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ISOLATION AND IDENTIFICATION OF SOME COMPOUNDS FROM *CYCLAMEN ROHLFSIANUM* (PRIMULACEAE) FROM LIBYA

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ABSTRUCT

Cyclamen rohlfsianum (Primulaceae family) is one of endemic plants growing in Libya (AL-Jabal AL-Akdar region), which is known locally as Rakf. It used in folk medicine in treatment of diabetic and the local Bedouin are using the tuber in the fermentation process of milk to produce cheese. This is the first chemical study on the plant. The chemical screening revealed the presence of phenolics, triterpenoids, saponins and steroidal compounds. Through chromatographic techniques of acetone extract (aerial parts), one compound was obtained and identified as Kaempferol in addition to four pure compounds obtained from hydrolyzed methanol extract (tubers), which were identified as Genistein, Hesperetin, Oleanolic acid and 7, 8, 4`-Trihydroxyflavone. The last compound is reported from primulaceae family for the first time. The structure of these compounds had been elucidated using spectroscopic methods (IR, ^{1}H, ^{13}C-NMR and MS). Two dimensions nuclear magnetic resonance technique (2D-NMR) and attached proton test technique (APT) had been used to confirm the structure of these compounds.

Key words: *Cyclamen rohlfsianum*, Primulaceae, Libya, Kaempferol, Genistein, Hesperetin, Oleanolic acid and 7, 8, 4`-Trihydroxyflavone.

INTRODUCTION

The primulaceae is a family of perennial or annual herbs, including a number of popular garden ornamentals, such as *Primulas* and *Cyclamen* and the familiar wild *primrose*, *primula veris*[1,2]. A family with 20 genera and about 1000 species, cosmopolitan in distribution but more abundant in North temperate regions[3]. The family is divided into the following tribes: Primuleae, Cyclamineae, Lysimachieae and Samoleae[4,5]. They are represented in Libya by 5 genera and 6 species, *Cyclamen* (1 species), *Samolus* (1 species), *Androsace* (1 species), *Asterolinon* (1 species) and *Anagallis* (2 species)[3]. Economic uses; Although economically the primulaceae is mainly of ornamental importance, it's worth noting that *cyclamen purpurascens* (*C.europaeum*) (*Common cyclamen*) contains the poisonous glycoside cyclamin, while *Anagllis arvenis* was once an important medicinal plant and contains a poisonous glycoside similar to saponins. *Lysimachia vulgaris* yields a yellow dye and also has been reported as a febrifuge. Flowers of *Primula veris* are used for home-made wine[2]. The flowers are particularly rich in flavonoids; saponins are present in some species also phenolic esters while alkaloids appear to be absent. A thiocyanin pigments are

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common, but not betacyanins or betaxanthins[6,7] *C. rohlfsianum* is one of endemic plants of AL-Jabal AL-Akdar region. It is known as locally *Rakf*. According to chemical abstract, this is the first chemical study on *C. rohlfsianum* but through personal communication with Dr. Abdurazag A. Auzi that he had published some work on this species[8,9].

C. rohlfsianum is very distinct species with a very cork and roots appearing all over the lower surface. The leaves appear in late irregular tuber which has growing points distributed over the upper surface summer, and are broadly kidney shaped with broad triangular dentate lobes with promient ribs. The upper surface is a shiny bright green, either plainaw with an irregular silvergrey marbling in an uneven band. The lower surface of the leaves is either purplish or red (Fig. 1)[8]. It is present in the wild of AL-Jabal AL-Akhder and adjoining area between Benghazi and Darna. It grows from sea level to 450 m rocky and scrubby habitats[8,9]. In folk medicine, the local Bedouin were using the tuber or subterranean (the underground pant of the plant) in the formation process of milk to produce cheese. Also the tubers were prescribed to diabetic patients[9].

