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Flavonoids and Tannins from *Anogeissus latifolia*

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Abstract: *Anogeissus latifolia* (Roxb. ex DC), Family Combretaceae, is considered as one of the most useful trees in India as the main source of the commercial Indian gum, known as ghatti gum and used for tanning processes. In traditional medicine it is reported for the treatment of many diseases like UTI infections, cardiac disorder and liver complaints and proved to have a wide range of biological activities due to its high content of tannins, appreciable quantities of flavonoids, terpenes and saponins.Two dimensional paper chromatography investigation of the 70% aqueous methanol leaf extract revealed the presence of high polyphenolic contents of versatile structures. This study describes the chromatographic isolation and identification of four flavonoids and three tannins metabolites for the first time from the leaf and stem methanol-soluble portions. Their structures were established as vitexin, isovitexin, isoorientin, orientin, gallagic acid bilactone (terminalin), puncalin and 2-*O*-galloylpunicalin based on the chromatographic properties, spectroscopic analyses (^{1}H and ^{13}C NMR) and HRESI-MS.

Key words: *Anogeissus latifolia*; Combretaceae; HRESI-MS; ^{1}H and ^{13}C NMR; Flavonoids; Tannins

1. INTRODUCTION

Combretaceae is a family of flowering plants in the Myrtales order, has over 600 species in 23 genera. Mostly found in tropical and subtropical Africa, but also in central and southern America, southern Asia, and northern Australia, it is made up of trees, shrubs, and lianas.[1]. *Anogeissus* is a very big genus in the Combretaceae family and is found all over the world in tropical and subtropical regions as trees, shrubs, and small trees. It has been used to cure a variety of ailments in traditional medicine, including diarrhoea, colic, stomach sickness, cough, and numerous skin conditions like ulcers, boils, psoriasis, and itching[2]. *A. latifolia* (Roxb. ex DC.) is a medium-sized deciduous tree that is indigenous to Sri Lanka, India, Myanmar, and Nepal. It is also referred to as an axle-wood tree, a button tree, a dindiga tree, a gum-ghatti tree, a baklee tree, a dhaura tree, and an Indian gum tree [3]. The herb has been employed in conventional medicine to treat liver issues, UTI infections, heart disorders, and anaemia [4]. It has been stated that the bark extract can be used to cure snake and scorpion bites, stomach disorders, colic, cough, diarrhoea, fever, and a number of skin conditions, including ulcers, boils, and itching [5]. Also the bark and the leaf extracts showed demulcent and astringent properties[6] alongside a wide range of biological activities, e.g. antiulcer, antimicrobial[7], antioxidant[8, 9], anthelmintic[10], antiplasmodial[11], antidiabetic[12, 13], anticonvulsant[14] and hepatoprotective[15] activities. Moreover, their ethanolic extracts have shown strong cytotoxicity against human cancer cell lines, including lung (A549), prostate (PC-3), breast (T47D), colon (HCT-16), and leukaemia (THP-1, and HL-60)[16]. From the phytochemical point of view, the plant was proved to be a rich source of tannins and having appreciable quantities of flavonoids, terpenes and saponins[4-6].Several tannins have been isolated from the plant as 3,4,3'-tri-*O*-methylellagic acid, 3,4,3'-tri-*O*-methylflavellagic acid, 3,3'-di-*O*-methylellagic acid 4'-β-D-xyloside, 3,3'-di-*O*-

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methylellagic acid 4'-β-D-glucoside[6], chebulic acid[17], corilagin[18] and trigallic acid[5,17]. Quercetin, myrecetin[19], luteolin 7-*O*-β-D-galactopyranosyl-(1'''→6'')-*O*-β-D-galactopyranoside[20] and quercetin 3-*O*-β-D-galactopyranosyl-(1'''→4'')-rhamnopyranoside[5] were the major flavonoids reported in the plant. Also 3-β-hydroxy-28-acetyltaraxaren and β-sitosterol were reported in the plant[4]. Although many different genera of family Combretaceae attracted the attention of several authors from phytochemical point of view, little work appears to have been reported on *A. latifolia*. Furthermore, phytochemical screening of the plant and 2D-PC investigation of the 70% aqueous methanol leaf extract revealed the presence of high polyphenolic contents of versatile structures. So it was interesting to carry out more detailed phytochemical investigation for the methanolic *A. latifolia* leaf extract.

