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Phytochemical and Biological Studies of *Ruellia brittoniana.*

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ABSTRACT

Ruellia brittoniana (Acanthaceae) is herbaceous ornamental perennial plant, used for variety of purpose in traditional medicine. Flavonoids compounds, fatty acid methyl esters and fatty acids were isolated by chromatographic methods (CC, PTLC and GLC). Phytochemical study revealed the presence of 5, 2′,3′ trihydroxy 7-O-glucoflavone, 5,7,4′, trimethoxy 3-O-Rhamnoflavone and 2,2′,4′,6′-tetrahydroxy-chalcone . GLC analysis of the unsaponifiable matter of *Ruellia brittoniana* revealed the presence of 21 components. The main sterol is β-sitosterol. GLC analysis of fatty acid methyl esters revealed that myristic acid is the main fatty acid, while arachidonic acid is found to be the least concentration fatty acids. The herbal extract is study for anti-inflammatory and antioxidant effects.

Keywords: *Ruellia brittoniana*, Acanthaceae, phytochemical, flavonoids, fatty acids, biological.

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INTRODUCTION

Acanthaceae (or Acanthus family) is a taxon of dicotyledonous flowering plants containing almost 250 genera and about 2500 species. Most are tropical herbs, shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions. The main centers of distribution are Indonesia, Malaysia, Africa, Brazil and Central America [1]. Acanthaceae considered as one of the families which are rich in flavonoids. *Ruellia* is a genus of flowering plants commonly known as ruellias or wild petunias, some are used as medicinal plants, but many are known or suspected to be poisonous. Regardless, their leaves are food for the caterpillars of several lepidoptera (butterflies and moths). *Ruellia brittoniana* is considered to be antioxidant plants according to the reported constituents.

MATERIAL AND METHODS

Plant Material:

The aerial part of *Ruellia Brittoniana* (10 kg), family Acanthaceae was purchased from El-Orman Botanical Garden, Giza, Egypt in October 2010 and the plant was kindly identified by agricultural engineer Terease Labib, El-Orman Botanical Garden. The voucher specimen of dried sample has been deposited to the deptartment of pharmacognosy, Egyptian Russian University.

Material for biology

Raw murine macrophage (RAW 264.7) was purchased from the American type culture collections. Cells were routinely cultured in RPMI-1640. Media were supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, containing 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulphate, and 250 ug/ml amphotericin B. Cells were maintained in humidified air containing 5% CO_2 at 37ºC. RAW 264.7 cells were collected by scraping. All experiments were repeated four times, unless mentioned, and the data was represented as (Mean ± S.D.). All cell culture material was obtained from Cambrex, BioScience (Copenhagen, Denmark).

Extraction and isolation

The air dried plant (600g) macerated in 70% aqueous methanol with occasional stirring at room temperature three times. The combined methanol extracts were concentrated and dried under vacuum to give dried residue (55 g). The dried residue was subjected to column chromatography using column (70 cm L x 5 cm D) packed with Silica gel 60 as adsorbent. The elution process started with 100% chloroform and gradually increasing the polarity of the eluent till reaching 100% methanol. The isolated fractions purified by PTLC. The lipoidal matter obtained by the extraction of 60 g of the air dried aerial part of *Ruellia brittoniana* with light petroleum ether (60-80º) was evaporated to yield 2.4 g residue. About 1 gm of the light petroleum ether residue was saponified by refluxing with 30% alcoholic potassium hydroxide for 5 hours. After distillation of the alcohol and dilution with 20 ml water, the unsaponifiable matter (USM) was extracted with ether (4 × 50 ml). The residue left after evaporation of ether was 0.02 g. It was subjected to GLC identification of sterols and hydrocarbons content of the USM fractions. The aqueous mother liquors left after removal of the USM were acidified with 10% hydrochloric acid to liberate the corresponding free fatty acids. The liberated fatty acids were extracted with petroleum ether (4 × 50 ml). The fatty acids residue was methylated and analyzed by GLC.

Compound I

Yellow powder (20 mg), with blue color under UV and R_f 0.82 using solvent system (chloroform: methanol: water, 70: 30: 5). [1] H NMR spectra in methanol, (500 MHz), the data is listed in table (1). The data of UV spectra are listed in table (3). Positive ESI-Ms spectra of compound I exhibited a molecular ion peak at m/z 449.051 $[M+H]^+$.