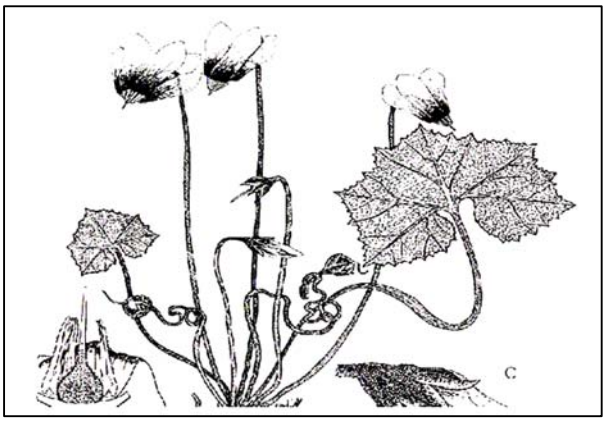

Fig. 1: Hand drawing of *cyclamen rohlfsianum*

EXPERIMENTAL

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer.^{1}H-NMR spectra were run at 300 MHz and ^{13}C-NMR spectra were run at 75.46 MHz in deutero dimethyl sulfoxide (DMSO-d_6). Chemical shifts are quoted in δ and were related to that of the solvents signals. IR (ν_{max} in cm^{-1}) spectra were recorded in KBr discs using Unicam Mattson FT-IR, 1000 series spectrometer. MS Finnigan mat SSQ7000 Ionization mode El eV 70 was used to record MS. Analytical thin layer chromatography (TLC) was carried out on precoated 0.25 mm silica gel plates with fluorescent indicator (Macherey-Nagel GF_{254}). Preparative TLC was conducted on glass plates (20 cm × 20 cm) coated with silica gel 60 and the spots were visualized either by UV light (254-366 nm) or I_2 vapor. Wet column chromatography was carried out using RDH silica gel S (230-400 mesh ASTM) and silica gel (70-230 mesh). The plant material of *cyclamen rohlfsianum* confined in the wild in AL-Jabal AL-Akhder and adjoining area between Benghazi and Darna, was collected from Ras Elhelal during April 2005, The plant was identified by Dr. Imhamed M. El-shareef (Botany Department of Benghazi University). Sample from the plants was deposited in the Herbarium Cyrenaica Benghazi University (CHGU), Benghazi. The aerial parts were allowed to dry in air and then grounded into a powder. Tuberous of the fresh plant were cut into small pieces and then kept in a covered bottles, with methanol.

Extraction of the tuberous *C. rohlfsianum*

The tuberous fresh (2 Kg) was extracted with methanol (6 L × 3) over 10 days at room temperature.

The combined extracts were evaporated under vacuum and concentrated using a rotator evaporator apparatus at 35°C to produce brown crude 45 g. The crude 40 g was dissolved in 500 mL of HCl (*2N*):MeOH (1:1) in a 1L round bottom flask (pH = 2-3.5) and kept for 24 hours. Then it was heated (under a reflex) on a water bath (70-80°C) for 8 hrs. The hydrolysed mixture was filtered and concentrated using a rotator evaporator at 35°C to produce 10 g crude. Extraction of the crude by chloroform and concentration by rotator evaporator at 35°C give 3 g crude. The crude was separated on preparative TLC to using solvent system CH_2Cl_2. The separation of the extract gave bands with different R_f values. The band at R_f = 0.35 was recovered. This band was washed by methanol and the methanol was evaporated to give yellow crude 0.6 g. This crude was subjected to column chromatography using silica gel 7 g (230-400 mesh) eluted with 100% *n*-hexane to give two compounds. The spectral data of Compound **N2** (4.6 mg) are as follows: **IR, ν_{max} (KBr):** OH (3500 cm^{-1}), C=O (1636.58 cm^{-1}), C=C (1581.12 cm^{-1}). **EI mass spectrum:** m/z = 302 (94.47 %) [$M^{+\bullet}$], 301 (25.71%), 153 (48.71%), 150 (61.23%), 124 (16.45%). ^1H and ^{13}C-NMR (3 mg) spectral data of compound **N3** were as follow: **IR, ν_{max} (KBr):** OH (3465.36 cm^{-1}), C=O (16362.27 cm^{-1}), C=C (1608.57 cm^{-1}). **EI mass spectrum:** m/z = 270 (96.18%) [$M^{+\bullet}$], 269 (5.94%), 152 (100%), 124 (6.1%), 118 (7.08%).

The base line of preparative TLC was recovered and washed with methanol. The methanol was evaporated to give a brown crude (2 g). This crude was subjected to column chromatography using silica gel 60 g (230-400 mesh) eluted with 100 % *n*-hexane followed by discontinuous gradient elution with *n*-hexane: CH_2Cl_2 (1:4-4:1) and 100% CH_2Cl_2 and the discontinuous gradient eluted with CH_2Cl_2: EtOAc (9.5:0.5-7:3) to give 16 fractions (100 mL each). Each fraction was examined by TLC using *n*-hexane:EtOAc (4:1, 7.5:2.5 and 3.5:1.5). Similar fractions were combined. Fractions (8-16) were combined. The mixture was separated by preparative TLC using *n*-hexane: EtOAc (7:3), CH_2Cl_2:EtOAc (9.5:0.5 and 1:1) as solvent system to give the compound **N5** (3 mg). The spectral data were as follows: **IR, ν_{max} (KBr):** OH (3406 cm^{-1}), C=O (1689 cm^{-1}), C=C (1465.12 cm^{-1}). EI mass spectrum: m/z = 456 (1.98 %) [$M^{+\bullet}$], 248 (100 %), 207(15.99 %), 203 (50.76 %), 189 (8.55 %). Use of EtOAc as system gave the compound **N1** (4 mg). Its spectral data are as follows: **IR, ν_{max} (KBr):** OH (3410 cm^{-1}), C=O (1653 cm^{-1}), C=C (1615 cm^{-1}). **EI mass spectrum:** m/z = 270 (100 %) [$M^{+\bullet}$], 118 (14.42 %), 153 (39.7 %), 269 (24.44 %), 242 (1.55 %).