2. METHODS

2.1. Plant material

A. latifolia (Roxb. ex DC.) leaves were collected from Giza, Zoo Garden, Egypt, in April 2019. The plant was identified by Mrs. Trease Labib, senior specialist of plant taxonomy, Orman Garden, Giza, Egypt, as well as by comparison with the reference herbarium specimens. Voucher specimen (code AL-1619) was deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.

2.2. Preparation of the crude extract

In a soxhlet apparatus, each of the air-dried powder of *A. latifolia* leaf (800 g) and stem (235 g) was exhaustively defatted with petroleum ether (40-60°C). The left plant marcs were individually extracted with 70% aqueous methanol under reflux (12 x 4L for leaf, and 12 x 3L for stem, 45°C, 3 days /each). Thereafter, the combined methanolic extracts were evaporated on a rotary evaporator at low temp. (45°C) and reduced pressure. The residues were separately taken with hot methanol under reflux (12 x 2L, for leaf, and 12 x 1L for stem, 45°C, 3 days /each) and the methanol-soluble portions of leaf and stem were then dried in vacuum at low temp.to produce 190 and 45g amounts, respectively.

2.3. Isolation and purification of the stem and leaf extracts

2.3.2. Leaf extract

Leaf methanol-soluble portion was fractionated on a polyamide column (120 x 5cm, 500g) using dist. H_2O followed by a gradient H_2O-MeOHwith decreasing polarity up to 100% MeOH. Then, similar fractions were combined according to the detection by UV-light and spray reagents on paper chromatography into four main collective fractions (A–D). Fraction B was subjected to cellulose column with 30% aqueous MeOH as an eluent to give three sub-fractions. Each sub-fraction was chromatographed on sephadex LH-20 column eluted with 30% MeOH followed by final purification on sephadex LH-20 column with MeOH to obtain chromatographically pure samples of **1** (23 mg) and a mixture of **2** and **3** (19 mg). Fraction C was subjected to a series of columns chromatography on sephadex LH-20 and /or cellulose with suitable solvent system in each case for the final purification to produce compound **4** (11 mg).

2.3.1. Stem extract

The methanol-soluble portion of stem was dissolved in the least amount of MeOH, and then precipitated with 300mL of EtOAc, followed by decantation. The precipitate was chromatographed on a series of sephadex LH-20 columns with a conventional solvent system in each case for the final purification to obtain three compounds **5** (46 mg), **6** (300 mg) and **7** (107 mg).

3. RESULTS

In this study, 4 flavone C-glycosides and 3 tannins metabolites were isolated from the methanol-soluble portions of the *A. latifolia* leaf and stem extracts for the first time, respectively using sequential column chromatographic fractionation on different conventional stationary phases e.g. polyamide, cellulose and/or sephadex. Theirstructures were

identified as vitexin **1**, isovitexin **2**, isoorientin **3**, orientin **4**, gallagic acid bilactone (terminalin **5**), puncalin **6** and 2-*O*-galloylpunicalin **7** on the basis of the chromatographic behavior and spectroscopic data (^{1}H and ^{13}C NMR) and negative HRESI-MS^2 mass spectrometry.

