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Full Length Research Paper

Flavone C-glycosides from *Montanoa bipinnatifida* stems and evaluation of hepatoprotective activity of extract

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The methanolic extract of the stems of *Montanoa bipinnatifida* was investigated for its phenolic compounds and hepactoprotective activity. A new flavon C-glycosides named as luteolin 6-C- apioside 8-C- glucoside (1) as well as apigenin 6-C-glucoside(2), apigenin 8-C-glucoside(3), apigenin 6,8- di-C-glucoside(4), luteolin 8-C-glucoside(5) luteolin 6,8- di-C-glucoside(6), luteolin 6-C- glucoside(7), apigenin(8) and luteolin(9) were isolated from the *M. bipinnatifida* stems aqueous alcoholic extract. Structures of the isolated compounds were established by chromatography, UV and 1D/2D $^{1}H/^{13}C$ spectroscopy. Hepatoprotective and antioxidant effects were investigated for the aqueous methanol extract of *M. bipinnatifida* stems (100 and 150 mg/Kg b.wt.) in normal and carbon tetrachloride –hepatic damaged rats. The hepatotoxic dose of CCl_4 (25 mg/Kg, orally) raised significantly ($P< 0.05$) the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels as compared to control values. The dose aqueous methanolic extract of *M. bipinnatifida* stems were able to prevent CCl_4-induced rise in serum enzymes.

Key words: *Montanoa. bipinnatifida*, Flavone-C-glycosides, CCl_4, hepatoprotective.

INTRODUCTION

Herbal medicines derived from plant extracts have been used for thousand years for the treatment of disease. More recently, there has been renewed interest in the use of natural products because of their reduced side effects on the human body in comparison to synthetic medicinal drugs. Liver is considered the key organ in the metabolism detoxification and secretary functions in the body and its disorders are numerous with no effective remedies however, the search for new medicines is still ongoing. Many folk remedies from plant origin have been long used for the treatment of liver diseases (Aktay et al., 2000; Gutierrez et al., 2008). It is our opinion that instead of random search of plant, a selective search based on traditional knowledge would be more focused, productive and certainly more economic. Plants from the Asteraceae family have been widely used in folk medicine. *Montanoa* plants have been used in the biocidal and spermicidal activities (Valencia et al., 1986). In traditional Mexican medicine Zoapatle, a decoction made from *Montanoa tomentosa* has been used for centuries in fertility regulation as an oral contraceptive (Sabanero et al., 1995; Southam et al., 1983; Waller et al., 1987; Wens et al., 1985). *Montanoa bipinnatifida* (Kunth) K. Koch family *Asteraceae* is a tree reaching 2 to 3 m in height. Previous phytochemical investigation of the genus *Montanoa* resulted in the isolation of sesquiterpene lactones as a major class of chemical compounds (Braca et al., 2001; Muller et al., 2004; Quijano et al. 1978, 1982, 1986, 1987; Oshima et al., 1986a) and flavonoids (Oshima et al., 1986 b). To the best of our knowledge, there is no scientific literature on phytochemical and hepatoprotective effect of

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the stems of *M. bipinnatifida*. The present study deals with the isolation and identification of the flavone C-glycosides from the stems of *M. bipinnatifida* and evaluation of hepatoprotective activity of their aqueous alcoholic extract.

MATERIALS AND METHODS

General

^1H and ^{13}C NMR spectra were recorded on a 500 MHz Bruker AMX and a Varian Unity Inova with TMS as an internal standard and DMSO-d6 as solvent. FAB mass spectra were obtained using a Finnigan MAT TSQ 700 spectrometer.

UV spectral data was measured on a Shimadzu 240 spectrometer in MeOH. Paper Chromatography Whatman 1, using solvent systems A (15% AcOH) and B (n-BuOH-AcOH-H_2O, 4:1:5, upper layer). Compounds were visualized by exposure to UV light (365 nm), before and after spraying with $AlCl_3$ and Naturestoff-polyethylene glycol reagents and sprayed with 2% $ZrOCl_2$ and 2% citric acid.

