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Steryl Glycosides, Lipoidal Matter and Volatile Constituents of *Urtica pilulifera*

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Abstract: Four steryl glycosides were isolated from the aqueous methanolic extract of *Urtica pilulifera* and characterized as β-sitosterol-3-*O*-β-D-glucoside, two stigmasterol-3-*O*-β-D-glucosides as well as stigmasterol-3-*O*-galactoside. The analysis of the unsaponifaible fraction showed three sterols; cholesterol, stigmasterol and β-sitosterol and the main hydrocarbons were nonane (17.80%), pentadecane (11.40%), dodecane (8.90%) and tridecane (5.30%) while GLC analysis of fatty acid methyl esters showed that the main fatty acids were linoleic acid (63.16%), oleic (18.75%), palmitic (8.69%) and stearic acid (3.81%) which constitute 94.41% of total fatty acids.

Key words: Fatty acids · Sterol · Steryl glycosides · *Urtica pilulifera* · Volatile components.

INTRODUCTION

Urtica pilulifera, family *Urticaceae,* is a weed annual herb with quadrangular stems, furnished with stinging hairs [1]. *Urtica spp.* were used in wound-healing antimicrobial ointment, skin regeneration ointment, hair regeneration and stimulation of hair growth [2], to treat both dental plaque and bleeding or inflammation of the gingiva [3]. *Urtica* herb extract was useful for bladder disorder, prevention against hemorrhagic and purulent inflammation following adenomectomy as well as inhibition of footpad swelling [3]. The leaf extracts of *Urtica dioica* and *Urtica urens* were members of most active anti-inflammatory substances that explain their clinical efficacy in rheumatoid arthritis [4, 5].They inhibit prostaglandins and leukotriene syntheses in *in-vitro* and *in-vivo* models. *Urtica spp.* used for various immune disorder, their flavonoids have immunomodulatory activity [6], they significantly increased reduced RBC, WBC, PCV and Hb levels [7]. In addition, *Urtica spp.* exhibit antiviral activity when tested against infection of feline immuno deficiency virus sharing numerous biological and pathogenic features with the human immuno deficiency virus [8]. *Urtica dioica* aqueous extract has an antioxidant activity as radical scavenger [9-11] and it was used as potential source of phytomedicines against hyperuricaemia and gout [12]. Its water extract showed antiulcer activity and analgesic effect [13, 14]. *Urtica pilulifera* showed anti-diabetic activity [15]. *Urtica dioica* had inhibitory effect on alpha glucosidase in case of diabetes type II [16] also induces a strong bradycardia [17] and showed hypotensive response [18]. The root extract was superior to the placebo for amelioration of lower urinary tract symptoms and was advantageous in obstructive and irritative urinary symptoms [19]. *Urtica* species contained many secondary metabolites, Phenylpropan and lignans [20], flavonoids and phenolic compounds [21], isoprenoid compounds [22], terpene compounds [20], alkaloids, polysaccharides and agglutinin [23]. The antioxidant and antitumor activity of aqueous methanolic and petroleum ether extracts of *Urtica pilulifera* herb were reported by authors [24, 25]. Therefore, this study aimed to isolate sterol, volatile constituents and lipoidal matter of *U. pilulifera* extract.

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MATERIALS AND METHODS

Plant Material: Young plants (50% flowering) of *Urtica pilulifera* grown in experimental farm of Faculty of Agriculture, Cairo University were collected in spring (1st April, 2005), air dried and powdered. *Urtica pilulifera* herb (about 600g) was exhaustively extracted by 20% aqueous methanol with shaking. The extract was evaporated under reduced pressure at 40ºC till free from solvent, 5g was produced.

Isolation of Steryl Glycoside: The crude extract was fractionated on alumina column using solvent systems as follows; petroleum ether (40-60°C), followed with gradient petroleum ether: ether systems (9: 1), (8: 2), (7: 3), (6: 4), (5: 5), (4: 6): (3: 7), (2: 8) and (1: 9), 100% ether and then ether: methanol (1: 0.1), respectively. Eight sterol fractions were collected and their colors were as follows; pink (1), brown (2), brown (3), pink (4), brown (5), brown (6), blue (7) and pink (8). The fractions number three, four, six and eight were used for isolation of steryl glycosides. They were purified on precoated silica gel sheets using solvent systems, benzene: ethyl acetate (6:2) and benzene: methanol (96:4) followed by benzene: chloroform (4:1). Fraction three gave pure brown single spot, fraction four gave one major pink single spot and fraction six gave one major spot as fraction eight together with few minor components. The four major isolated sterols were tested by Molish test for glycosides and all of them gave positive results so they were partially hydrolyzed to identify the sugar moiety. The isolated steryl glycosides were analyzed by infrared, proton-nuclear magnetic resonance (Broker WH at 300 MHZ) and mass spectroscopy (Finningen SSQ 7000, filament emission current: 400, electron energy: 70eV, ion source temp.: 150, mass range: 40- 600D).