3.1. Characterization and identification of compound 1

Yellow amorphous powder (23mg); R_f-values: 0.43 (BAW), 0.29 (15% AcOH) on PC; deep purple spot, turned to faint yellow fluorescence in ammonia vapors and $AlCl_3$alongside greenish yellow with NP/PE (360 nm) and green color with $FeCl_3$; Negative HRESI-MS,: *m/z* 431.0963 $[M–H]^-$*calcd*. for 431.09783, 341.0663 $[M–H–90]^-$, 311.0551 $[M–H–120]^-$ and 283.0610 $[M–H–120–CO]^-$; ^{1}H NMR (400 MHz, DMSO-d_6),: δ ppm 13.60 (minor), 13.16 (each br s, 2 x H-bonded OH-5_{flvs}), 8.02 (d, J= 8.8 Hz, 2H, H-2'/6'$_{Vit}$), 7.92 (d, J= 8.4 Hz, 2H, H-2'/6'$_{Isov}$), 6.95 (d, J= 8.8 Hz, 2H, H-3'/5'$_{Vit}$hidden by minor d of H-3'/5'$_{isov}$), 6.77 (s, 1H, H-3_{vit}), 6.39 (s, 1H, H-6_{Vit}), 4.69 (d, J= 10 Hz, 1H, H-1"$_{Vit}$), 4.59 (d, J= 9.6 Hz, 1H, H-1"$_{Isov}$), 4.02 (t-like, J= 8.8 Hz, 1H, H-2"$_{Isov}$), 3.85 (t-like, J= 8.8 Hz, 1H, H-2"$_{Vit}$), 3.80 – 3.10 (m, remaining sugar protons); ^{13}C NMR (100 MHz, DMSO-d_6),: δ ppm182.5 (C-4), 164.8 (C-2), 163.1 (C-7), 161.6 (C-5), 161.0 (C-4'), 156.3 (C-9), 154.5 (C-9_{isov}), 129.5 (C-2'/6'), 122.2 (C-1'), 116.5 (C-3'/5'), 105.1 (C-8), 104.3 (C-10), 102.8 (C-3), 98.7 (C-6), 83.7 (C-5"$_{isov}$), 82.3 (C-5"), 79.0 (C-3"), 73.9 (C-1"), 71.8 (C-2"), 71.2 (C-4"), 61.8 (C-6").

3.2. Characterization and identification of compounds 2 and 3

Yellow amorphous powder (19mg); R_f-values: 0.57, 0.43 (BAW), 0.55, 0.39 (15% AcOH) on PC; two brown-deep purple spots, turned to yellow fluorescence in ammonia vapors, $AlCl_3$, and greenish-yellow/orange fluorescence in NP/PE (360 nm) and green color with $FeCl_3$; ^{1}H NMR (400 MHz, DMSO-d_6), Figure 2: δ ppm 13.60 (br s, 1H, OH-5_{Isoor}), 13.56 (br s, 1H, OH-5_{Isovit}), 7.93 (d, J= 8.8 Hz, 2H, H-2'/6'$_{Isovit}$), 7.41 (m, 2H, H-2'/6'$_{Isoor}$), 6.94 (d, J= 8.8 Hz, 2H, H-3'/5'$_{Isovit}$), 6.91 (d, J= 8.8 Hz, 1H, H-5'$_{Isoor}$), 6.78 (s, 1H, H-3_{Isovit}), 6.67 (s, 1H, H-3_{Isoor}), 6.53 (s, 1H, H-8_{Isovit}), 6.45 (s, 1H, H-8_{Isoor}), 4.60 (d, J= 9.6 Hz, 2H, 2x H-1"), 4.05 (t-like, J= 9.2 Hz, 2H, 2x H-2"), 3.80 – 3.10 (m, 10H, remaining sugar protons); ^{13}C NMR (100 MHz, DMSO-d_6), Figure 3: δ ppm182.6 (C-4_{Isov}), 163.8 (C-2_{Isov}), 161.8 (C-2_{Isov}), 160.9 (C-5_{Isov}), 156.7 (C-9_{Isov}), 128.9 (C-2'/6'$_{Isov}$), 121.5 (C-1'$_{Isov}$), 116.5 (C-3'/5'$_{Isov}$), 109.3 (C-6_{Isov}), 103.8 (C-10_{Isov}), 103.2 (C-3_{Isov}), 94.2 (C-8_{Isov}), 82.0 (C-5"$_{Isov}$), 79.4 (C-3"$_{Isov}$), 73.5 (C-1"$_{Isov}$), 71.0 (C-2"$_{Isov}$), 70.7 (C-4"$_{Isov}$), 61.9 (C-6"$_{Isov}$).