Plant material

Stems of *M. bipinnatifida* (Kunth) K. Koch were collected in March 2006 from Orman garden, Giza., Egypt. Identification of the plants was confirmed by the Department of Flora Agricultural Museum, Ministry of Agriculture and Herbarium of the Department of Botany, Faculty of Science, Cairo University. Voucher Specimens were kept in herbarium, National Research Centre, El-Tahrir Str., Dokki, Cairo, Egypt.

Extraction and isolation

Powder of the air-dried stems of *M. bipinnatifida* (1 Kg) was defatted with $CHCl_3$ (3 x 1 L) and extracted with CH_3OH: H_2O (7:3, 5 x 3 L) at room temperature.

The combined extracts were filtered, evaporated under reduced pressure and lyophilized (150 g). Ten grams of the dry residue was used for hepatoprotective study. Weighed samples of *M. bipinnatifida* extract were used to prepare the solutions, which were diluted with distilled H_2O to the appropriate concentration for experiment.

The rest of the dry extract was redissolved in 2 L H_2O and extracted with EtOAc (5 x 2 L). After evaporation of solvents, the EtOAc extract and the remaining H_2O phase gave dark brown solids 25 and 105 g, respectively. The EtOAc extract was loaded on a polyamid 6S column chromatography (50 x 3 cm). The column was eluted with H_2O, and then H_2O-EtOH mixtures of decreasing polarity and 10 fractions (1 L, each) were collected. The major flavonoid fractions obtained were combined into four fractions after chromatographic analysis. Fraction 1 (3.5 g) was fractionated by column chromatography on Sephadex LH-20 with aqueous EtOH (0 to 70%) for elution to give compounds **1** (17 mg) and **7** (25 mg). Fraction 2 (3 g) was subjected to column chromatography on cellulose and n-BuOH saturated with H_2O as an eluent to give two major subfractions, then each of them was separately fractionated on a Sephadex LH-20 to yield pure samples **4** (22 mg) and **5** (18 mg), using the same procedure fraction 3 (2.5 g) gave chromatographically pure samples **2** (15 mg) and **3** (38 mg). Fraction 4 (1.5 g) was chromatography on Sephadex LH-20 using aqueous acetone (0 to 25%) for elution to give pure sample **6** (20 mg), **8** (25 mg) and **9** (30 mg).

Luteolin 6-C- apioside 8-C- glucoside (1)

UV, λ_{max}, in MeOH: nm 348, 271, 256; (MeOH+ NaOMe): 407, 345 sh., 280, 266, 240 sh; (MeOH+NaOAc); 398, 327, 281, 272 sh; (MeOH+NaOAc+ H_3BO_3): 430, 383, 286, 267; (MeOH+$AlCl_3$): 430, 332, 281; (MeOH+$AlCl_3$+ HCl): 384, 358, 297 sh, 279, 265 sh ; FAB-MS: m/z = 579.22 [M-H] , ^1H NMR of **1** (DMSO-d_6): δ 7.54 (1H, dd, *J* = 8.2, 2.1 Hz, H-6'); 7.52 (1H, d, *J* = 2.1 Hz, H-2'); 6.86 (1H,*d, J* = 8.2 Hz, H-5'); 6.48 (1H, *s*, H-3); 5.19 (1H,*d, J* = 3.1 Hz, H-1''); 4.58 (1H, d*, J* = 9.4 Hz, H-1'''); 4.18 (1H, d, *J* = 3.1 Hz, H-2''); 4.16 (1H, d, *J* = 9.6 Hz, H-4''a), 4.13 (1H, d, *J* = 9.6 Hz, H-4''b; 3.15-3.72 (overlapped the rest sugar protons): ^{13}C NMR: δ 182.02 (C-4), 164.26 (C-2) , 161.34 (C-7), 159.83.(C-5), 156.52 (C-9), 148.49 (C-4'), 145.26 (C-3'), 121.18 (C-1'),120.77 (C-6'), 115.69 (C-5'), 115.51 (C-2'), 110.34 (C-6), 105.94 (C-8), 101.12 (C-10), 79.69 (C-1''), 70.69 (C-2''), 79.46 (C-3''), 72.24 (C-4''), 63.9 (C-5''). 74.14 C-1'''), 73.57 (C-2'''), 79.46 (C-3'''), 69.99 (C-4'''), 80.69 (C-5'''), 60.22 (C-6''').

