+	-
	2- *Micrococcus luteus*	+	++	+	+	++	+	-
	3- *Bacillus megaterium*	+	+	++	+	+	+	-
	4- *Staphylococcus aureus*	++	+	+ +	+	+	+	-
	5- *Streptomyces sp*	++	+	++	+	+	+	-
	6- *Bacillus cereus* ATCC-9634	+ +	++	+	+	+	+	-
Gram negative bacteriana	7- *Serratia Mar*	-	+	+	-	-	+	-
	8-*Pseudomonas aeruginos* ATCC - 6NA10245	+	+	+	-	-	+	-
	9-*Escherichia coli* B- 3704	+	+	+	-	-	+	-
	10- *Salmonella sp.*	+	+	+	-	-	+	-
	11- *Pseudomonas sp.*	++	-	+	-	-	+	-
Fungi	12- *Sacharomyces cerevisia*	+	+	+	-	-	-	+
	13- *Candida albicans* IMRU3669	+	+	+	-	-	-	+
	14- *Aspergillus flavus* S-C 43 (3/3)	+	+	+	-	-	-	+

Diameter of the inhibition zones were: high (+++) (20-16 mm), moderate (++), (15-12 mm), slight (+), (11-1mm), no zone of inhibition (negative) (-).

cereus as assayed by disc susceptibility tests (Table 2) .

While **1, 2** and **3** revealed antimicrobial activity against all organisms under investigation, Gram negative organisms including *Serratia Mar, Pseudomonas aeruginosa, E. coli, Salmonella sp.* and *Pseudomonas sp.* revealed weaker susceptibility than Gram positive for most of the tested compounds. Weak responses were exhibited by the three fungi (Table 2), compounds **5** and **10** were incapable of inhibiting the growth of each of examined Gram negative bacteria and fungi. Commercially available antibiotics including ampicillin and nystatin were used a positive controls and for comparison of antibiotic efficacy. Further studies will elucidate the mechanism of action of these antibiotics to determine whether their activity is bactericidal or inhibitory to bacterial growth.

PLANT MATERIAL

Aerial parts of *Acacia ehrenbergiana* Hayne were collected from Aswan in March 2004 and identified by Dr. M. Younis, Botany Department, Faculty of Science, South Valley University, Aswan, Egypt. Voucher specimens (14792) are deposited at the Herbarium of National Research Centre, Cairo, Egypt.

EXTRACTION AND ISOLATION

Air-dried aerial parts of *Acacia ehrenbergiana* (600 gm) were defatted with petroleum ether (40-60º), then extracted on hot with 70% EtOH under reflux. The dry residue was extracted with EtOAc to give 57 g dry extract. The EtOAc extract was fractionated on polyamide 6 (Flucka) column and eluted with H_2O–MeOH with gradual increasing portions of MeOH, yielding seven fractions (I-VII). Fraction I (eluted with H_2O-10% MeOH, 3.5g) was found to be phenolic free on the basis of its chromatographic properties on PC. Fr. II (20% MeOH, 4 g) was applied on a microcrystalline cellulose (E. Merck) column and *n*-BuOH saturated with H_2O, then the two major subfrs. were separately fractionated on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column with EtOH as an eluent to give compounds **1** (12 mg) and **2** (23 mg). Fr. III (30-40% MeOH, 3.2 g) was chromatographed on Sephadex column for twice with *n*-BuOH-Isopropyl alcohol-H_2O (BIW, 4:1:5 organic layer), and then with EtOH to afford pure sample from compounds **3** (30 mg) and **4** (35 mg). Fr. IV (50% MeOH, 265 mg) was subjected to a cellulose column using 20-50% aqueous EtOH to give two major subfrs, which were in turn fractionated separately on a sephadex column with MeOH as eluent resulting in a pure sample of **5** (18 mg) and **6** (32 mg). Fr. V (60-70% MeOH, 680 mg) was separated on a sephadex column for twice with BIW and then 40% aqueous EtOH; the two major subfrs., thus obtained were separately purified once again with BIW *n*-BuOH-Acetic acid -H_2O (BAW, 4:1:5 organic layer), on preparative paper chromatography (PPC) to yieled pure samples of **7** (13 mg) and **8** (22 mg). Fr. VI (90-95%, MeOH, 159 g) was applied conc. MeOH soln. on preparative paper chromatography (PPC) and the two eluted bands were then separately purified once on a sephadex column and MeOH as an eluent, giving pure samples of **9** (11 mg), and **10** (8 mg). A part from Fr. VII (MeOH, 79 mg) was purified once on a sephadex column with EtOH for elution to give 10 mg pure sample from component **11**. All separation processes were followed up by 2D-PC and CoPC using Whatman No.1 paper with (S_1) (BAW) and S_2 (15% acetic acid) as solvent systems.