Extraction of the aerial parts

The *C. rohlfsianum* aerial parts powder (400 g) was extracted with acetone (1L, 3 times) for 72 hours. The acetone extract was concentrated by rotary evaporator at 30°C. The crude mixture obtained (28 g) was chromatographed on silica gel (70-230 mesh) flash column chromatography[99,100]. Eluted with petroleum ether followed by discontinuous gradient elution with petroleum ether:$CHCl_3$ Petroleum ether (100%) to petroleum ether: $CHCl_3$ (1:1) fraction. It was separated by preparative TLC using $CHCl_3$ (100%) to give compound **N4** (3 mg). Its spectral data are as follows: **IR, ν_{max} (KBr):** OH (3316.62 cm^{-1}), C=O (1661.91 cm^{-1}), C=C (1612.95 cm^{-1}) **EI mass spectrum:** m/z = 286 (100 %) [$M^{+\bullet}$], 285 (31.93 %), 152 (5.58 %), 134 (3.09 %).

RESULTS AND DISCUSSION

Identification of compound N1

The IR spectrum revealed a band at 3410 cm^{-1} for the presence of hydroxyl group and strong interamolecular hydrogen bonding giving the resulting absorption at 3050-3150 cm^{-1}. The presence of intense absorptions at 1653, 1615.4 and 1570.3 cm^{-1} in the IR spectrum of the compound indicated the presence of a cross conjugated carbonyl, olefinic and aromatic functions in the molecule. The ^1H-NMR spectrum for the purified compound **N1** showed the resonance peak for aromatic protons and phenolic protons in the region of δ_H 6.22-8.23 ppm and δ_H 12.96-9.64 ppm, respectively. In addition, most of the

peaks were in the low field region except for peak at δ_H 3.34 for H_2O and peak at δ_H 2.5 for deuterodimethylsulfoxide (DMSO-d_6). The ^{1}H -NMR spectrum confirmed *AB* system with meta coupled protons identifying a tetra substituted benzene ring A, two doublets at δ_A 6.22 ppm (d, ^4J = 2.19Hz) and δ_B 6.36 ppm (d, ^4J = 2.19Hz) with one hydrogen each representing protons at H-6 and H-8, respectively, along with a singlet signal at δ_H 8.23 ppm characteristic of isoflavone H-2 on the ring C and with 5, 7-ioxygenated ring A[10,11]. The spectrum also revealed the benzenoid proton resonances arise from two sub spectra, an *AA`XX`* system ($\delta_{AA`}$ = 6.82 ppm, $^3J$ = 8.6 Hz) ($\delta_{XX`}$ = 7.37 ppm, ^3J = 8.6 Hz) representing protons at H-3,5` and H-2,6`, respectively. The *AA`XX`* part of the spectrum indicates a para-disubstiluted benzene ring, and locates one hydroxyl group in the 4`-position of the phenyl ring B. The spectrum showed the absorption of three hydroxyl groups at 9.5, 10.57 and 12.9 ppm. The absorption of hydroxyl group at 12.9 ppm indicates the presents of hydroxyl at C-5, Fig. 3 shows ring system by ^{1}H-NMR. The ^{13}C-NMR spectrum confirmed the presence of 15 carbon atoms from thirteen signals, all of them were sp^2 carbons. They include eight quaternary and seven secondary from APT technique. The resonance peaks in the low field region were assigned to aromatic groups. The ^{13}C-NMR spectrum displayed signal at δ_C 179.95 ppm, which is characteristic for C-4. It confirms the 5-hydroxyisoflavone structure. Signal at δ_C 153.45 ppm is representative of C-2 and signal at δ_C 122.14 ppm is distinctive of C-3. The flavonoid ring junctions appeared at δ_C 157.36 and 104.32 ppm for carbons C-9 and C-10 respectively. The other aromatic carbons attached to hydroxyl groups appeared at C-5, C-7 and C-4` at $\delta_C$ 161.79, 164.00 and 157.18 ppm, respectively. Other non-substituted aromatic carbons appeared at C-6, C-8, C-3,5` and C-2,6` at $\delta_C$ 98.77, 93.43, 114.87 and 129.85 ppm, respectively and other aromatic carbon quaternary appeared at C-1` at δ_C 121.07 ppm. APT spectrum exhibited a signal at δ_C 114.87 ppm typical of two carbons C-3` and C-5` (=C-H aromatic) and signal at δ_C 129.85 ppm is typical of C-2` and C-6` (=C-H aromatic), Fig. 3 shows ring system by ^{13}C-NMR[12,13]. The carbons signal at δ_C 114.87 and 129.85 ppm described a direct connectivity with proton signals at δ_H 6.82 and 7.37 ppm in the HMQC spectrum, respectively and the signals at δ_C 98.77 and 93.43 ppm, illustrated a direct connectivity with proton signals at δ_H 6.36 and 6.22 ppm, respectively. The EI-mass spectrum of compound **N1** showed a molecular ion peak [$M^{+\bullet}$] at m/z = 270 (100%) which corresponds to the molecular formula $C_{15}H_{10}O_5$.