Isolation of Herb Volatile Fraction: Isolation of volatile fraction from herb of *Urtica pilulifera* was carried out according to Macleod and Cave [26].

Preparation of Volatile Fraction: Fresh herb at flowering stage (100g) of *Urtica pilulifera* was subjected to hydrodistillation in modified Likens and Nikerson apparatus which allowed the simultaneous extraction of the volatile compounds in an organic solvent (pentane). The pentane layer was collected and analyzed by GC/MS. Analysis of the volatile constituents were carried out on gas chromatography apparatus directly coupled to mass spectrometer (Finningen SSQ7000) applying the following conditions; Capillary column of DB-5 fused silica, 30 m in length, 0.25 mm internal diameter and 0.25ìm thickness, carrier gas; helium at a rate of 10ml/min, temperature programming; 40-250°C increased at a rate of 3°C/min, chart speed was 0.5 cm/min and ionization detector (70eV) was used. The identification of the constituents was performed by comparing their retention times and mass fragmentation patterns with those of authentic and available references [27, 28].

Preparation of Lipoidal Matter: Five grams of *Urtica pilulifera* powdered seeds were extracted with petroleum ether (40- 60°C) in a continuous extraction apparatus (Soxhlet). About 0.5 g lipoidal matters were obtained and it was saponified. Preparation of saponifiable and unsaponifiable matter was prepared according to Tsuda *et al.* [29]. The unsaponifiable fraction was subjected to gas-liquid chromatography analysis. Fatty acids were prepared as methyl esters and they were then analyzed by "Gas-liquid chromatography technique" for separation and identification of the fatty acids. The qualitative identification of the fatty acids was achieved by comparing the retention time (R_t) of their peaks with those of the expected authentic chromatographed under the same conditions.

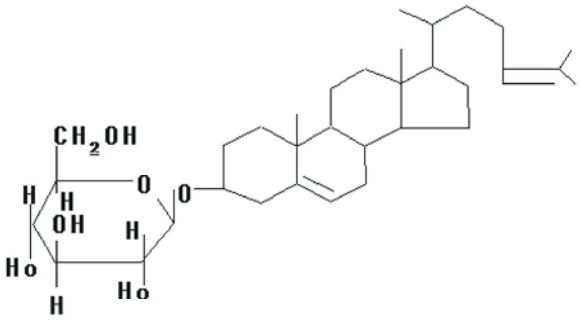
Condition of Fatty Acids GLC Analysis: (Hp-Gas-liquid chromatography 6890); Column, Hp-INNowax polyethylene glycol. Length, 60m with internal diameter 320ìm and film thickness, 0.25μm. Oven initial temperature was 70°C and Initial time was 2 min. Temperature program was rate, 4°C/min and 240°C as final temp, final time, 60 min while the injection temperature was 250°C. Detector temperature was 280°C (flame ionization detector). Gasses flow rates were N_2 40 ml/min, H_2 40 ml/ min and Air 350 ml/min.

Conditions of Unsaponifiable Lipoidal Matter GLC Analysis: Column: capillary column. Hp-5 phenylethyl, length, 30m, diameter, 320ìm, film thickness, 0.25ìm. Oven: initial temperature, 80°C, initial time, 1 min. Temperature program: rate 8°C/ min, final temp. 240°C, final time, 30 min. Injection temp.: 250°C. Detector temp.: 300°C (flame ionization detector). Gasses flow rates, N_2 30 ml/ min, H_2 30 ml/ min, Air, 350 ml/ min.

RESULTS AND DISCUSSION

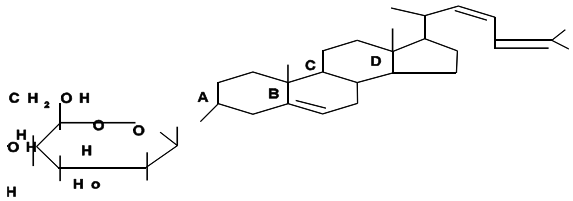
Urtica pilulifera Steryl Glycosides: The purification of the four fractions gave four major sterols that gave brown (compound 1) and pink (compounds 2, 3 and 4) coloration on spraying with sulphuric acid (10%) and heating at 105°C for 10 seconds. They constituted 5%, 1%, 1% and 2%, respectively, as compared to aqueous methanolic extract. The investigation of sugar moiety by partial hydrolysis revealed the sugar moiety to be glucose for all steryl glycosides except compound (3) where the sugar moiety was galactose.