MYRICETIN 3-*O*-(3'''-*O*-GALLOYL)-β-D-RUTINOIDE (1)

Yellow amorphous powder. Chromatographic properties: R_f.-values, 0.1 (S_1), 0.64 (S_2), in systems BWA and 15% AcOH respectively, dark purple fluorescence under UV, changing to yellow with ammonia vapor. It gives orange-red with NP/PE [Diphenyl boric acid, Ethanolamine ester complex (Natural product reagent)] (Hiermann, A. 1982), and deep green with $FeCl_3$ spray reagents. UV spectral data λ_{max} nm; (MeOH): 354, 300 (sh), 269; $AlCl_3$: 426,304,273; $AlCl_3$ +HCl: 403, 359, 275; NaOMe: 332, 267, 256 sh, NaOAc: 358, 322, 272. Acid hydrolysis with 2N HCl gave myricetin (UV identical with pure material), gallic acid, rhamanose and glucose are identical with authentic samples. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.16 (2H, s, H-2'/6'), 6.91 (2H, s, H-2"/H-6"), 6.35 (1H, d, J =1.8 Hz, H-8),6.17 (1H, d, J =1.8 Hz, H-6), 5.38 (1H, d, J =7.2 Hz, H-1"), 4.40 (1H, br s, H-1'"), 4.13 (1H, br d, H-3'"), 3.70 (1H, br d, H-6"$_a$), 3.5-3.2 (remaining sugar protons hidden by H_2O-signals), 0.99 (3H ,d, J =6 Hz, CH_3-6'"). ^{13}C- NMR (75 MHz, DMSO-d_6): δ ppm are given in Table 1. Negative ESI-MS *m/z:* 778 [M-H] , 479.1 [M-galloylrhamnosyl]$^-$, 317 [myricetin-H], 316 [myricetin -2H], 299.3 [myricetin-OH]$^-$, 288 [myricetin-CO], 271.3[myricetin-2 OH] and 168.9 [galloyl-H]$^-$.

ANTIMICROBIAL ACTIVITY

Microrganisms

The microorganisms list species and genus for each and group alphabetically by gram status. No (1, 4, 6, 8, 9, 13 and 14) were obtained from the culture collection of the United States Department of Agriculture, Northern Regional Research Laboratory (Peoria, Illinois. USA), while others were obtained from Botany Department, South Valley University, Aswan, Egypt and were identified according to key given by Buchanan and Gibbon,1974. *Sacharomyces cerevisiae* was identified as ascosporogenous.

Media

For the disc-diffusion bioassay, nutrient broth containing 3 g/L beef extract, 5 g/L peptone and adjusted to pH 7.0 was used for cultivating the bacteria. Czapek-Dox's agar medium containing 30 g/L sucrose, 2.0 g/L $NaNO_3$, 0.5 g/L KCl, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.4 mg/L $FeSO_4$, and 20 g/L agar, adjusted to pH 7.0-7.2 was used for cultivating the fungi. Malt extract agar containing 20 g/L malt extract, 1 g/L peptone, 20 g/L dextrose, and 20 g/L agar, adjusted to a pH, 7.0-7.2 was used for cultivating the yeast.