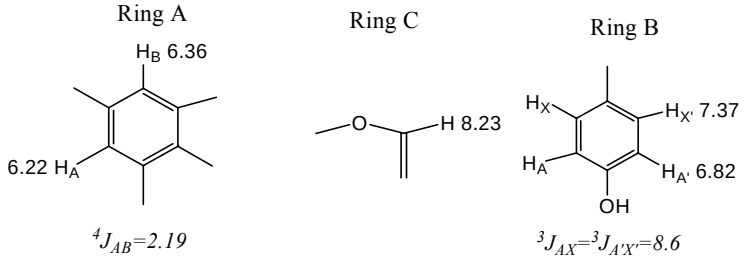

Fig. 2: Ring system by ^{1}H-NMR Compound N1

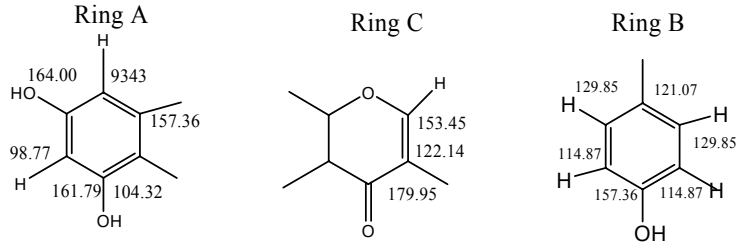

Fig. 3: Ring system by ^{13}C-NMR Compound N1

The **N1** compound undergoes Retro Diel's-Alder fragmentation (RDAF) to give two important peaks at m/z = 118 [C_8H_5O] (14.42%) and 153 [$C_7H_5O_4$] (39.7%). Other important fragments are m/z = 269 [$M^{+\bullet}$-H] (24.44%) and 242 [$M^{+\bullet}$-CO] (1.55%). On the basis of the spectral data discussed above, and by comparative literature analysis[14,15], the compound was identified as 5,7,4`-trihydroxyisoflavone "Genisteinn". This is the first report in the *cyclamen* genus.

Identification of compound N2

The IR spectrum showed absorption for hydroxyl group at 3500 cm^{-1}, a strong interamolecular hydrogen bonding giving the resulting absorption at 2500-3100 cm^{-1} (broad), C-H stretching sp^3 at 3117.77 cm^{-1}, carbonyl group 1636.58 cm^{-1} and aromatic double bond 1581.12 cm^{-1}.[15] The ^{1}H-NMR spectrum exhibited five signals for hydrogen sp^2 aromatic at δ_H 5.4-6.94 ppm and six sp^3 at 2.7-5.45 ppm; thus, the pattern indicated flavanone[16]. *AB* system with two meta coupled proton identifies a tetra substituted benzene ring A at δ_A 5.89 ppm (d, ^4J = 2.19 Hz) and at δ_B 5.91 ppm (d, ^4J = 2.19 Hz). The ring C at H-2 doublet of doublet at δ_H 5.40 ppm (^3J = 3.3, ^3J = 12.10 Hz), doublet of doublet at 2.7 ppm (^3J = 3.3, ^2J = 17.13 Hz) and doublet of doublet at 3.2 ppm (^3J = 12.10, ^2J = 17.13 Hz) due to two hydrogen at C-3. These data implied flavanone with 5, 7-dioxygenated ring A. The spectrum also showed signals at 6.86-6.94 ppm and their integration appeared as three protons representing 2`,5` and 6` for ring B protons. This pattern indicts a 3`,4`-disubstitued ring B[17]. The spectrum illustrated the absorption of three hydroxyl groups at 8.9, 10.6 and 12 ppm. The absorption at 12 ppm was assigned for the hydroxyl group present at C-5 and a singlet represent three hydrogen at 3.78 for O-CH_3. It shows ring system by ^{1}H-NMR[18]. The spectrum indicated sixteen carbons including nine quaternary, five secondary, one triplet and one methoxyl group from APT technique. The C^{13}-NMR spectrum confirmed signals at δ_C 195.72, 77.96 and 41.88 ppm typical of C-4, C-2 and C-3, respectively, which confirms the flavanone structure. The chemical shifts at δ_C 162.53, 166.40 and 146.35 ppm are for three aromatic carbons connected to hydroxyl groups at C-5, C-7 and C-3, respectively. The flavonoid ring junctions appeared at δ_C 163.24 and 101.65 ppm for carbons C-9 and C-10, respectively Fig. 5 shows ring system by ^{13}C-NMR.

