Phytochemical and Microbiological studies of *Petreavolubilies*L

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Abstract: The aim of the present study was to evaluate the phytochemical screening of Petreavolubilies L for volatile oils, polyphenols and/or tannins, sterol and/or triterpenes, flavonoid aglycones and/ glycosides, carbohydrates and /or glycosides, and iridoid glycoside It is free from anthraquinone glycosides, alkaloid, saponins, resins and oxidase enzyme. Phenylethanoidsverbascoside, eukovoside and cistanoside D were isolated and identified by co-chromatographic and specteoscopic methods. Antimicrobial properties of n-butanol fraction and pure compound were evaluated against Escherichia coli ATCC 14169, *Pseudomonas aeruginosa* ACCT 9027, *Staphylococcusaureus* ATCC 6538, *Bacillus subtulis* ATCC 6633, *Micrococcus leutus* ATCC9341, *Aspergillusniger* and *Candida albicans* ATCC 10231 by the disc diffusion method.

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Keywords: verbascoside, eukovoside, cistanoside-D and antimicrobial properties.

1. Introduction

Iridoids have interesting and multidirectional pharmacological properties, as purgative, antibacterial, antifungal, ant hepatotoxic, hypotensive, hypoglycemic, antitumor, antioxidant, and immune-stimulating activities (Jain, 1977, Jianun and Guiqui, 1997). An impressive number of modern drugs have been isolated from natural sources; many of them have been used in traditional medicine. Plant-based traditional medicine system plays an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicine for their primary health care (Owolabiet et al., 2007).

According to World Health Organization, medicinal plants would be the best source to obtain a variety of drug. Therefore, medicinal plants should be investigated to better understand their properties, safety and efficacy (Nascimento et al., 2000).

The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Various studies have indicated that there are many substances such as peptides, unsaturated long chain aldehydes, alkaloidal constituents, some essential oils, phenols, ethanol, chloroform, methanol and butanol soluble compounds are found and isolated from some plants. These compounds are emerged with their potentially significant therapeutic application against human pathogens, including bacteria, fungi and virus (El Astal et al., 2005 and Doughari et al., 2008).

A *Petreavolubilies* L. (Verbenaceae, local name, nilmonilota) is a vinous plant found in a scattered manner in various regions of the world. It is comparatively little known, and there are a few scientific studies on its pharmacological properties and phytochemical constituents. *P. volubilies* is used in the folk medicinal system of Bangladesh for treatment of diabetes *P. volubilies* L can be used as a source of antibiotic substances for possible treatment of bacterial and fungal infections, including gonorrhea, pneumonia, urinary tract and some mycotic infections (Gouda, 2009.), ant-hyperglycemic potential of leaves of *P volubilies* showed dose-dependent and significant reduction of serum glucose levels in mice (MahabubaRahman et.al., 1995). The plant is comparatively little known and there is a total absence of any significant scientific studies on its pharmacological properties, and phytochemical constituents in Egypt. The aim of the present study was to evaluate the phytochemical screening of *Petreavolubilies* L., regarding volatile oils, polyphenols, sterols, flavonoid aglycones, carbohydrates and /or glycosides, and iridoid glycoside.

2. Materials and methods

The column Chromatography (CC) was performed with Silica gel 30- 70 (Merck), and/or Polyamide 6, bulk density: 0.25 g/ml, particle size 50-160 µm (Fluka). Analytical and preparative thin layer chromatography (TLC) were carried out on silica gel 0.2 mm layer thickness of Schleicher &Schuell (F 1500/LS 254 20 × 20 cm) and Silica gel 60 F 254 (Merck). Nuclear magnetic resonance (NMR) spectra were recorded on 500MHz, D2O (Oxford 500 NMR spectrometer, National Research Center, Giza, Egypt,

2.1. Plant material

Aerial parts of the *Petreavolubilies L* were collected from El-Zohrea Zoo in Giza, Egypt, in June 2009, during the flowering stages. Voucher specimens were deposited at Herbarium of Phytochemistry

Department, Applied Research for Medicinal Plant Center (NODCAR).