Animals

Forty-eight male Sprague- Dawley rats weighing (120 to150 g) were purchased from the Animal House of National Research Centre, Dokki, Cairo, Egypt.

They were housed at standard environmental condition and were allowed free access to tap water and pellet diet. Rats were divided into eight groups, each of six.

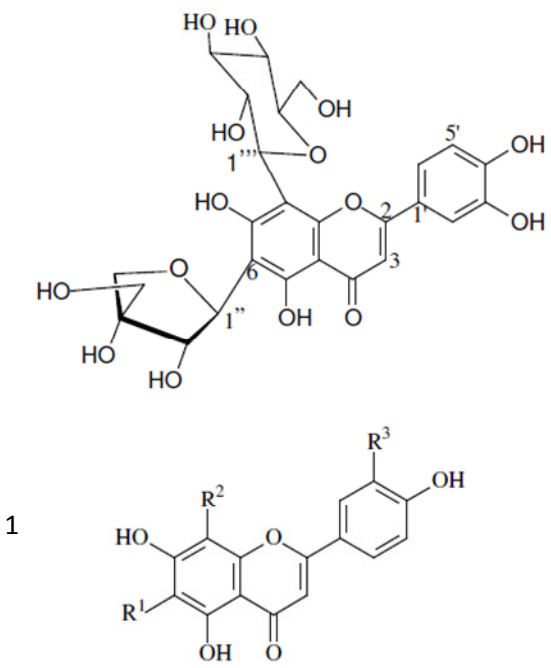
The methanolic extract of the stems *M. bipinnatifida* was dissolved in normal saline (0.9% NaCl) prior to oral administration to experimental animals. Group I served as control, Group II rats were administrated with 20 mg/Kg of silymarin, Group III rats were treated with 100 mg/kg of aqueous methanol extract of *M. bipinnatifida* stems, Group IV rats were treated with 200 mg/Kg aqueous methanol extract of treated *M. bipinnatifida* stems, Groups V, VI, VII and VIII were intoxicated with CCl_4 (2.5 ml/Kg) and then Groups VI and VII treated orally with methanolic extract of *M. bipinnatifida* stems (100 and 150 mg/Kg, respectively), Group VIII treated with silymarin for 15 following days, daily once by gastric gavage needle. 60 min post- administration of the last dose on day 15 blood samples were withdrawn from retro-orbital venous plexus into plane test tubes from all groups (6 rats in each group) and sacrificed by overdose of diethyl ether for determination of liver function tests. The alive rats of all groups were challenged with 50% CCl_4 in liquid paraffin v/v (2.5 ml /Kg bw. per Os) to induce hepatic injury. The study has got the approval from the Local Ethical Committee, in the National Research Centre.

Biochemical analysis

The activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum samples were assayed by the method of Reitman and Frankel (1957). Liver of each rat was promptly removed and a part was used to determine the levels of lipid peroxidation (MDA) (Buege and Aust, 1978) and reduced glutathione (GSH) according to Ellman (1959). Glutathione peroxidase (GPx) activity was determined according to the method of Lawrence and Burk (1976).

Statistical analysis

Data were analyzed by one- way analysis of variance (ANOVA) followed by least significant difference (LSD) using Excel 2003 Microsoft Corp (11.5612.5606), Redmond, WA software package. Results were presented as means ± standard error of the means (X±SE). P-values < 0.05 were ranged as statistically significant.