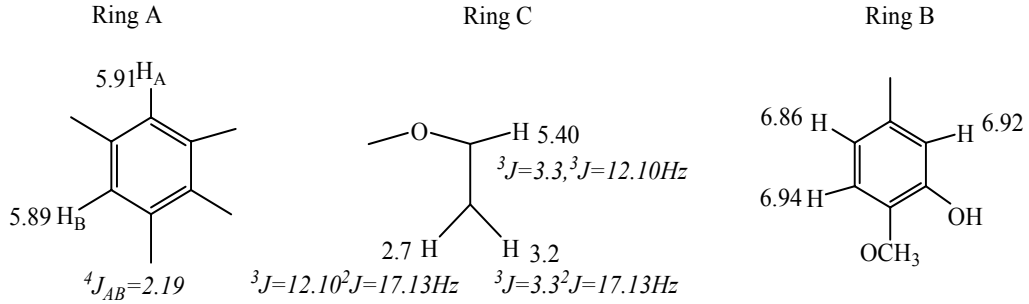

Fig. 4: Ring system by ^{1}H-NMR Compound N2

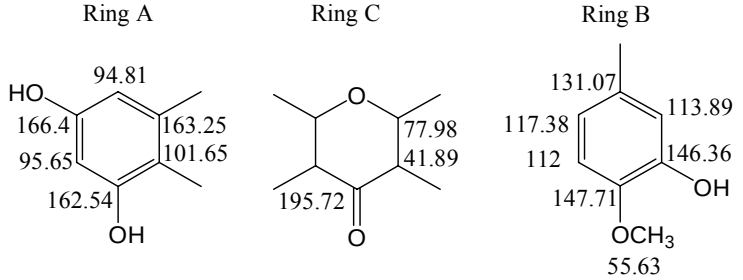

Fig. 5: Ring system by ^{13}C-NMR Compound N2

The ^{13}C-NMR spectral data of compound **N2** compared with previously reported data of Hesperetin (II) showed great similarity[18,19]. The EI-mass fragmentation pattern of compound **N2** was in full agreement with 5,7,3`-trihydroxy-4`-methoxyflavanone structure, which gave a molecular ion peak at m/z = 302 (94.47%) and base peak at m/z = 137 (100%), which showed the Retero Diel's-Alder Fragmentation (RDAF) of ring C to produce the ion at m/z = 153 [$C_7H_5O_4$] (48.71%), 149 [$C_9H_9O_2$] (4.48%). The RDA fragment at m/z 153 arising from ring A was observed, which indicates the existence of two hydroxyl group on the ring, and an important fragment at m/z = 301[$M^{+\bullet}$-H] (25.71%)[20]. With the help of the spectral data discussed above, the compound was identified as 5, 7, 3`-trihydroxy-4`-ethoxyflavanone "Hesperetin". The common Hesperetin was never been reported from the *cyclamen* genus.

Identification of compound N3

^{1}H-NMR and ^{13}C-NMR spectra indicated one compound of well defined and separated signals. The IR spectrum exhibited strong absorption bands at 1632.27, 1608.57 and 1573.5 cm^{-1} due to the presence of carbonyl, olefinic and aromatic functions respectively. The appearance of broad absorption at range 2500 - 3465.3 cm^{-1} indicated the presence of hydroxyl function in the structure[21]. ^{1}H-NMR signals illustrated one olefinic and six aromatic protons at range δ_H 6.7-7.98 ppm and the singlet signal at δ_H 6.7 ppm due to the H-3. The ^{1}H-NMR spectrum showed *AB* system with ortho coupled proton, which identifies a tetra substituted benzene in ring A at δ_B 7.4 ppm (d, ^3J = 8.54Hz). The spectrum also revealed the benzenoid proton resonances arising from two sub spectra, an *AA`XX`* system $\delta_{XX`}$ 7.98 ppm (d, $^3J$ = 8.78 Hz). The spectrum also showed signals at 6.91-6.94 ppm and their integration appeared as three protons representing 6,3`and 5` for rings B and A protons. The *AA`XX`* part spectrum indicates a para-disubstituted benzene ring, and locates one hydroxyl group in the 4`-position of the phenyl ring B. The three hydroxyl groups also exhibited signals at δ_H 10.22, 9.35 and 10.19 ppm. Fig. 6 viewed ring system by ^{1}H-NMR. The C^{13}-NMR spectrum confirmed the presence of 15 carbon atoms from thirteen signals; all of them were sp^2 carbons. APT spectrum showed a signal at δ_C 115.69 ppm, which is characteristic of two carbons C-3` and C-5`. Signal at δ_C 128.16 ppm is characteristic of two carbons C-2` and C-6` and signals at δ_C 103.80, 114.94 and 113.67 ppm were typical of C-3, C-5 and C-6, respectively.