2.2. Extraction and Isolation

Isolation of compounds I &II and III from *Petreavolubilies*

The air-dried and powdered aerial parts of P. volubilies (500g) were extracted, twice with MeOH 80% (2 × 2 l) at 45°C in a percolator. The combined methanolic extracts were evaporated until dryness in a vacuum (25 g, yield 5 %), suspended in H_2O and partitioned successively, between ether, ethyl acetate and n- butanol, respectively. The n-BuOH fraction (7 g) which was fractionated over polyamide 6 (CC) and eluted with H_2O-MeOH gradient (0-100% MeOH) yielded four main fractions. Fraction B2 wassubjected to silica gel (HPTLC), solvent system MeOH: EtOAc: $CHCl_3$ (1: 0.5: 0.5) to give compounds I, II and III (30, 25, 20mg, respectively).

2.3. Antimicrobial studies

The effect of n-butanol fraction extracted from Petrea was used to assay the antimicrobial activity. Different Gram positive (*Micrococcus leutus* ATCC9341), *Staphylococcus aureus*, ATCC 6538, and *Bacillus subtulis*, ATCC 6633), and Gram negative (*Escherichia coli*, ATCC 14169, *Pseudomonas aeruginosa,* ACCT 9027), yeast (*Candida albicans*, ATCC 10231) and mold (*Aspergillusniger*) were used. The micro-organisms were kindly supplied from the Microbiology Department of the Applied Research Center for Medicinal plants, (NODCAR) Cairo, Egypt. The cultures were stored in refrigerator at 5 ^oC, and reactivated on a suitable media for each microorganism.

2.3.1. Preparation of the microbial suspension

The cultures were grown in nutrient broth (Difco, MI,USA) for 48 hrs at 37^{o}C. Serial dilutions in sterile saline solution were prepared to obtain a suspension containing 10^5 cell/ml. Fungi were grown on slants of Sabouraud dextrose agar (SDA) medium, and incubated at 28 ^oC for 7 days. Spores were harvested by adding sterilized solution (Tween 80, 0.42 v/v), and filtered through several layers of cotton sheet. The number of spores was estimated by hemocytomter and suspension was adjusted to contain ~ 10^5spore / ml (Padwal et al., 1976).

2.3.2. Agar diffusion method (Perez et al., 1990)

The tested organism was spread plated onto the surface of an appropriate nutrient agar medium. Four cups of 1 cm diameters were placed in each plate, three cups impregnated with different concentrations of the extract (0.0, 200 and 400 ppm), and one for the control solution, (diethyl sulphoxide, DMSO). The plates were incubated at 37 ^oC for 24 hrs, for bacteria and 28 ^oC for 5 days for fungi. The plates were examined for inhibition zones the lowest concentration of the extracts required to inhibit the growth of the tested microorganism was distinguished as the minimum inhibition concentration (MIC).

2.3.3. The disc agar diffusion method (Bauer et al., 1966)

This method was used for testing antimicrobial activity of the volatile constituents and pure compounds. Volatile oil was dissolved in diethylether 0.1 %, while the pure compounds in DMSO 0.1 %, and 4 mm discs of filter paper (Whitman No. 0.42) were impregnated with each compound. The discs were placed onto the surface of appropriate culture seeded with the tested microorganisms, and the plates were incubated at 37 ^oC for 24 hrs, for bacteria and at 28 ^oC for 5 days for fungi. The plates were examined for inhibition zones, the lowest concentration required to inhibit the growth was distinguished as the minimum inhibition concentration (MIC).

3. Results and discussion

Preliminary phytochemical screening of *Petra* shows that it contains volatile oil, polyphenols and/or tannins, sterols and/ or triterpenes, flavonoidaglycones, carbohydrates and /or glycosides, and iridoid glycoside. However it is free from anthraquinone glycosides, alkaloid, saponins, resins and oxidase enzyme. There compounds of phenylethanoids were isolated from *Petreavolubilies* L, and the phytochemical properties of each compound were discussed.