2	R1 = glucoside	R2 = R3 = H
3	R2 = glucoside	R1 = R3 = H
4	R1 = R2 = glucoside	R3 = H
5	R1 = glucoside	R2 = H R3 =OH
6	R1 = R2 = glucoside	R3 = OH
7	R1 = R2 = glucoside	R3 = OH
8	R1 = R2 = R3 = H	
9	R1 = R2 = H	R3 = OH

Figure 1.The isolated and identified nine compounds

RESULTS

Fractionation of the EtOAc extract resulted in the isolation and identification of nine compounds **1-9** (Figure 1). The structure of the isolated compounds was established through chromatography as well as conventional chemical and spectroscopic methods of analysis (UV, MS, 1/2 D NMR).

The results given in Table 1 showed that, when CCl_4 was injected the level of ALT and AST enzymes were significantly elevated compared with the normal control, which can be considered due to the oxidative damage in the structural integrity of the liver (Brent and Rumack, 1993). Administration of 20 mg/kg of Silymarin induced a significant a reduction in serum ALT and AST compared to that shown in CCl_4 group. Treatment with 100 mg/Kg of the extract of the *M. bipinnatifida* stems showed significant change in serum ALT and AST level, also a high dose of 150 mg/Kg causes a more significant protection, evidenced by the reduction in ALT and AST values (Table 1). Administration of CCl_4 exerted a significant increase in lipid peroxides and reduction of GPx and GSH. Treatment with Silymarin significantly reversed these elevated parameters. The treatment with the low and high dose (100 and 150 mg/kg) of the extract of the stems of *M. bipinnatifida* improved lipid peroxides, GPx and GSH levels in the liver.

DISCUSSION

Compound **1**, obtained as a yellow amorphous powder showed chromatographic properties (dark purple spot on paper chromatogram under UV light turning yellow when fumed with ammonia vapour) and colour reaction (a lemon yellow with Naturestoff and a bright-yellow colour with 2% $ZrOCl_2$ which disappeared on addition 2% citric acid and water) (Liu et al., 1997) characteristic of 4' –oxygenated flavonoids bearing a free hydroxyl at 5-position. UV spectral properties of **1**, in methanol and after addition of usual shift reagents revealed the presence of free 7-hydroxy group; free di-orthohydroxy groups reagents (Harborne and Williams, 1975; Mabry et al., 1969). This followed from the recognizable bathochromaic shift with NaOAc, $NaOAc/H_3BO_3$. On negative FAB-MS analysis component **1** exhibited a molecular ion m/z $[M-H]^-$ = 579.22 corresponding to molecular mass of 580 and molecular formula $C_{26}H_{28}O_{15}$.

The ^1H NMR spectrum of **1** (DMSO-d_6, room temperature) showed aromatic signals at δ ppm 7.54 (dd, J = 8.2, 2.1 Hz), 7.52 (d, J = 2.1 Hz) and 6.86 (d, J = 8.2 Hz) assignable to H-6', H-2' and H-5', respectively together with a singlet at δ ppm 6.48 for H-3. The ^1H NMR spectrum of **1** also showed two distinct signals for anomeric protons, a hexose anomeric proton resonance at δ ppm 4.58 (1 H, d, J = 9.8 Hz) and a pentose proton resonance at δ ppm 5.19 (1 H, d, J = 3.1 Hz), indicating the presence of two sugars with β-configuration. It is found that one the sugars was β-D-glucose in the pyranose form and the other an apiose moiety where the $J_{1,2}$ ~ 3-4 Hz confirmed the more stable β-D-erythro furanoside form of the apiose (Ranganathan et al., 1980; Silva et al., 2000). The absence of the two meta coupled protons of H-6 and H-8 in the ^1H NMR spectrum of **1** together with the downfield shifts of their carbon signals in the ^{13}C NMR spectrum to δ ppm 110.34 and 105.94 respectively, due to the glycosylation of the aglycone at these positions (Agrawal, 1989). ^{13}C DEPT experiments showed three methylene, eleven methane and twelve quaternary carbons together with molecular formula $C_{26}H_{28}O_{15}$. The ^{13}C- NMR spectrum showed a singlet at δ 180.02 assigned to the carbonyl carbon. Assignments of the aglycone carbons were aided by comparison with the reported values for luteolin 6, 8-C-di-glycosides (Markham and Mohan, 1982). However, unambiguous assignment could be achieved by HMQC and HMBC which proved that the signals at 164.26, 161.34, 159.83, 156.52, 148.49 and 145.26 are assignable to C-2, C-7, C-5, C-9, C-4' and C-3' respectively. In the HMBC spectrum (Figure 2) the pattern of ^1H-^{13}C correlation was observed

