Oleanane-Type Triterpenoid Saponins and Rosmarinic Acid from *Clinopodium Umbrosum*

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ABSTRACT: *Clinopodium umbrosum from Lamiaceae family has been used widely in traditional medicine as an herbal medicine. Due to a lack of phytochemical reports and limited studies on C. umbrosum, this plant species was selected for further phytochemical analysis. The grounded aerial parts of the plant were extracted with petroleum ether, chloroform, and methanol, subsequently. The methanol extract was fractionated via Solid Phase Extraction (SPE), Vacuum Liquid Chromatography (VLC), and reversed Phase High-Performance Liquid Chromatography (HPLC), respectively. 1D and 2D NMR spectral analyses were applied for structure elucidation of the purified compounds. The Gas Chromatography-Mass Spectrometry (GC-MS) technique was used for the analysis of the essential oil of C. umbrosum which was achieved through hydrodistillation. In addition, free radical scavenging activity together with total phenolics and the flavonoid content of the methanol extract was assessed. Structure elucidation of the purified compounds revealed the presence of a caffeic acid derivative and two triterpene saponins in C. umbrosum methanol extract. Based on the GC-MS analysis of the essential oil results tolualdehide, palmitic acid and acetophenone were the main components of the essential oil of this plant. Moreover, high percentage of phenolic and flavonoid components of the extract seems to be responsible for its antioxidant, and free radical trapping activity. Conclusion: Overall, the phytochemical analysis of C. umbrosum showed the presence of rosmarinic acid, buddlejasaponin IVa and buddlejasaponin IV as the main constituents of the methanol extract.*

KEYWORDS: *Clinopodium umbrosum; Lamiaceae, Rosmarinic acid; Buddlejasaponin Iva; Buddlejasaponin IV.*

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INTRODUCTION

Genus *Clinopodium* from the family Lamiaceae is one of the worthy celebrity genera in Iran, which has 2 endemic species, and also about 13 species worldwide. *C. umbrosum* called (Reyhanake-saye-pasand) by locals is a perennial herbaceous plant that grows in north and northwest of Iran and also is distributed in Afghanistan, Pakistan, the Caucasus, the Himalayas, Assam and Burma. It is widely used in the traditional medicine of Iran and other countries for the treatment of hypertension, pain, and inflammation [1], and as wounds healing, scratches, and cuts. Natives also take advantage of the plant as an astringent, anti-bloating, and blood purifier, moreover, its leaves are used for gastric problems [2, 3]. From the pharmacological aspect, plants in the genus *Clinopodium* have been reported to have antibacterial [4, 5], antifungal [5], antiviral [1], antioxidant [6-9], antitumor [10], anti-inflammatory [11, 12], and CNS and acetylcholinesterase inhibitory properties [13-15].

According to the phytochemical studies on the genus *Clinopodium*, we might classify the reported phytochemicals into flavonoids, saponins, polyphenols, terpenes, sterols and volatile oil. Previous researches indicated that various phytochemicals such as phenols and flavonoids were abundant in genus *Clinopodium.* For instance, in a study from China methanol extract of *C. urticifolium*, flavonoids such as apigenin, didimin, hesperidin, narirutin, luteolin-7-O-β-D-glocopyranosid, luteolin, isosakuranrtin, apigenin-7-O-β-D-methylglucopyranosid and purn-in were isolated and identified [16]. Numerous flavonoids were extracted and identified from ethanolic extract of *C. chinense* aerial parts including purnin, homoeriodictol, hesperidin-7-O-β-D-glucopyranosid, kaempferol-3-O-rhamnopyranoside [17]. In another study conducted by *Migliag et al*., six known flavonoid compounds, apigenin, luteolin, neoeriocitrin, naringenin, narirutin and didymin, were isolated from aqueous extracts from the aerial parts of *C. chinense* [18].