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Table 1: [1]H NMR chemical shifts of flavonoids in methanol:

Cpd. I		Cpd. II		Cpd. III	
No. H	**δ H ppm**	**No. H**	**δ H ppm**	**No. H**	**δ H ppm**
6-H	6.6, d, *J* = 2 Hz	**6-H**	6.8, d, *J* =2 Hz	**H-3**	7.05, d, *J* = 8.3, 1.9 Hz
8-H	6.95, d, *J*= 2 Hz	**8-H**	7.2, d, *J* =2 Hz	**H-4**	7.38, m
4′-H	7.21, dd, *J* = 8.1, 1.5 Hz	**2′,6′-H**	7.69, dd, *J* =8.7 Hz	**H-5**	7.3, d, *J* = 7.6, 1.8 Hz
5′-H	7.61, dd, *J*= 8.1, 7.9 Hz	**3′,5′-H**	7.61, dd, *J* =8.7 Hz	**H-6**	7.68, dd, *J* = 8.7, 1.7 Hz
6′-H	7.71, dd, *J* = 7.9, 1.5 Hz	**4′-OMe**	3.56, s	**H-3′**	6.8, d, *J* = 2.3 Hz
1′′-glucose	5.2, d, *J* = 7.4 Hz	**5-OMe**	3.57, s	**H-5′**	6.7, d, *J*= 2.3 Hz
-	-	**7-OMe**	3.57, s	**H-α**	7.85, d, *J* = 15.7 Hz
-	-	**1′′ Rhamnose**	4.13, d, *J* =1.6 Hz	**H-β**	8.12, d, *J* =15.7 Hz
-	-	**Me-Rhamnose**	1.1	-	-

Compound II

Yellow amorphous powder (0.18 g), with purple color under UV and R_f 0.93 using solvent system (chloroform: methanol: water, 70: 30: 5). [1]H NMR and [13]C NMR spectra in methanol, (500 MHz), the data are listed in table (1&2). The data of UV spectra are listed in table (3). Positive ESI-Ms spectra of compound II exhibited a molecular ion peak at m/z 475.32 $[M+H]^+$.

Table 2: [13] C NMR chemical shifts of flavonoids in methanol:

Cpd. II				Cpd. III	
No. C	**δ C ppm**	**No. C**	**δ C ppm**	**No. C**	**δ C ppm**
2	145	**5′**	128.7	**2**	160
3	132.6	**6′**	129.29	**4**	132.1
4	176	**5-OMe**	56.3	**5**	121
5	164	**7-OMe**	55.6	**6**	129.17
6	98.08	**4′-OMe**	55.5	**co**	193
7	168.06	**1-Rh**	116	**2′**	167.5
8	96.2			**4′**	166
9	159			**6′**	161
10	105.86			**c-β**	132.2
1′	132.09			**c-α**	129.17
2′	129.29			**1′**	102.4
3′	128.7			**3′**	98
4′	130.16			**5′**	97

Compound III

Yellow powder (0.20 g), with yellow color under UV and R_f = 0.76 using solvent system (chloroform: methanol: water, 70: 30: 5). [1]H NMR and [13] C NMR spectra in methanol, (500 MHz), the data are listed in table (1&2). The data of UV spectra are listed in table (3). Positive ESI-Ms spectra of compound III exhibited a molecular ion peak at m/z 275.04 $[M+H]^+$.

Table (3) UV Spectral data (λ_{max} nm) for isolated compounds

	compound I	compound II	compound III
MeOH	256,268sh,349	259,270sh,356	253, 311sh, 370
NaOAc	260,266sh,366sh,404	263,295sh,372	272, 346, 412sh
$NaOAc/H_3BO_3$	260,371	265,295,378	274, 312sh, 374
$AlCl_3$	275, 297 sh, 330, 435	263, 271sh, 358	270, 384, 424
$AlCl_3$/HCL	274, 293sh, 357, 386	262, 272sh, 355	270, 384, 424

Biological Screening

Assay for anti oxidants is based upon the use of the stable free radical 2, 2-diphenyl-1-picryl hydrazyl (DPPH) which is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole. The delocalisation also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present)[2-4]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers.