Table 1. Effect of the ethanolic extract of the stems of *M. bipinnatifida* (100 and 150 mg/ Kg) and Silymarin (20 mg/kg) on serum enzymes of liver (ALT and AST) and on oxidants (GSH, GPx and LPx) in normal and carbon tetrachloride-treated rats.

Parameters/Groups	ALT unit/ml	AST unit/ml	GSH mol/mg protein	GPx mol/mg protein	LPx n moles of MDA /mg protein
Control	23.82 ± 0.69	49.33 ± 1.48	12.55 ± 0.56	0.98 ± 0.07	331.87 ± 2.23
Silymarin (20 mg/Kg.b.wt.)	17.99 ± 0.32	26.33 ± 0.92	17.38 ± 0.69	1.35 ± 0.04	275.37 ± 3.16
M.bipinnatifida I (100 mg /Kg.b.wt.)	19.77 ± 0.58	34.17 ± 1.09	13.83 ± 0.58	1.07 ± 0.02	327.38 ± 2.69
M.bipinnatifida II (150 mg /Kg.b.wt.)	18.78 ± 0.88	33.42 ± 1.09	15.07 ± 0.47	1.17 ± 0.05	319.70 ± 2.02
CCl_4 (2.5 ml/Kg b.wt)	48.35 ± 1.66	91.50 ± 2.26	5.12 ± 0.26	0.43 ± 0.03	543.45 ± 7.18
CCl_4+*M. bipinnatifida* I	33.83 ± 1.58	64.00 ± 1.41	8.65 ± 0.26	0.75 ± 0.06	430.62 ± 3.10
CCl_4+*M. bipinnatifida* II	30.83 ± 0.95	54.17 ± 1.17	9.88 ± 0.35	0.84 ± 0.05	398.03 ± 5.21
CCl_4+ Silymarin	23.17 ± 1.10	45.33 ± 1.26	16.60 ± 1.66	1.28 ± 0.08	288.55 ± 4.19

$P < 0.05$ Each value represents the mean ± SEM, n= 6 rats/group.

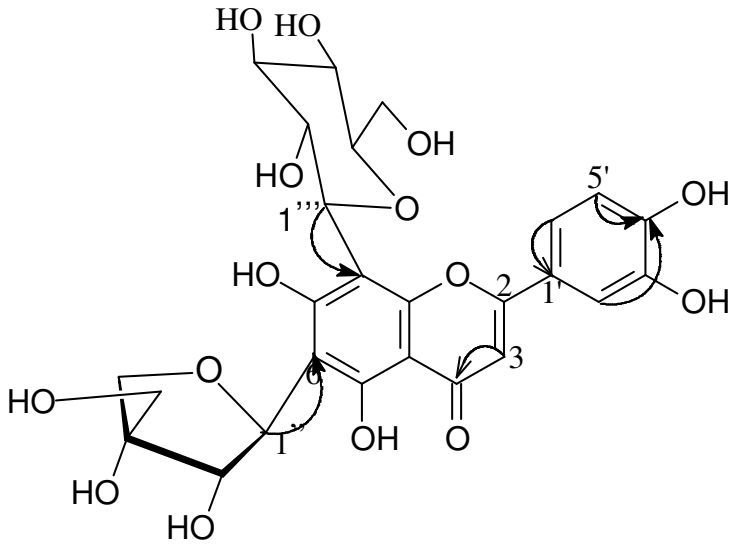


Figure 2. HMBC correlation (H→C)