Compound 1

Compound 1 is yellow amorphous powder with melting point ranged from 149-151°C, UV λ max nm (logε) methanol (MeOH): 340 (0.67), 330 (0.72) and 290 (0.54); + NaOH: 380 (0.91), 300 (0.35) and260 (0.24); + AlCl3: 361 (0.53), 320 (0.37), and 268 (0.42); + AlCl3/HCl: 330 (0.53), 243 (0.35) and 220 (0.62). ^{1}H-NMR and C13-NMR spectral data (500 MHz, DMSO - d 6), for the compounds are shown in Table (1). NMR data with the results obtained by Co-chromatography with reference samples (different monosaccharaides and phenolic acids), suggest that this compound has three posseses:1) the structure 2-(3,4-dihydroxy-β-dihydroxyphenyl), 2) ethanol-1-O-α-L-rhamnopyranosyl-(1→3)-β-D-(4-O-caffeoyl) , and 3) glucopyranoside, verbascoside, acteoside and kusaginin, respectively (El-Hela et al.,2008, and Owen et al.,2003). Reviewing the current literature, the compound verbascoside was isolated before (Figure 1) from Verbena supine L. (Ramdan, 2008 and Gouda, 2009).

Compound 2

Compound 2 is yellowish-green amorphous powder its UV spectrum (EtOH) at λ max 322, 228 and 202 nm with the strong bathochromic shift at 385 nm. Addition of sodium acetate (EtONa) indicates, the presence of phenolic hydroxyl groups, in the molecule

NMR spectral data (500 MHz, DMSO - d 6), (Table,2). [1]H-NMR, C13-NMR data together with the result obtained by Co-chromatography with reference samples (different monosaccharaides and phenolic acids) suggests that the compound 2 possesses the structure4'''-O-methyl2-(3,4-dihydroxy-β-dihydroxyphenyl) ethanol-1-O-α-L-rhamno-pyranosyl-(1→3)-β-D-(4-O-feruoyl), glucopyranoside (Eukovoside), as showing in Fig. (1). Reviewing the current Literature compound, Eukovoside was isolated before from different species of Verbena (El-Hela et al., 1998 and 2008, Lahloub et al., 1990, Owen et al., 2003 and Gouda, 2009).

Compound 3

Compound 3 is yellowish-green amorphous powder, the TLC spot acquired brownish-red color after heating for 1 min, at 105oC. UV: MeOHλmax; 322,228, 202; (NaOMe): λ max 385. NMR spectral data are shown in Table (3), H1 NMR, C13 NMR data together with the result obtained by Co-chromatography with reference samples (different monosaccharides and phenolic acids) suggests that the compound 3 possesses the structure 3, 3'''-O-dimethyl 2-(3,4-dihydroxy-β-dihydroxyphenyl) ethanol-1-O-α-L rhamnopyra-nosyl-(1→3)-β-D-(4-O-caffeoyl) glucopyrano- side (Cistanoside D) as showing in Fig. (1). Reviewing the current literature the compound was not isolated before from different species of Verbena (El-Helaet al., 1998 and Gouda, 2009).

Table 1. ^{1}H and ^{13}C-NMR spectral data of compound 1

Position	^{1}H(J in Hz)	^{13}C
Aglycone		
1	-	131.11
2	6.66 d,(2.4 Hz)	112.30
3	-	146.15
4	-	146.43
5	6.63 d, (7.8 Hz)	116.38
6	6.49 dd, (7.8/2.4 Hz)	119.38
β	2.73 m, (6.9 Hz)	35.02
α	3.69 m, 3.82 m	70.57
Ferulic acid		
1	-	124.50
2	7.03 d, 2.0 Hz	111.09
3	-	146.63
4	-	148.12
5	6.78 d, (8.1 Hz)	112.45
6`	6.81 dd, (8.1/2.0 Hz)	122.50
B	7.49 d, (15.6 Hz)	145.63
α	6.33 d, (15.6 Hz)	115.71
CO	-	165.86
Glucose		
1	4.34 d, (7.2 Hz)	102.37
2	3.40 d, (9.6 Hz)	74.65
3	3.84 t, (9.0 Hz)	79.24
4	4.70 t,(9.0 Hz)	70.12
5	3.24 m	74.55
6a	3.28 dd,(10.2/6.3 Hz)	60.83
6b	3.86 dd, (10.2/6.3 Hz)	
Rhamnose		
1	5.02 brs	101.26
2	3.72 d,(3.0 Hz)	69.1
3	3.10 dd, (4.5/4.9 Hz)	70.49
4	3.63 t, (9.6 Hz)	71.77
5	3.66 m	68.77
6	0.97 d, (6.3 Hz)	18.14
3- OCH_3	3.79 s	55.81
3- OCH_3	3.89 s	55.68