Oleananeolic acid and ursolic acid are common triterpene compounds in the Lamiaceae family that their presence in *Clinopodium* genus such as *C. urticifolium* and *C. vulgare* [10, 16]. Elsewhere, a phytochemical analysis on methanol extract of *C. urticifolium* led to the isolation and identification of six new triterpenoid saponins called Clinopodiside (I-V) and Clinoposaponin Ga [16]. In a study on aqueous extract from *C. gracile*, five new triterpene saponins were extracted, along with two known saponins buddlejasaponin IV and saikosaponin [19]. Phytochemical analysis of 80% ethanolic extract of whole plant *C. polycepharum* resulted in the isolation of a new triterpenoid saponin [20]. Studies on the *C. chinense* have led to the isolation and identification of triterpene saponins called clinopodisides [21-23]. Phytochemical analysis of methanol extract of whole plant *C. chinense var.parviflorum* in 1994 resulted in the isolation and purification of three new saponins called clinoposaponins [24]. Besides, studies by *Tisushi et al.* on the aqueous extract of *C. micranthum* resulted in the isolation and identification of two new triphenyl saponin oleananes, clinoposaponins, along with five known saponins saikosaponin a, saikosaponin b1, saikosaponin b3, 6-O-acetyl-saikoponin a and buddlejasaponin IV, and two artifact saponins buddlejasaponin IVa and clinoposaponin VIIa [25].

Aoshima et al. extracted and identified eight new caffeic acid oligomers called clinopodic acid (J-C) with seven known compounds rosmarinic acid [26], clinopodic acid I, clinopodic acid E, 8-epiblechnic acid, lithospermic Acid, salvialonic acid A and salvialonic acid B From the 80% acetonic extract of *C. gracil*. These compounds had also shown to inhibit hyaluronidase activity and anti-inflammatory and immunomodulatory properties [27]. Studies on the leaves of *C. bolivianum* resulted in the sequestration of hydroxycinnamic acid derivatives, including caffeic acid and rosmarinic acid, which exhibited strong antioxidant effects [7].

Moreover, in a recent study, fourteen oleanane-triterpenoid saponins were isolated from *C.gracile* all of them were inactive to both ATP-citrate lyase and nuclear factor kappa B. one of the compounds is the first oleanane-triterpenoid saponin with a 16-ketone moiety from the *Clinopodium* genus [28]. The largest group in the triterpene compounds are oleanane triterpenoids that encompass a huge number of bioactive phytochemicals all of which have six-membered pentacyclic rings. The naming of the ring structures and carbon atoms in oleanane is the same as in steroids. They are classified based on their structure as olean-12-ene and 11-keto-olean-12-ene, directly derived from the oleanane skeleton and contains a number of different methyl groups that vary in orientation between different oleananes.

Isolated compounds from *C. chinese* were evaluated for their effect on insulin resistance and cytotoxic activity

against the A_{549} and HepG2 cancer cell lines. No cytotoxic compound was found while some compounds showed promising activities for reducing insulin resistance [29]. It was reported that saponins and flavonoids consist a majority of the *C.chinese* total extract. Total Extract of *C.chinese* (TEC) showed promising effects in the treatment of rats with abnormal uterine bleeding. The possible mechanism was introduced to be anti-inflammatory effects on the uterus tissue, down regulation and up regulation of the expression of MMP-2/9 and VEGF and TGF-beta [30]. Essential oil compositions *of C. nepeta subsp. glandulosum* were analysed for their antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus cereus* and *Streptococcus sanguinis* ATCC using broth microdilution method. Major components of essential oils were identified as piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%). The lowest antibacterial activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL)[31]. Elsewhere, moderate antioxidant, alpha amylase and alpha glucosidase inhibitory activity of *C. vulgare* was studied [32].

Considering lack of phytochemical reports and limited studies on *C. umbrosum*, except for one report at 1993, this species was selected for further phytochemical analysis and is the first report on *C. umbrosum* native to Iran.

RXPERIMENTAL SECTION

Plant material

The aerial parts of C. umbrosum were collected during June from the Kheyroud forest near Noshahr, Mazandaran, Iran. After authentication of the plant, herbarium specimen with number TBZFPH1969 was kept at the herbarium of Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran.

Extraction, fractionation and isolation

After collection of the aerial parts of C. umbrosum, the collected material was washed, dried and powdered by a blender (Moulinex- France). The grounded plant materials (46.5g) were subjected to solvent extraction by means of a Soxhlet apparatus using 2L petroleum ether, 2L chloroform and 2L methanol (MeOH), correspondingly. The yielded extracts were dried by a rotary vacuum evaporator (Heidolph, Germany) at 40°C under reduced pressure.