between δ_H 4.58 (H-1''') with δ_C 105.94 (C-8) and δ_H 5.19 (H-1'') with δ_C 110.34 (C-6) indicating a glucosyl moiety is attached to C-8 and an apiosyl moiety is attached to C-6. Based on the above data, **1** is deduced to be luteolin 6-C-apiosyl- 8-C-glucoside. To the best of the authors' knowledge, this is the first report of isolation of this compound from any natural source.

Increasing evidences support the hypothesis that CCl_4 induced tissue damage may be a consequence of oxidative stress and nutritional deficiencies. In addition, the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Suja et al., 2004). Accordingly, CCl_4-mediated hepatotoxicity was chosen as the experimental model. The ability of a hepatoprotective drug to reduce the liver injury or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects (Yadav and Dixit, 2003). The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee et al., 1995). The decrease in GSH and GPx level in liver tissues was observed in the CCl_4-treated groups. The increased value of GSH and GPx level in liver tissues of the rats treated with two doses of methanolic extract of stems of *M. bipinnatifida* and Silymarin may be due to *de novo* GSH synthesis or GSH regeneration (Sanmugapriya and Venkataraman, 2006).

Moreover, lipid peroxide (LPx) level is a measure of membrane damage and alterations in structure and

function of cellular membranes. In the present study, elevation of lipid peroxidation in the liver of rats treated with CCl_4 was observed. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Ashok et al., 2001). Treatment with *M. bipinnatifida* stems extract and silymarin significantly reversed these changes. Several studies have reported the inhibitory effects of flavonoids on lipid peroxidation (Husain et al., 1987; Videla et al., 1985). Lipid peroxidation could be inhibited by flavonoids possibly by acting as strong $\mathbf{O}^{\bullet}_2$ scavengers and $^1O_2$ quenchers. However, $\mathbf{O}^{\bullet}_2$ itself does not appear to be capable of initiating lipid peroxidation H $\mathbf{O}^{\bullet}_2$ (the protenated form of $\mathbf{O}^{\bullet}_2$) (Halliwll and Chirice, 1993).

Sulfhydryl compounds such as glutathione (GSH) are well known to be an antioxidant substance in organisms, playing a critical role against CCl_4-induced injury by covalently binding to $CCl^{\bullet}$. This is considered as the initial reactant in the chain reaction of oxidation, and then result in the lipid peroxidation and the cell membrane disruption (Barttin et al., 1985). Treatment with *M. bipinnatifida* stems extract (100 and 150 mg/kg) resulted in elevating the content of liver GSH compared with the control group. Several diseases have been associated with the changes in GSH levels, and reduced the resistance to the oxidation stress. The level of GSH and the GPX were used to minitor the balance of oxidative stress and chemopreventive ability (Hatono et al., 1996). In our study, the *M. bipinnatifida* stems extract exhibited protective effectives against liver damage from $CCl_3^{\bullet}$. Furthermore the GSH-related antioxidant system has been improved.

In conclusion, the treatment with *M. bipinnatifida* stems extract could reduce damage induced by CCl_4. The mechanism of protection including the inhibition of lipid peroxidation, increasing the content of GSH, elevating the expression of antioxidant enzymes, all of which result in recuperation of biological parameters. Furthermore, the relationship of their antioxidant effects to the hepatoprotective mechanism, such as changes in antioxidant enzyme activity and the effects and mechanisms of the pure compounds from this plant requires further study.

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