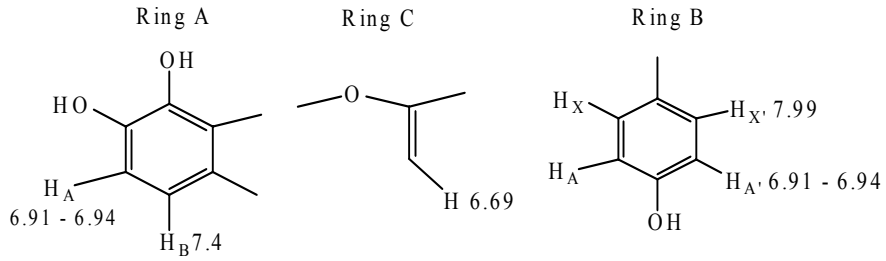

Fig. 6: Ring system by ^{1}H-NMR of Compound N3

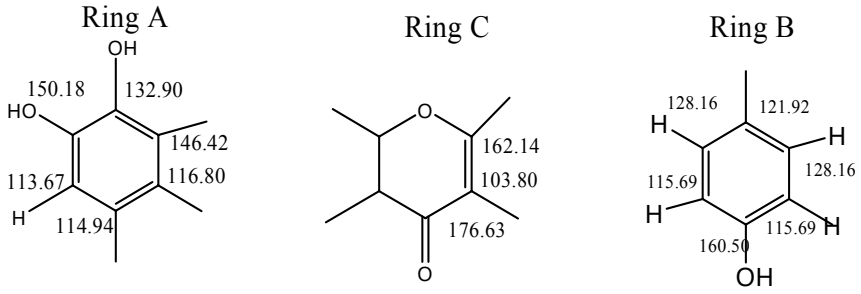

Fig. 7: Ring system by ^{13}C-NMR of Compound N3

The spectrum gave signal at δ_C 176.63 ppm, which is characteristic of C-4. The flavonoid ring junctions appeared at δ_C 146.42 and 116.80 ppm for carbons C-9 and C-10, respectively. The other aromatic carbons attached to hydroxyl groups appeared at C-7, C-8 and C-4` at δ_C 150.18, 132.90 and 160.50 ppm, respectively. A comparison of carbon resonances of isolated flavone and the literature data[22] has been made, Fig. 7 explains the ring system by ^{13}C-NMR. In HMQC spectrum, it showed δ_C 116.80 and 128.16 ppm and a direct connectivity with proton signals at δ_H 6.9 and 7.99 ppm, respectively. The signals at 103.80, 114.94 and 113.67 ppm showed a direct connectivity with proton signals at 6.7, 7.4 and 6.86 ppm. The EI-mass spectrum of compound **N3** revealed a molecular ion peak [$M^{+\bullet}$] at m/z = 270 (96.18%), which is corresponding to the molecular formula $C_{15}H_{10}O_5$. The compound **N3** undergoes Retero Diel`s-Alder fragmentation (RDAF) to give important peaks at m/z = 118 [C_8H_5O] (7.08%), 153 [$C_7H_5O_4$] (21.53%) and 152 [$C_7H_4O_4$] (100%). The spectrum also displayed other major fragments at m/z = 242 [$M^{+\bullet}$-CO] (4.12%) and 241[$M^{+\bullet}$-HCO] (4.81%). The spectroscopic evidence and comparative study was in conformity with structure and compound was identified as 7, 8, 4`-trihydroxyflavone. This is the first report of 7, 8, 4`-trihydroxyflavone from the primulaceae family.