The efficiency of extraction by each solvent (petroleum ether, chloroform and MeOH) were 2.2, 1.7 and 11.57%, respectively. Subsequent to preliminary phytochemical analysis using a Thin Layer Chromatography (TLC) approach, the MeOH extract (11.57% w/w) was chosen for additional assessment. The chemical vanillin- sulfuric acid spray reagent was used at 110 °C until the spots of the saponins became obvious on TLC plate. Fractioning of the MeOH extract was carried out *via* Solid Phase Extraction (SPE) procedure. In this regard, 2g of the extract was dispersed at the least possible amount of (10:90) mixture and loaded on Sep-Pak C_{18} cartridge (Waters, Ireland). The sample was eluted through Sep-Pak column with a gradual rise 10 to 20% in portions of MeOH (MeOH-H_2O: 10:90, 20:80, 40:60, 60:40, 80:20 and 100:0, each of them 200mL), affording 6 sep-pak fractions. The whole process were over repeated in order to acquire satisfactory quantities of the fractions. The fractions were dried in the rotary vacuum evaporator at 45°C in vaccue. According to the results of TLC using ethyl acetate:formic acid:glacial acetic acid:water 100:11:11:26 (V/V/V/V) as eluent for SPE fractions, fractions of 20% and 80% containing the prevailing constituents were selected for subsequent analyses. Primary purification of the compounds from 20% Sep-Pak fraction (165.8 mg) were accomplished by reversed phase preparative HPLC (Shimadzu, Japan) using acetonitrile as the mobile phase from 10 to 25% in run time of 0 –50 min and flow rate of 10mL/min utilizing a Shim-pack ODS column (250mm L. x 50mm I.D.) and a UV/PDA detector affording compound 1 (49mg; t_R= 21 min).

Furthermore, 80% Sep-Pak fraction (482.6mg) was divided into twenty subfractions (A-T) by vacuum liquid chromatography (VLC) on silica gel 40 (70-230 mesh ASTM, Merck) column using MeOH and chloroform as the eluents with an ongoing 5 percent rise in portions of MeOH, within MeOH- chloroform mixtures (5:95, to 100:0; each 100 mL). Thereafter, subfractions (A-T) were monitored by TLC under 254 and 366 UV wavelengths and those with similar spots were united and dried in the rotary evaporator. Next, subfraction G, MeOH-chloroform (35:65), was exposed to the same preparative HPLC condition with mobile phase of acetonitrile from 30 to 70% in run time of 0 –50 min to isolate and purify

compound 2 (33 mg; t_R= 21.5 min) and compound 3 (42 mg; t_R= 24.0min). 1D and 2D NMR spectral analyses were applied for structure elucidation of the purified compounds in DMSO-d6 with a Bruker DRX 400 MHz NMR spectrometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR).

Gas chromatography–mass spectrometry (GC-MS) analyses

Grounded plant materials (10g) for hydrodistillated for about 3 hours with Clevenger-type instrument was utilized. Since the amount of essential oil was low in quantity, xylene was used for trapping the oil. Analysis of the essential oil was done by GC-MS (Shimadzu, GC-MS-QP 5050A gas chromatograph). DB1 capillary column with a methyl phenyl syloxane film, 60 m x 0.25 mm i.d., and 0.25 µm thickness was employed for separation of the essential oil components. The carrier gas (helium) with a rate of flow 1.3mL/min and split ratio of 1:10 along with 1 µL of the essential oil injection was adjusted for the analysis condition. Temperatures of 220°C and 260°C were set for injector and interface whilst column temperature program were arranged in 3 min at 50°C, 50-260°C in keep time of 9°C/min. Regarding GC–MS detection, an electron ionization procedure with ionization potential energy of 70 eV with other parameters set at; ion source temperature; 260°C; quadrupole 100°C, Solvent delay 2 min, scan speed 2000 amu/s and scan range 30-600 amu, EV voltage 3000 volts for acquiring components mass spectra. Retention indices for the reported compounds in Table 1 were calculated according to the kovats retention indices (KI) via the known formula using retention times of n-alkanes standards series (C_8–C_{20}) in the same temperature programmed and GC-MS conditions with *C. umbrosum* essential oil. Final affirmation of the identified compounds was fulfilled via comparison of the KIs and mass spectral data with those for the standards and also by automatic program matching with the NIST 21, NIST 107 and WILEY229 library of the computer.