In addition, they have a metal chelation potential. In our body, a number of biochemical reactions involves the generation of reactive oxygen species (ROS). Under normal conditions, the balance between the generation and diminishing of ROS is controlled by the antioxidant defense system, which includes both enzymes with antioxidants activities and non-enzymatic factors. The former includes superoxide dismutase, catalase and glutathione peroxidase. The latter includes some low molecular weight compounds such as antioxidant vitamins (ascorbic acid, tocopherol and carotenoids) and other antioxidant micronutrient from diet [5]. This study is deal with the in vitro determination of its antioxidant activity using the stable free radical DPPH. Study of anti-inflammatory is based on nitrite accumulation used as an indicator of nitrous oxide (NO) production using a microplate assay based on the Griess reaction. The Griess reaction is based on a two-step diazotization reaction in with acidified nitrites generate a nitrosating agent that reacts, which sulfanilic acid to form diazonium ion. This ion is then coupled to N- (1- naphthyl) ethylenediamine to produce the chromophoric pink azo-derivative that can be determined spectrophotometrically at 540 nm[6].

RESULTS AND DISCUSSIONS

Identification of unsaponifiable and saponifiable matters (fatty acid methyl esters):

GLC analysis of the unsaponifiable matter, table (4), of *Ruellia brittoniana* revealed the presence of 21 components. The main sterol was β-sitosterol (8.51%). GLC analysis of fatty acid methyl esters revealed that Myristic acid (57.25%) is found to be the main fatty acid, while Arachidonic acid (0.35%) is found to be the least concentration fatty acids, table (5).

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Table 4: GLC analysis of hydrocarbons and sterols of *Ruellia brittoniana*

Hydrocarbons and sterols					
RT	**%**	**Components**	**RT**	**%**	**Components**
14.697	0.43533	Tetradecane	25.703	2.36479	Hexacosane
15.514	0.48497	Pentadecane	26.590	3.19923	Heptacosane
16.097	0.25008	Hexadecane	27.591	2.56945	Octacosane
17.830	1.74648	Octadecane	28.383	3.37349	Nonacosane
19.222	2.28452	Nonadecane	28.744	3.00238	Triacontane
20.042	1.26856	Eicosane	30.828	2.59901	Cholesterol
21.071	1.60377	Heneicosane	31.942	2.37175	Campasterol
22.051	1.81139	Docosane	32.495	3.15181	Stigmasterol
23.006	3.22973	Tricosane	34.214	8.5122	β -sitosterol
23.588	4.74047	Tetracosane	38.644	1.55130	α - amyrine
24.827	6.49394	Pentacosane			

Table 5: GLC analysis of fatty acid methyl esters of *Ruellia brittoniana*

Fatty acid methyl esters		
RT	**%**	**Components**
12.432	57.25838	Myristic acid
15.669	1.28817	Palmetic acid
17.354	1.87772	Stearic acid
18.916	5.11215	Oleic acid
20.797	0.84011	Linoleic acid
22.775	0.55732	Linolenic acid
24.838	0.35367	Arachidonic acid
27.269	1.79234	Lignoceric acid
32.734	16.02539	Hexadcosanoic acid

Identification of flavonoid compounds:

The ^{1}H NMR spectra of compound I revealed the presence of metacoupled doublets at (δ 6.6 and 6.95), each integrating for one proton, were assigned to H-6 and H-8, respectively. The three double of doublets at (δ 7.615, 7.714 and 7.21) corresponding to (H-2′, H-3′, and H-4′), The anomeric proton at δ 5.2 of glucose with 7.4 Hz coupling constant indicating β-configuration and It was confirmed by direct comparison with authentic sample after acid hydrolysis. UV spectra in methanol, showed two characteristic absorption bands at λ_{max} 349 nm band I and 256 nm band II, no bathochromaic shift in band II on addition of NaOAC was diagnostic for occupied 7-OH group. The effects of the remaining UV-shift reagents and ^{1}H NMR data are agree with 5, 2′, 3′ trihydroxy 7-O-glucoflavone. Positive ESI spectra of compound I exhibited a molecular ion peak at m/z 449.051 $[M+H]^{+}$, corresponding to M. wt of 448.14 and molecular formula $C_{21}H_{20}O_{11}$ to support the evidence of 5, 2′, 3′ trihydroxy 7-O-glucoflavone. Thus by comparing the obtained data ^{1}H NMR, UV Spectra and Positive ESI- MS with reviews reported. Compound I was identified as: 5, 2′, 3′ trihydroxy 7-O-glucoflavone. The best of our knowledge it is isolated for the first time from family Acanthaceae[7].