Compound 1: ^{1}H- NMR spectrum of compound 1 showed a complex aliphatic part similar to sitosterol [30]. Methylene proton of (C_6) appeared at δ5.30 ppm. Singlets from 3.17 ppm to 4.12 ppm were for glucose and dublet at δ5.38 ppm with *J*= 0.06 for H- 1 anomeric proton of glucose in *β*-position. Multiplet from1.8 ppm to 2.20 ppm was for sterol methylene group (-CH_2) while, the singlet at δ1.23 ppm was for proton of C_{19}- CH_3. Multiplet at 0.85 ppm was for proton of C_{18}- CH_3. Singlet at 0.93 ppm was for C_{21}-H, singlet at 0.77 ppm for C_{29}-H also multiplet at 0.82 ppm was for C_{27}-H. These data were in parallel with the data obtained by mass- spectrum which showed $[M]^+$ at m/z 576 for *β*-sitosterol glucoside and base peak at m/z 396 due to removal of glucose. Fragments at 329, 255, 213, 187, 147, 105 and 81 were identical to fragmentation pattern of *β*- sitosterol as compared with Chaurasia and Wichtle [30]. The characteristic bands of methyl and methylene groups of sitosterol were observed at IR- spectrum; they gave band at 2851.23 cm^{-1} for -CH_2 group and band at 1463.90 $cm^{-1}$for -CH_3 group while bands at 3421.02 $cm^{-1}$and 1049.87 cm^{-1}were for hydroxyl groups while the double bond in sitosterol cycle gave band at 1541.64 cm^{-1}. From the above data the isolated compound was identified as β-sitosterol- 3-*O*- *β*-D-glucoside.



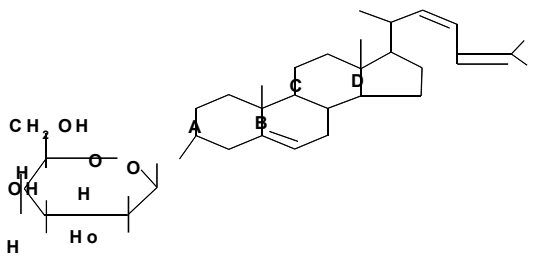
β- sitosterol- 3- *O*- *β*- D- glucoside

Compound 2: It gave pink color when sprayed with H_2SO_4 (10%) and heated at 105°C. The sugar moiety was glucose as compared with authentic sugars. The spectral data of compound (2) were as follows; the proton of C-$_{18}$ on steryl nucleus gave multiplet from 0.85 ppm to 0.87 ppm while methyl group at C_{19} gave a sharp singlet at δ1.24 ppm. The singlets of C_{18} and C_{19} were characteristic of steryl nucleus. Proton of C_{21} gave a singlet at 0.90 ppm while singlet at 0.86 as well as duplet at 0.77 and 0.78 ppm with *J*= 3 was f or C_{29}-H. Singlet at 0.83 ppm was for C_{27}-H. The methylene groups gave the familiar hump at 1.50 - 2.05 ppm. The multiplet at 4.09 to 4.39 ppm was for protons of side chain. The sugar moiety gave its characteristic singlet of C_1-H in *β*-position as duplet at 5.37- 5.39 ppm with *J*=6 also its hydroxyl groups gave singlets from 3.24- 3.80 ppm. The mass spectral data showed that $[M]^+ + 2$ of this compound was at m/z 576 which indicates the presence of glucose molecule bound to steryl nucleus, then cleavage of sugar moiety gave fragment at m/z 394. The cleavage of glucose was followed by cleavage of ring- D then removal of side chain to give base peak fragment at m/z 149 in ionic form. The IR- spectrum showed the presence of methylene group at 2852 cm^{-1} and methyl group at 1465.10 cm^{-1} also band at 3423.01 cm^{-1} for sugar hydroxyl groups. The above explanation of spectral data showed that compound 2 was stigmasterol glucoside.



Stigmasterol - 3- *O*- *β*-D-glucoside

Compound 3: Compound 3 gave pink color when sprayed with sulphuric acid (10%) on pre-coated silica gel with heating at 105°C. The partial hydrolysis of this compound showed it was galactose. ^{1}H-NMR spectrum of compound 3 showed the same characteristic data for stigmasterol. The protons of galactose gave singlets at 3.59 ppm to 3.60 ppm and 3.63 ppm to 3.64 ppm as well as characteristic multiplet of ^{1}H-galactose at 5.52 ppm [31]. The other singlets were as the same of compounds 1 and 2 which in accordance with Chaurasia and Wichtl [32]. The mass spectral data of compound (3) showed that it had the same fragmentation pattern of compound (2) with the same IR spectrum. All obtained data indicated that compound 3 was stigmasterol- 3- *O*-galactoside.