Antioxidant, total flavonoids and total phenolics assays

The in-vitro antioxidant activity of *C. umbrosum* MeOH extract was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method that established the capacity of the MeOH extract to scavenge the free radicals of DPPH [33]. Besides, the total flavonoid content of the MeOH extract was assessed in a colorimetric method using $AlCl_3$ reagent and quercetin as the standard compound [34]. Additionally, the Folin-Ciocalteu method was used to determine the total phenolics in *C. umbrosum* MeOH extract in which gallic acid was used as the standard reference compound [35, 36].

RESULTS AND DISCUSSION

The obtained essential oil from *C. umbrosum* which was little in amount (<0.1%) was analyzed by GC-MS and it was established that in the essential oil of this plant 17 compounds were present corresponding to the 74.68% of the total oil (Table 1). Mostly, the identified components were classified into the non-terpenes (69.06%), oxygenated diterpenes (3.67%) and hydrocarbon monoterpenes (1.95%). Among the non-terpene compounds, tolualdehyde (29.16%), palmitic acid (17.57%) and acetophenone (13.44%) were the foremost constituents. Additionally, phytol and L-limonene were the leading components of their groupings in *C. umbrosum* essential oil.

There is a considerable difference in the comparison of volatile oils extracted from *Clinopodium* species.

In the previous studies, sesquiterpenes and monoterpens were the main component of the volatile oil but in this study non-terpenoid compounds consist the majority components of our essential oil. We might relate this differences to various reasons including specie type, geographical location, climate, and humidity of the area plant was grown up [6, 8, 37].

Phytochemical analysis of the *C. umbrosum* MeOH extract led to the isolation and identification of a caffeic acid derivate and two triterpenoid saponins. The chemical structures of the purified compounds were elucidated via 1D and 2D NMR spectra, compliant with the published NMR data on the reported compounds. Following data were pulled out from the relative ^{1}H and ^{13}C NMR spectra in conjunction with HSQC and COSY spectroscopic data (Supplementary file) for each compound:

Compound (1): Rosmarinic acid; ^{1}H-NMR (400 MHz , DMSO d6): δH 7.06 (1H, s, H-2), 6.77 (1H, d, J= 8 Hz, H-5), 6.97 (1H, d, J= 8 Hz, H-6), 7.40 (1H, d, J=16 Hz, H-7), 6.21 (1H, d, J= 16 Hz, H-8), 6.69 (1H, s, H-2'), 6.63 (1H, d, J= 7.6 Hz, H-5'), 6.51 (1H, d, J=7.6 Hz, H-6'), 3.05 (1H, d, J= 12.4 Hz, H1-7'), 2.80 (1H, m, H2-7'), 4.92 (1H, d, J= 8.4 Hz, H-8').

^{13}C-NMR (125 MHz, DMSO d6): δC 125.9 (C-1), 115.0 (C-2), 145.3 (C-3), 148.8 (C-4), 116.3 (C-5), 121.6

Table 1: Chemical composition of the aerial parts of C. umbrosum essential oil with their corresponding percentages, retention time and indices, molecular weights and formulas

No.	Compound name	Molecular formula	Molecular weight	Retention time (min)	KI [a]	Percentage
1	1-octene-3-ol	$C_8H_{16}O$	128	17.819	-	0.74
2	Decane	$C_{10}H_{22}$	142	19.692	187.9	0.34
3	L-limonene	$C_{10}H_{16}$	136	20.84	209.3	1.95
4	Acetophenone	C_8H_8O	120	21.53	222.2	13.44
5	M-tolualdehyde	C_8H_8O	120	21.78	226.9	29.16
6	Decanal	$C_{10}H_{20}O$	156	29.401	295.9	0.38
7	Dodecane	$C_{12}H_{26}$	170	30.177	310.8	0.61
8	Tridecane	$C_{13}H_{28}$	184	35.133	323.7	0.53
9	N-dodecanol	$C_{12}H_{26}O$	186	42.406	374.3	2.24
10	Beta-Ionone	$C_{13}H_{20}O$	192	42.736	381.8	0.68
11	Hexadecane	$C_{16}H_{34}$	226	48.439	457.9	0.42
12	Hexadecanal	$C_{16}H_{32}O$	240	52.239	499.8	0.82
13	1-hexadecanol	$C_{16}H_{34}O$	242	58.472	566.5	0.79
14	Methyl palmitate	$C_{17}H_{34}O_2$	270	60.045	566.6	0.37
15	Palmitic acid	$C_{16}H_{32}O_2$	256	61.322	603.9	17.57
16	Phytol	$C_{20}H_{40}O$	296	66.428	-	4.64
Total identified						74.68
Non-terpenes						68.09
Monoterpenes						1.95
Oxygenated diterpenes						4.64