Identification of compound N4

Compound **N4** was isolated in an amorphous form from the acetone crude extract of the aerial part. The confirmation of its identity as kaempferol was mainly based on comparison of its spectral data with those reported in literature. The presence of absorption bands at 1661.91, 1612.95 and 1568.5 cm^{-1} in the IR spectrum of the compound were indicative of carbonyl, olifinic and aromatic double bonds in the molecule. The broad absorption at 2500-3500 cm^{-1} appeared due to the hydroxyl function. The H^1-NMR data for compound **N4** is similar to literature values for kaempferol[23,24]. Signals for six sp^2 aromatic protons (δ_H 6.2-8.03 ppm) and its pattern indicated flavonol. The ^{1}H-NMR spectrum of compound **N4** explained *AB* system ring A with meta coupling, two doublet protons at δ_A 6.19 ppm (H-6, ^4J = *2.06* Hz) and δ_B 6.43 ppm (H-8, ^4J = *2.06* Hz), the protons at C-6 and C-8 of flavonols, which contain the common 5,7-dihydroxy substitution pattern giving rise to two doublets in the range 6.0-6.5 ppm. The H-6 doublet occurs consistently at higher field than the signal for the H-8. In the spectrum at δ_H 6.93 ppm, another two proton signals were also observed, which coupled and bonded to C-5` and C-3` atoms. Thus, doublet protons 3`,5`-H have coupling constant of 8.78 Hz .Two doublet protons were also recorded in the NMR spectrum at 8.03 ppm 2`,6`-H with a coupling constant of 8.78 Hz. The spectrum was showed the benzenoid proton resonances arise from two sub spectra, an *AA`XX`* system, and indicates a para-disubstituted benzene ring, and locates one hydroxyl group in the 4`-position of the phenyl ring B. Additionally singles were recorded at 9.5-10.8 and 12.4 ppm, which represent four hydroxyl groups bonded with four different carbon atoms, Fig. 8 explains the ring system by ^{1}H-NMR. The C^{13}-NMR spectrum of this compound exhibits thirteen signals representing fifteen carbons skeleton including eight quaternary and seven secondary from APT technique. There were overlapping of four non-substituted aromatic carbons at δ_C 129.21 and 115.20 ppm being in the same environment.

The signal at δ_C 129.21 ppm were assigned to carbons C-2` and C-6 and the signal at $\delta_C$ 115.20 ppm were assigned to C-3` and C-6`. The flavonoid ring junctions appeared at $\delta_C$ 155.98 and 121.46 ppm for carbons C-9 and C-10, respectively. Other aromatic carbons attached to hydroxyl groups appeared at C-3, C-5, C-7 and C-4` at δ_C 135.35, 160.48, 163.66 and 158.94 ppm, respectively. The other aromatic carbons non-substituted appeared at C-6 and C-8 at δ_C 98 and 93.25 ppm, respectively, and other quaternary aromatic carbons appeared as C-1` at δ_C 121.46. The carbonyl carbon, C-4 resonates at around δ_C 180-182 ppm, when the carbonyl is not hydrogen bonded, but in the presence of hydrogen-bonding to a 5-hydroxyl group, it moves down field to about δ_C 175-178 ppm. When 3-hydroxyl is present as well as a 5-hydroxyl, the

resonance returns to about δ_C 176 ppm, but with the 3-hydroxyl alone, the resonance appears at about δ_C 171-173 ppm[25,26]. A comparison of carbon resonances of isolated flavonol was made with the literature data of kaempferol[27-29], Fig. 9 illustrated ring system by ^{13}C-NMR.

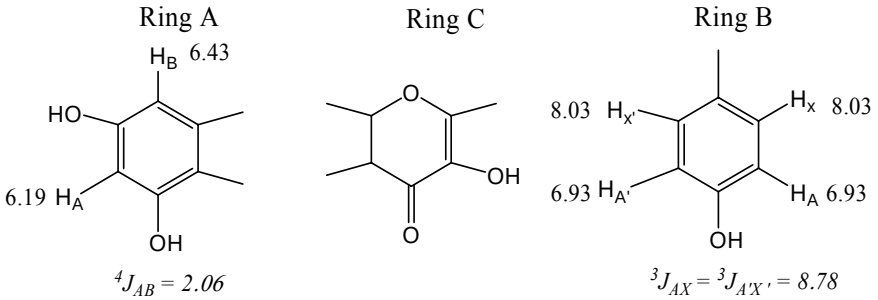

Fig. 8: Ring system by ^{1}H-NMR of Compound N4.