The ^{1}H NMR spectra of compound II revealed the presence of three singlets at (δ 3.56, 3.57 and 3.57) corresponding to three aromatic methoxyl groups, were assigned to OMe-4′, OMe-5 and OMe-7, respectively. Two metacoupled doublets at (δ 6.8 and 7.2), each integrating for one proton, were assigned to H-6 and H-8, respectively. The paracoupled double doublets at (δ 7.61 and 7.69) corresponding to four protons at positions (H-2′, H-6′) and (H-3′, H-5′), and the anomeric proton at δ H 4.13 for sugar moiety (rhaminose). It was confirmed by direct comparison with authentic sample. UV spectra in methanol, showed two characteristic absorption bands at λ_{max} 356 nm band I and 259 nm band II, no bathochromaic shift in band II on addition of NaOAC was diagnostic for occupied 7-OH group. The UVdata, ^{1}H NMR data and ^{13}C NMR data are agreed with 5, 7, 4′ trimethoxy 3-O-Rhamnopyranosid. Positive ESI spectra of compound II exhibited a molecular ion peak at m/z 475.32 $[M+H]^{+}$,corresponding to M wt of 474.18 and molecular formula $C_{24}H_{26}O_{10}$ to support the

evidence of 5, 7, 4′ trimethoxy 3-O-Rhamnopyranosid. Thus by comparing the obtained data ^{1}H and $^{13}CNMR$ with those reported [8-10]. Moreover UV Spectra and Positive ESI- MS, Compound II was identified as: 5, 7, 4′ trimethoxy 3-O-Rhamnopyranosid, and for the best of our knowledge it is isolated for the first time from family Acanthaceae.

The ^{1}H NMR spectrum of compound III showed a pair of AB doublets (J=15.7 Hz) at δ 7.85 and 8.12 consistent with trans-olefinic protons of a chalcone moiety[11]. A set of meta-coupled doublets (J=2.3 Hz) at δ 6.8 and 6.7, each integrating for one proton, were attributed to H-3′ and H-5′. The β-carbon in C-2 unsubstituted chalcones usually resonates around 144 ppm. However, in compound III it appeared at132.2 ppm, which is unusually upfield, indicating the presence of C-2 oxygenation[12], and this mean presence of hydroxyl group due to the absence of data for methoxy in [13]C and [1]H NMR, The presence of four aromatic proton signals at δ 7.05, 7.38, 7.3 and 7.68 in the [1]H NMR spectrum of III were assigned to protons at 3, 4, 5 and 6 positions of ring B, respectively. UV spectra in methanol, showed two characteristic absorption bands at λ_{max} 370 nm band I and 253 nm band II of chalcone nucleus, on addition of NaOAC, bathochromaic shift in band II was diagnostic for free 4'-OH group. The effects of the remaining UV-shift reagents, [1]H NMR data and ^{13}C NMR data were agreed with 2, 2′, 4′, 6′-tetrahydroxy-chalcone. Positive ESI spectra of compound III exhibited a molecular ion peak at m/z 275.04 $[M+H]^{+}$, corresponding to M wt of 274.09 and molecular formula $C_{15}H_{14}O_5$ to support the evidence of 2, 2′, 4′, 6′-tetrahydroxy-chalcone. Thus by comparing the obtained data [1]H and [13]C NMR with those reported from Andrographis paniculata[13]. Moreover UV Spectra and ESI- MS, The structure of compound III was elucidated as 2, 2′, 4′, 6′-tetrahydroxy-chalcone and for the best of our knowledge it is isolated for the first time from genus *Ruellia*.

Biological activities

Ruellia brittoniana is considerable to be among antioxidant effective plants according to the reported constituents. The sample concentration was 100µg/ml and it gives 4.2% antioxidant activity using positive control and vitamin C with Sc 50 value 4.8µg/m**.** The rise in the interest in role of antioxidants in human health has prompted research in the fields of food science and horticulture to assess fruit and vegetable antioxidants[14]. Accordingly, supplement of dietary antioxidant vitamins and micro-nutrients may enhance the antioxidant capacity of the body. This will help to attenuate the damage of the body induced by oxidative stress, thus can be used as potential therapeutic or preventive drugs for disease associated with oxidative stress. Recently, the antioxidant properties of natural flavonoids and related phenolic compounds extracted from dietary of herb plants have aroused much attention. There are approximate 5000 known phenolics of plant origin and model studies have demonstrated that many of them have antioxidant activity. The 70% methanolic extract of the aerial parts of *Ruellia brittoniana* cultivated in Egypt showed an antioxidant effect 4.2% at 100 µg/ml concentration level against the stable free radical DPPH.