Table2. ^{1}H and ^{13}C-NMR spectral data of compound 2

Position	^{1}H(*J* in Hz)	^{13}C
3,4- dihydroxyphenylethyl		
1	-	129.16
2	6.63 d,(1.8 Hz)	115.61
3	-	145.57
4	-	143.67
5	6.61d, (7.8 Hz)	116.46
6	6.49 dd, (7.8/1.8 Hz)	119.51
β	2.50 t, (7.5 Hz)	35.07
α	3.65 t, (7.5 Hz)	70.58
Iso-ferulic acid		
1	-	123.20
2	7.26 d, 2.0 Hz	111.18
3`	-	145.12
4	-	148.02
5`	6.81 d, (8.4 Hz)	116.46
6	7.07 dd, (8.4/2.0 Hz)	123.20
β	7.55 d, (15.9 Hz)	145.57
α	6.41 d, (15.9 Hz)	115.61
CO	-	165.81
Glucose		
1	4.36 d, (7.8 Hz)	102.81
2	3.31 dd, (8.4/7.8 Hz)	74.55
3	3.39 t, (9.6 Hz)	79.23
4	4.71 t,(9.6 Hz)	70.30
5	3.16 m	74.55
6a	3.68 m	60.81
6b	3.89 m	
Rhamnose		
1	5.03 brs	101.26
2	3.68 d,(4.5 Hz)	69.21
3	3.31 d, (9 Hz)	70.58
4	3.13 t, (9.6 Hz)	71.76
5	3.32 m	68.77
6	0.97 d, (6.0 Hz)	18.14
4-OCH_3	3.79 s	55.71

Table3.^{1}H and ^{13}C-NMR spectral data of compound 3

Position	^{1}H(*J* in Hz)	^{13}C
3,4- dihydroxyphenylethyl		
1	-	129.16
2	6.63 d,(2.1 Hz)	115.54
3	-	145.03
4	-	143.59
5	6.61d, (7.8 Hz)	116.38
6	6.50 dd, (7.8/2.1 Hz)	119.56
β	2.69 t, (7.5 Hz)	35.06
α	3.61 t, (6.9 Hz)	70.57
Caffeic acid		
1	-	125.54
2	7.02 d, 2.0 Hz	113.62
3	-	145.62
4	-	148.53
5	6.77 d, (8.1 Hz)	115.86
6	6.95 dd, (8.1/2.0 Hz)	121.43
β	7.48 d, (15.9 Hz)	145.62
α	6.21 d, (15.9 Hz)	114.77
CO	-	165.72
Glucose		
1	4.36 d, (7.8 Hz)	102.34
2	3.21 dd, (8.4/7.8 Hz)	74.57
3	3.38 t, (9.6 Hz)	79.15
4	4.70 t,(9.6 Hz)	70.29
5	3.44 m	74.57
6a	3.55 m	60.81
6b	3.88 m	
Rhamnose		
1	5.02 brs	101.25
2	3.69 d,(4.5 Hz)	69.22
3	3.30 d, (9 Hz)	70.47
4	3.13 t, (9.6 Hz)	71.75
5	3.32 m	68.76
6	0.96 d, (6.3 Hz)	18.20

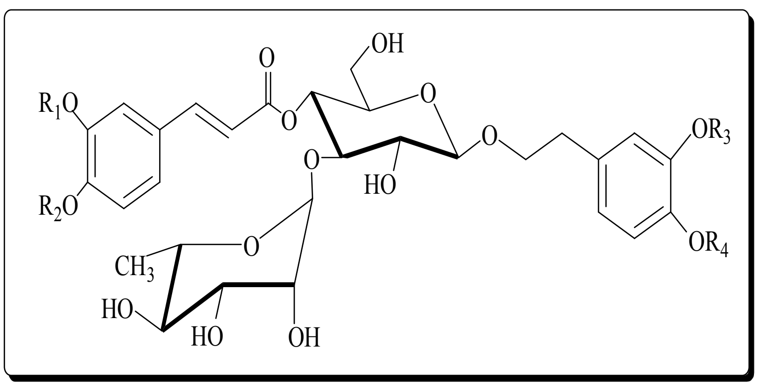

Fig.1. The structure of Phenylethanoid compounds isolated from *Petrea*

Name of the compound	R-Groups			
	R_1	R_2	R_3	R_4
Verbascoside (Acteoside)	H	H	H	H
Eukovoside	H	H_3	H	H
Cistanoside D	CH_3	H	CH_3	H

Anti-microbial activity of *n*-butanol fraction

Table (4) shows that the methanolic extracts of ***petra***have the greatest effect (inhibition zone) on *Escherichia coli, Staphylococcus aureus,Aspergillusniger* and *Candida albican,*.