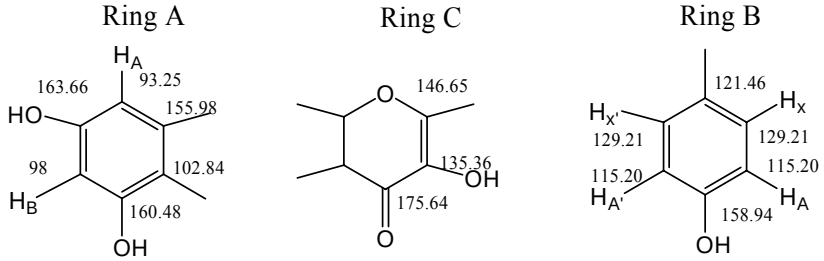

Fig. 9: Ring system by ^{13}C-NMR of Compound N4

The ^{1}H-NMR and ^{13}C-NMR spectra confirmed that isolated compound **N4** was 3, 5, 7, 4`-tetrahydroxyflavonol "Kaempferol". However, the carbons signal at δ_C 98 and δ_C 93.25 ppm corroborated a direct connectivity with proton signals δ_H 6.19 ppm and δ_H 6.43 ppm in the HMQC spectrum, respectively and δ_C 129.21, 115.20 ppm corroborated a direct connectivity with proton signals at δ_H 8.03 ppm and δ_H 6.93 ppm, respectively. The mass spectrum of the compound **N4** gave a molecular ion peak at m/z = 286 MHz (100 %) [$M^{+\bullet}$]. The peak at m/z 285 (31.93%) was due to the loss of H radical from the [$M^{+\bullet}$]. Another peaks at m/z = 258 (8.21%) and 257 (10.55%) resulted from the loss of C=O carbonyl group and the loss of C=O carbonyl with hydrogen, respectively. The spectrum also indicated that Retero Diel`s-Alder fragmentation (RDAF) gave two important peaks at m/z = 152 (5.58%) and 135 (3.20%)[30-32].

The compound was identified as 3,5,7,4`-tetrahydroxyflavonol structure known as kaempferol, The common kaempferol was never reported from the *C. rohlfsianum*, although it was reported from many *cyclamen* species.

Identification of compound N5

The IR spectrum of the compound revealed the presence of an acidic hydroxyl, carboxyl and an olefinic functions in the molecule supported by absorption broad at 3406-2859 cm^{-1}, 1689 and 1465 cm^{-1}. The ^{13}C-NMR spectrum was indicated 30 carbon resonances. The APT technique suggested the presence of five methines, ten methylenes, seven methyl groups and eight quaternary carbon atoms. The two olefinic signals, the singlet at δ_C 143.74 and the doublet at 121.46 ppm are certainly due to C-13 and C-12, respectively. The carbon signal due to C-18 at δ_C 40.83 ppm, is characteristic of olean-12-ene. The carbonyl carbon signal due to C-28 carboxyl carbon at δ_C 178.31 ppm and the carbon signal due to C-3 was observed at δ_C 76.85 ppm. A comparison of carbon resonances of **N5** and oleanolic acid[32-33] revealed a complete agreement in all data. The ^{1}H-NMR spectrum showed a one olefinic proton as

triplet δ_H 5.16 ppm (^3J = 3.3Hz) due to the H-12, which fully supports the presence of unsaturation in the structure. Another downfield signal appearing as a triplet at δ_H 3.01 ppm (^3J = 6.9Hz) was assigned to H-3 on the basis of literature report[34-36]. One proton double doublet resonated at δ_H 2.76 ppm (^3J = 13.8 and 3.9Hz) was assigned to H-18 on the basis of its chemical shift value, as well as on multiplicity pattern reported for H-18 with (β)-stereochemistry. The resonance from 1.16 to 2.0 ppm was assigned as multiple to the ($-CH_2$) groups. Additionally, one single proton was recorded at 12 ppm, which represents hydroxyl group of carboxylic acid. However from HMQC correlation and the significant feature of ^{1}H-NMR spectrum of this compound was the presence of seven tertiary methyl signals resonated as sharp singlet at δ_H 1.10, 0.88, 0.85, 0.67, 0.71, 1.08, 0.86, and 0.86 ppm and these were correlated to C-14, C-23, C-24, C-25, C-26, C-27, C-29 and C-30, respectively. The signals at δ_H 2.76, 5.16 and 3.01 ppm were correlated to C-18, C-12 and C-3. The mass spectrum of the compound displayed molecular ion peak at m/z = 456, which was in agreement with molecular formula $C_{30}H_{48}O_3$. The position of hydroxyl group, methyl groups and a substituted olefinic bond (through NMR analysis), suggests that the compound might be olean-12-ene. It is further supported by the characteristic retro-Diel's-Alder fragmentation of Δ^{12}-pentacylic triterpene skeleton giving rise to peaks at m/z = 207 and 189, which indicate the presence of one hydroxyl group in rings A/B. Also two peaks at m/z = 248 and 203 strongly indicates the presence of carboxylic group on ring D/E. The fragmentation pattern was consistent with the oleanolic acid structure.

On the basis of the spectral data discussed above, and by comparative literature analysis[37-39], the compound was identified as oleanolic acid structure. It was never reported from the *C. rohlfsianum*, although it was reported from many *cyclamen* species.

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