Preparation of bacterial suspensions

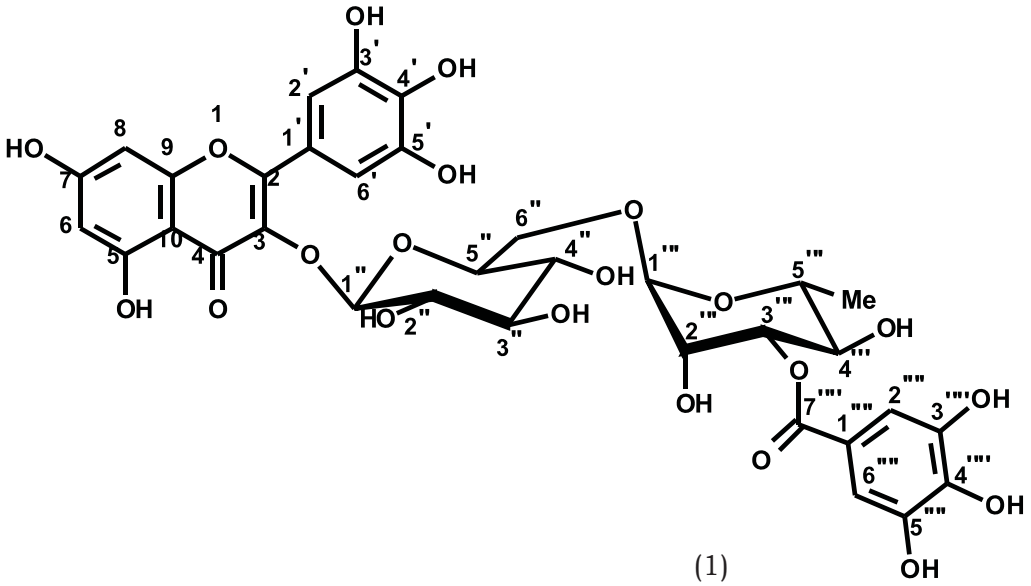
Suspensions of the microorganisms were prepared by suspending each bacteria in 5 ml sterile nutrient broth media, using a standard loop, then incubating the inoculated nutrient broth at 37 °C for 2h. One ml of each suspension was added to the center sensitivity testing plate. A sterile dry cotton wool swap was used to spread the inoculum on the media and then allowed to dry for a few minutes. Five compounds were tested as 200 µg/ml (W/V) solutions in sterile DMSO. Discs of 6 mm diameter of filter paper were placed in petri dishes (each one contains 10 discs) and then sterilized in a hot air oven at 180 °C for 1h. After cooling, 1 ml of the chemical solution was added onto each 10 discs to make 20 µg concentration per one disc. The discs were dried in the incubator at 35-37 °C, for 1h, or dried over phosphorous pentaoxide (P_2O_5) in a dissector under vacuum. Then distributed on the inocula by sterile forceps. Each disc should be pressed down on the medium and should not be moved once in place. The plates were incubated at 37 °C overnight. The diameters of the clear zones of inhibition were measured to the nearest 0.5 mm, compared to DMSO, under the same standardized conditions. The data obtained are expressed as size (mm) of inhibition zone. Diameter of the inhibition zones were: high (+++) (20-16 mm), moderate (++), (15-12 mm), slight (+), (11-1mm), no zone of inhibition (negative) (-).

Disc-diffusion method for three tested fungi

For the disc-diffusion method (Wilkins *et al.*, 1972), a standard concentration (100 µg/disc) of each of the five tested compounds was appropriately placed on the surface of an agar plate freshly seeded with standard inoculum of young culture (2 days old). The plates were kept at 5°C for 1 hr to allow diffusion of the compounds through the agar media. The plates of fungal test organisms were maintained at 30 °C for 48 hr. At the end of the incubation period, the inhibition zones were measured in mm.

Standard antimicrobial and antifungal:

The preliminary antimicrobial activity, was recorded in comparison to standard antibacterial ampicillin (10 mg/ml) and antifungal nystatin (10 mg/ml) in dist. water (Reeves and White, 1983).



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