[a] KI: Kovats retention index

(C-6), 146.2 (C-7), 115.3 (C-8), 166.6 (C=O), 129.8 (C-1'), 117.0 (C-2'), 145.1 (C-3'), 144.1 (C-4'), 115.8 (C-5'), 120.2 (C-6'), 37.4 (C- H1-7'), 37.4 (C- H2-7'), 75.7 (C-8'), 186.4 (C=O).

Compound (2): Buddlejasaponin Iva; ^{1}H-NMR (400 MHz , DMSO d6): δH 1.07- 1.79 (2H, H-1), 1.62-1.72 (2H, H-2), 3.61 (1H, H-3), 1.20 (1H, H-5), 1.45 (2H, H-6), 1.59 (1H, H-9), 3.76 (1H, dd, J = 3.2-8.4 Hz, H-11), 3.12 (3H, s, H-OCH3), 5.33 (1H, d, J = 3.2 Hz, H-12), 4.10 (1H, m, H-16), 2.33 (1H, dd, J = 4-13.5 Hz, H-18), 1.02-1.61 (2H, H-19), 1.11 (2H, s, H-21), 1.42-1.90 (2H, H-22), 3.05-3.6 (2H, H-23), 0.63 (3H, s, H-24), 1.13 (3H, s, H-25), 0.95 (3H, s, H-26), 1.22 (3H, s, H-27), 3.15-3.48 (2H, H-28), 0.89 (3H, s, H-29), 0.90 (3H, s, H-30), 4.68 (1H, d, J = 8 Hz, H-1'), 3.65 (1H, H-2'), 3.45 (1H, H-3'), 3.05 (1H, H-4'), 3.48 (1H, H-5'), 1.02 (3H, s, H-6'), 4.32 (1H, d, J = 7.6 Hz, H-1''), 3.73 (1H, H-2''), 3.64 (2H, H-6''), 4.49 (1H, H-1''').

^{13}C-NMR (125 MHz, DMSO d6): δC 39.4 (C-1), 26.1 (C-2), 83.2 (C-3), 42.6 (C-4), 46.8 (C-5), 17.7 (C-6), 31.0 (C-7), 39.4 (C-8), 51.0 (C-9), 35.8 (C-10), 75.5 (C-11), 53.7 (C-OCH3), 122.2 (C-12), 147.8 (C-13), 43.3 (C-14), 37.5 (C-15), 64.6 (C-16), 43.2 (C-17), 43.4 (C-18), 46.6 (C-19), 32.8 (C-20), 33.9 (C-21), 23.9 (C-22), 62.9 (C-23), 12.9 (C-24), 17.2 (C-25), 18.3 (C-26), 26.2 (C-27), 65.2 (C-28), 33.5 (C-29), 24.2 (C-30), 102.3 (C-1'), 75.4 (C-2'), 80.9 (C-3'), 71.3 (C-4'), 69.8 (C-5'), 17.6 (C-6'), 103.5 (C-1''), 74.9 (C-2''), 77.3 (C-3''), 71.1 (C-4''), 76.8 (C-5''), 62.1 (C-6''), 104.0 (C-1'''), 74.2 (C-2'''), 77.1 (C-3'''), 70.5 (C-4'''), 77.3 (C-5'''), 61.5 (C-6''').