Stigmasterol- 3-*O*- galactoside

Compound (4): ^{1}H- NMR spectral data showed the multiplet from 3.61 ppm to 3.63 ppm which was characteristic for stigmasterol. The other singlets were the same of compounds (2) and (3) with the characteristic singlets of glucose in β-position. The mass and IR spectral data showed a characteristic fragment of stigmasterol nucleus. The mass spectral data showed that $[M]^{+}+2$ of this compound was at m/z 936 which indicates the presence of multi glucose molecules bound to steryl nucleus, then cleavage of sugar moiety gave fragment at m/z 394, this indicated the cleavage of three glucose units. The cleavage of glucose was followed by cleavage of ring- D then removal of side chain to give base peak fragment at m/z 149 in ionic form. These mentioned spectral data showed the isolated compound was stigmasterol bound to three glucose molecules. Schilcher and Effenberger [33] isolated Scopoletin and β-sitosterol from *Urtica dioica* roots extract also Chaurasia and Wichtl [30] isolated sitosterol 3-β-D-glucoside as well as 7-β and 7-α-(OH)-sitosterols, (6`-*O*-palmitoyl)-sitoserol-3-*O*-β-D- glucoside and 24R-ethyl-5-α-cholestane-3β, 6α-diol were also isolated from *Urtica dioica* root extract.

Volatile Constituents of *Urtica Pilulifera* Herb at Flowering Stage: The major amount of volatile principle was oxygenated compounds which constituted 82.81% of total volatile constituents. The major oxygenated compounds were; benzyl n-valerate (63.22%), Cedrol (4.2%), Allyl cyclohexane propionate (1.33%), 1-hexadecanol acetate (1.31%), octadecanal (1.26%), allyl n-nonanoate (1.24%), lauryl alcohol (1.07%) and isobutyl n-heptanoate (1.03%). The oxygenated compounds constituted alcohols, aldehydes, ketones, esters and amide which represented 5.51%, 1.52%, 1.48%, 72.77% and 0.22%, respectively. The major alcohols were cedrol (4.2%) and lauryl alcohol (1.07%). The major aldehydes were octadecanal (1.26%) and citral (0.18%) where the major ketones were allyl- ionone (0.88%) and α-ionone (0.39). The major esters were benzyl n-valerate (63.22%), 1 -hexadecanol acetate (1.31%), citronellyl n-propionate (1.28%), allyl n-nonanoate (1.24%) and isobutyl n-heptanoate (1.03%). The volatile fraction contained one compound in amide form which was octadecenamide (0.22%). The major hydrocarbons were 10- pentyl heneicosane (5.35%), heptacosane (3.87%), triacontane (2.48%) and 3- ethyl- 5(2`- ethyl butyl) octadecane (1.27%). The hydrocarbons constituted cyclic and acyclic compounds which represented 1.21% and 15.98%, respectively, in volatile fraction. The major cyclic compound was caryophyllene (0.7%) while the major a- cyclic compounds were 10- pentyl heneicosane (5.35%), heptacosane (3.87%) and tri acontane (2.48%). The volatile fraction contained two types of terpenes, monoterpenes and sesquiterpenes. They constituted 8.02% of *Urtica pilulifera* volatile constituents. The monoterpenes were 4- terpeniol (0.011%), dihydrocarveol (0.02%), ipsenol (0.04%), z-citral (0.06%), citral (0.18%), isopentyl-isovalerate (0.06%), hydroxyl citronellol (0.02%), citronellyl formate (0.004%), α1-ionone (0.39%), citronellyl acetate (0.11%), citronellyl n- propionate (1.28%), citronellyl-isobutyrate (0.03%) and allyl ionone (0.88%) while sesquiterpenes constituted two compounds, caryophyllene (0.7%) and cedrol.

***Urtica pilulifera* Seed Fixed Oil:** The fixed oil content of *U. pilulifera* was 15%. The analysis of fatty acid methyl esters by GLC showed that the main fatty acids were linoleic acid (63.16%), oleic (18.75%), palmitic (8.69%) and stearic acid (3.81%) which constituted 94.41% of total fatty acids.

GLC analysis of Unsaponifiable Matter of *Urtica Pilulifera*: Identification of the compounds was carried out by matching the relative retention times of the detected peaks with those of pure available authentic samples. The unsaturated fatty acid constituted 82.95% of all fatty acids content while the saturated fatty acids constituted 17%. *Urtica* fixed oil was a source for ε-6 fatty acid, linoleic acid (63.20%) and linolenic acid (1.30%).

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