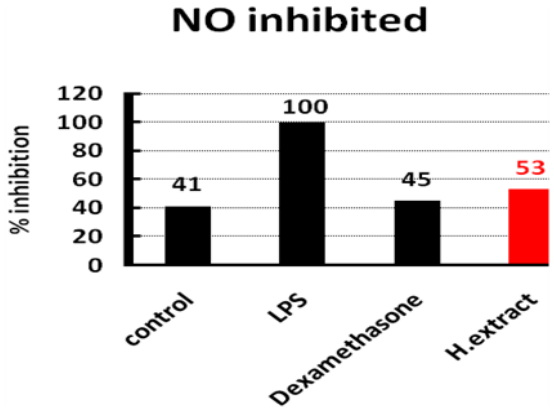

Figure (2) The percentage of inhibition of Nitric oxide in LPS-stimulated RAW 264.7 cells supernatant after the treatment with the samples (50µg/ml) for 24 hours compared LPS treated cells, as measured by Griess assay.

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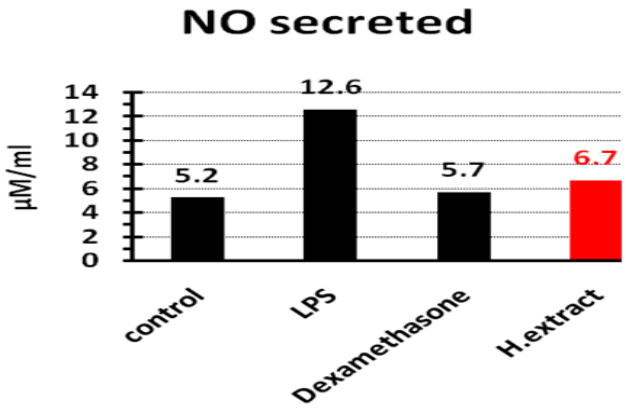
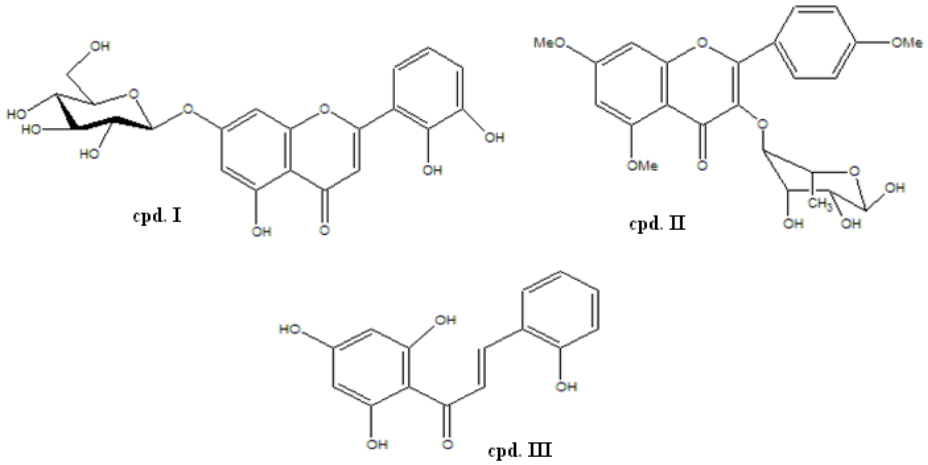


Figure (1): The level of Nitric oxide in RAW 264.7 cells supernatant after the treatment with the samples (50µg/ml) for 24 hours compared with LPS-treated cells (100µg/ml), as measured by Griess assay.



The anti-inflammatory activity, a standard curve relating NO in µM of sodium nitrite to the absorbance was constructed. From which the NO level in the cell supernatant is computed by interpolation. The results indicated that LPS (100 µg/ml) induced nitric oxide production up to 140 % more than the control, while that the potent anti-inflammatory Dexamethasone (50 ug/ml) inhibited nitric oxide production to 5.7 µM/ml compared to 12.6 µM/ml of that of the LPS with level of 45 % inhibition, very close to the control cells with 5.2 µM/ml NO. The herbal extract showed anti- inflammatory effect as shown in the amount of NO produced with a level of 6.7 µM/ml leading to 53 % inhibition, in comparison to the potent anti-inflammatory drug Dexamethasone (45 % inhibition), it is close to its inhibitory effect (fig. 1&2).

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