Data obtained through ^{1}H-NMR and ^{13}C-NMR spectra for compound 1, indicated presence of a polyphenolic structure. In ^{1}H-NMR spectrum, two double peaks at δ 7.41 ppm and 6.21 ppm (J= 16 Hz), each with an integration of one proton, designated presence of a trans-cinnamic acid derivative. A doublet

peak at 6.97 ppm with J=8 Hz, one singlet peak at 7.06 ppm and a doublet peak at 6.77 ppm with J=8 Hz, revealed presence of an aromatic ring with three substituent covered at the positions 1,3 and 4, respectively. Moreover, singlet peak at 6.69 ppm and a doublet peak at 6.63 ppm with J=7.6 Hz and a doublet peak at 6.51 ppm with J=7.6 Hz specified presence of another aromatic ring with three substituent covered at 1', 3' and 4' positions, respectively. Observing a doublet peak at 3.05 ppm with J=12.4 Hz and a multiple peak at 2.80 ppm shows the dissimilar position of two protons of 7' position. Also observing a doublet peak at 4.92 ppm with J=8.4 Hz showed the presence of an esteric group in the structure. In ^{13}C-NMR spectrum, signals of 146.1 ppm and 115.3 ppm were linked with alkene carbons of 7 and 8. Peak at 166.6 ppm, showed the carbonyl of the esteric group and peak at 186.3 ppm verified the presence of carboxylic group. Also, signals of alkanic carbons 7' and 8' at 37.3 ppm and 75.7 ppm were perceptible. Peaks related to tertiary carboms (2, 5 and 6) and quaternary carbons (1, 3 and 4) of benzene ring in the structure of caffeic acid derivative were observed at 115, 116.3, 121.5, 125.9, 145.3, and 148.8 ppm, respectively. Moreover, signals related to tertiary carbons (2', 5' and 6') and quaternary carbons (1', 3' and 4') of benzene ring of alpha hydroxy di hydrocaffeic acid derivative were perceived at 117.0, 115.8, 120.2, 129.8, 145.0 and 144.0 ppm, correspondingly.

Determining the exact position of data related to ^{1}H-NMR and ^{13}C-NMR spectra, were established through the data obtained from COSY and HSQC, 2D-NMR spectra. Eventually, based on the carbon and hydrogen spectrum data, the proposed structure for compound 1 was rosmarinic acid.

In addition, analyzing the ^{13}C-NMR and ^{1}H-NMR spectra for compound 2 revealed existence of 49 carbons at the chemical structure that might be indicating a saponin aglycon with 30 carbons, three 6- carbonic glycons and a methoxy group. In ^{13}C-NMR spectrum, two peaks at chemical shifts of 122.2 ppm and 147.8 ppm could be seen which might be related to the alkenic double bond between carbons number 12 and 13 of an oleanane saponin aglycon. Correlation of carbon 12 and hydrogen 12 signals at chemical shift 5.3 ppm is easily trackable in HSQC spectrum of this structure (supplementary file). Moreover, in ^{13}C-NMR spectrum, 7 methyl groups at 12.8 ppm, 17.1 ppm, 18.3 ppm, 26.2 ppm, 33.5 ppm, 24.1 ppm chemical shift was observed which seems that 6 of those methyls are related to positions number 24,25,26,27,29 and 30 of the oleanane aglycon structure and the remaining one at chemical shift 17.5 is related to 6' position of glycon. In analyzing the ^{1}H-NMR and HSQC spectra of this compound, signals related to 7 methyl groups each with an integration worths 3 protons in chemical shifts 0.63 ppm, 1.13 ppm, 0.95 ppm, 1.22 ppm, 0.89 ppm, 0.90 ppm, 1.02 ppm were related to protons at the positions 24,25,26,27,29,30 and 6', respectively. Signals of carbons in chemical shifts of 61.5 ppm, 62.1 ppm, 62.9 ppm, 65.2 ppm shows the existence of 4 hydroxy methyls at the chemical structure. Among those signals, it seems that the first two signals were related to carbons number 6'' and 6''' of two glucose glycon moiety and the two last signals were linked to carbons 23 and 28 of oleanane aglycon. The corelation between carbons at the positions 23 and 28 with hydroxy methyl protons at chemical shift 3.05 ppm, 3.60 ppm, 3.15 ppm, 3.48 ppm was approved via HSQC spectrum. Carbon signal at chemical shift of 64.6 ppm revealed existence of hydroxyl group on this carbon number 16. Carbons signal at 53.7 ppm and its correlation with peak of proton at the chemical shift of 3.12 ppm with an integration of three protons shows the existence of a methoxy at the structure. Carefully, analyzing the ^{13}C-NMR spectrum of this compound, existence of several peaks at the chemical shift between 60-80 ppm (carbons of sugar moiety), three peaks at chemical shifts 102.3 ppm, 103.5 ppm, 104.0 ppm (anomeric carbons) and one peak at chemical shift 17.5 ppm (sugar methyl) approved the existence of three sugar moiety in the structure. In ^{1}H-NMR spectrum, existence of anomeric protons at 4.68 ppm 4.32 ppm and 4.49 ppm each with an integration worthed for one proton and a bulk of covered peaks at the range of 3-4 ppm ascertained existence of three suger in the structure. Conclusively, finding the exact type, position of data related to ^{1}H-NMR and ^{13}C-NMR spectrum and bondings among glycones and aglycon were determined based on the data from the COSY and HSQC 2D-NMR spectra and also comparing them with data extracted data from the literature online [38]. As a final point, the proposed structure for compound 2 was an oleanane saponin named buddlejasaponin IVa. The chemical structures of the three identified compounds are depicted in Fig. 1.