Table 4. Antibacterial action of *n*-butanol fraction

Type of organism	Inhibition zone		
	0	100 µl	200µl
Esherichia coli, *ATCC 14169*	0	10	18
Staphylococcus aureus, ATCC 6538	0	18	24
Bacillus subtulis, *ATCC 6633*	0	8	15
Pseudomonas aeruginosa, ATCC 9027	0	6	10
Candida albicans, *ATCC 10231*	0	10	20
Aspergillusniger	0	12	30

Antimicrobial activity of the pure compounds

Table (5) showsCistanoside Dhaveasignificant inhibition effect on bacteria (*Bacilluscereus, Staphylococcus aureus*, *Micrococcus leutus*, *Bacillus subtulis*, *Esherichia coli*),yeast & fungi (*Candida albicans* and *Aspergillusniger*), and no effect on *Pseudomonas areuginosa*. Eukovosidehas a significant inhibition effect on bacteria (*Staphylococcus epidermidis, Staphylococcus aureus*, *Micrococcus leutus*,*Bacillus subtulis*, *Escherichia coli*),yeast & fungi (*Candida albicans* and *Aspergillusniger)*,and without any effect on *Pseudomonas areuginosa*. Verbascosidehas asignificant effect inhibition onbacteria (*Bacillus* cereus*, Bacillus subtulis*), *Candida albicans*and fungi (*Aspergillusniger)* and no effect *Staphylococcus aureus*, *Micrococcus leutus*, *Bacillus subtulis* and *Esherichia coli.*

Table 5. Antimicrobial activities of the compounds isolated from *Petrea*

Microorganisms	Extracted compounds/ Concentration, mg/ml									Standard		
	Cistanoside D			Eukovo side			Verbascoside					
Aspergillusfumigatus	5	2.5	1	5	2.5	1	5	2.5	1	5	2.5	1
	++	+	-	+	+	-	+	+	+	+++	+++	++
Candida albicans, ATCC 10231	+	+	+	+	+	-	-	-	-	++	++	++
Staphylococusaureus, ATCC 6538	++	++	++	+	+	+	-	-	-	++	++	++
Pseudomonas aeruginosa, ACCT 9027	-	-	-	-	-	-	-	-	-	+++	+++	++
Bacillus subtilis ATCC 14169	++	+	+	+	+	-	+	-	-	+++	+++	++
Escherichia coli, ATCC 14169	-	-	-	-	-	-	-	-	-	++	++	++

*Reference standard; chloramephenicol as antibacterial standard and terbenalin as antifungal standard.
-Inhibition zones not detected.**,** + Inhibition zone 0.1-0.5 cm beyond control.
++Inhibition zones = 0.6-1.0 cm beyond control, +++Inhibition zone 1.1-1.5 cm beyond control

The results obtained in the present study also validate the folk medicinal uses of both plants for treatment bacterial and fungal organisms are pathogenic for animals and human and some others cause damage to plants, some of these microorganisms, Escherichia coli raised from water pollution and cause urinary tract infection, diarrhea and gastroenteritis. Some of *Pseudomonassp*. cause human ears and eyes diseases. *Salmonella sp.* causes septcimia, typhoid and food poisoning. These species considered dangerous because they cause death in few hours.*Staphylococcus sp*. causes food poisoning (intoxication) that is characterized by severe diarrhea and vomiting. Such organisms act as carrier to provide the reservoir for the spread of staphylococcal infections, most frequently by hands. *S. aureus* is also a major cause of impetigo, either alone or in conjunction with group A Streptococci. Such infections are seen most frequently in school children often in the face, sometimes causing pites and carbuncles (Lippicott, 1991 andEl astal*et al*., 2005).

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