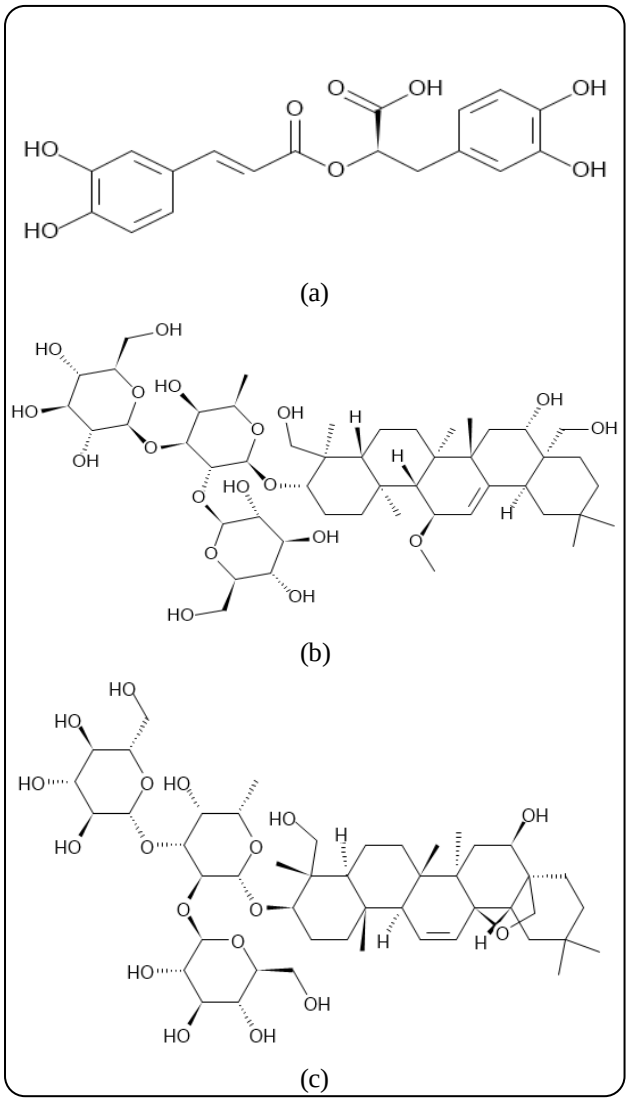

Fig. 1: Chemical structure of compounds 1-3 isolated from aerial parts of C. umbrosum: (a) Rosmarinic acid, (b) Buddlejasaponin IVa, (c) Buddlejasaponin IV.

Likewise, analyzing the ^{1}H-NMR and ^{13}C-NMR spectra for compound 3 was also indicative of a saponin aglycon with 30 carbons and three 6- carbonic glycons, totally 48 carbons at the chemical structure. In ^{13}C-NMR spectrum, two peaks at chemical shifts of 132.0 ppm and 130.7 ppm were related to the alkenic double bond between carbons number 11 and 12 of an oleanane saponin aglycon. If we do not consider some exceptions, the NMR data of this compound was really close to the previous structure identified as buddlejasaponin IVa. Nonetheless, we might mention the lack of methoxy group at carbon 11 and relocation of double bond from carbons 12 and 13 to carbons 11 and 12 resulting in formation of an epoxy group between carbons 13 and 28. The existence of a peak related to carbon 13 at chemical shifts of 83.12 ppm and carbon 28 at 72.2 ppm, verifies the existence of the mentioned epoxy group (furan group consisted of carbons number 13,17,18 and 28). Also, in ^{13}C-NMR spectrum, 7 methyl groups were observed at 12.2, 18.4, 19.8, 20.6, 33.8 and 24.0 ppm corresponding to 6 methyl groups at positions 24, 25,26,27,29 and 30 of oleanane aglycon structure and the last methyl at 17.1 ppm was related to 6' position of glycon. Same as the compound 2, multiple peaks at 60-80 ppm (sugar moiety region), and three peaks at δ 102.3, 103.5 and 104.0ppm (anomeric carbons) and one peak at δ 17.1 ppm (rhamnose methyl) shows the existence of three sugar moiety in this structure. Exact position of other data related to carbon and proton signals in ^{1}H-NMR and ^{13}C-NMR spectra related to this structure were retrieved from COSY and HSQC 2D spectra and the ultimate elucidated structure for compound 3 was an oleanane saponin, buddlejasaponin IV. As far as we know, determination of carbohydrates covalent structure between C–C, C–H, and O–H bonds defining the ordering of these atoms within a molecule through 1D-NMR (^{1}H-NMR & ^{13}C-NMR spectra) remains a challenging analytical problem. Thus 2D-NMR spectra especially heteronuclear multiple bond coherence spectroscopy (HMBC) seems to be mandatory even if for determining the planar structure of the unknown sugar moiety. Besides, one should apply nuclear overhauser spectroscopy (NOESY) to assign through-space connections, configuration at stereocenters and geometrical isomerism. However, here in this study we could not establish the mentioned HMBC and NOESY spectra. Fortunately, the suggested saponin structures with the defined sugar moieties were previously reported in the published article by *Yamamoto et al.*, matching the obtained spectroscopic data with those published in the literature [25] affirmed the elucidated structures.

The only phytochemical analysis on *C. umbrosum* has been conducted by Lee *SM et al.* at 1993 in which they have studied different fractions of 95% ethanolic extract of whole plant in Taiwan, fifteen crystalline components were isolated and identified, including five steroids (α-spinasterone, β-sitosterol, stigmasterol, α-spinasterol, and α-spinasteryl-3-O-β-glucopyranoside), four triterpenoids (3β - hydroxyurs - 11 - en - 28.13 - olide, betulinic acid, oleananeolic acid, ursolic acid), four flavonoids (luteolin, luteolin - 7 - O - β- glucopyranoside, apigenin - 7 - O - β -

glucuronide, and apigenin - 7 - O - β - methylglucuronate) and two lignolic acids [3 - (3,4 - dihydroxyphenyl) - lactic acid and rosmarinic acid] [39].

Total flavonoid and total phenolic contents of the *C. umbrosum* MeOH extract were calculated as 4.25 mg of equivalent to quercetin standard and 5.14mg of equivalent to gallic acid standard in 100g dry plant material, respectively. In addition, DPPH free radical scavenging potential of the MeOH extract inhibiting 50% of the DPPH radicals was calculated as 38.52µg/mL and for quercetin it was 6.73µg/mL. Analyzing the results of antioxidant assay showed that low concentrations of MeOH extract revealed a high radical scavenging activity and as the concentration grows high, trapping the DPPH free radical also increased. It seems that the mentioned antioxidant activity is in line with the high percentage of phenolic and flavonoid components in the MeOH extract of *C. umbrosum*. Previous studies upon other species of this genus such as *C. vulgare C. bolivianum* and *C. chinese* showed a direct relationship between the antioxidant activity and percentages of phenolic and flavonoid compounds in the extract.

CONCLUSIONS

The phytochemical analysis of *C. umbrosum* aerial parts showed presence of an ester of caffeic acid and two triterpenoid saponins as the foremost abundant components of the MeOH extract from this plant. Gathering all the data together, we might claim that three compounds namely rosmarinic acid, buddlejasaponin Iva and buddlejasaponin IV were structurally elucidated as the major components of the MeOH extract for the first time in *C. umbrosum*. Likewise, high content of phenolic and flavonoid compounds in the MeOH extract was detected corresponding for the extracts high potential trapping free radicals of DPPH.

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