3.3. Characterization and identification of compound 4

Yellow amorphous powder (11 mg); R_f-values: 0.29 (BAW), 0.21 (15% AcOH) on PC; deep purple spot, turned to yellow fluorescence in ammonia vapors and $AlCl_3$alongside orange with NP/PE (360 nm) and green color with $FeCl_3$; ^{1}H NMR (400 MHz, DMSO-d_6), δ ppm 13.60 (br s, 1H, H-bonded OH-5), 7.51 (br d, J= 8.0 Hz, 1H, H-/6'), 7.47 (br s, 1H, H-2'), 6.87 (d, J= 8.4 Hz, 1H, H-5'), 6.62 (s, 1H, H-3), 6.27 (br s, 1H, H-6), 4.71 (d, J= 9.6 Hz, 1H, H-1"), 4.00 – 3.10 (m, remaining sugar protons hidden by H_2O signal).

3.4. Characterization and identification of compound 5

Yellow amorphous powder (46 mg); R_f-values: 0.57(BAW), 0.27(15% AcOH) on PC; deep mauve spot that turned to blue color with $FeCl_3$ and yellow fluorescence in ammonia vapors, $AlCl_3$ and NP/PE (360 nm); Negative HRESI-MS, Figure 4: *m/z* 601.0485 $[M–H]^-$; ^{1}H NMR (400 MHz, DMSO-d_6), Figure 5: δ ppm 7.52 (s, H-4/26); ^{13}C NMR (100 MHz, DMSO-d_6), Figure 6: δ ppm159.7 (C-6/28), 158.4 (C-14/18), 148.5 (C-3/25), 146.5 (C-13/19), 140.2 (C-2/24), 140.0 (C-1/23), 136.8 (C-12/15), 136.3 (C-7/20), 123.2 (C-8/21), 113.1 (C-11/16), 112.7 (C-9/22), 110.6 (C-4/26), 107.8 (C-5/27), 106.7 (C-10/17).

3.5. Characterization and identification of punicalin 6

Yellow amorphous powder (300 mg); R_f-values: 0.39 (BAW), 0.45 (15% AcOH) on PC; deep purple spot in UV-light (360/254 nm), turned to yellow fluorescence in ammonia vapors, $AlCl_3$ and NP/PE (360 nm), indigo-red color/$KIO_3$and deep blue color/$FeCl_3$; Negative HR-ESI-MS, Figure 7: *m/z* 781.1450 $[M–H]^-$ (*calcd*. for $C_{34}H_{22}O_{22}$–H, 781.0525 and MW 782.0503), 390.1761 $[M–2H]^{-2}$; ^{1}H NMR (400 MHz, DMSO-d_6), Figure 8: δ ppm 2.00 (br t, J = 11.2 Hz, Glc-H-6β), 2.41 (br t, J = 10 Hz, Glc-H-5β), 2.89 (br t, J = 10 Hz, Glc-H-6α), 2.96 (br t, J = 7.6 Hz, Glc-H-5α), 3.14 (m, Glc-H-2β,2α), 3.48 (t, J = 11.2 Hz, Glc-H-3β), 3.73 (t, J = 6.6 Hz hidden by H_2O signal, Glc-H-3α), 3.83 (t, J = 7.2 Hz, Glc-H-6β), 3.93 (t, J = 12.4 Hz, Glc-H-6α), 4.04 (m, Glc-H-1β,4α), 4.14 (t, J = 8.8 Hz, Glc-H-4β), 4.70 (br s, Glc-

H-1α), 6.46 (s, GG-H-4β), 6.73 (s, GG-H-4α), 6.76 (s, GG-H-6β), 6.92 (s, GG-H-6α);^{13}C NMR (100 MHz, DMSO-d_6), Figure 9: δ ppm 64.2 (Glc-C-6α), 64.6 (Glc- C-β6), 70.9 (Glc-C-3α), 71.2 (Glc-C-5α), 71.3 (Glc-C-2α), 72.0 (Glc-C-5β), 73.6 (Glc-C-4α), 74.0 (Glc-C-4β), 75.0 (Glc-C-2β), 75.2 (Glc-C-3β), 91.0 (Glc-C-1α), 96.7 (Glc-C-1β), 109.0 (GG-C-6"α), 109.2 (GG-C-6"β), 109.6, 109.7, 110.1, 110.2 (GG-C-6/6'α,β), 112.8 (GG-C-2'β), 112.9 (GG-C-2'α), 113.6, 114.1, 114.2, 114.3, 114.4 (GG-C-2/2"α,β), 117.8, 117.9 (GG-C-2"'α,β), 120.9, 122.1, 122.5, 122.6 (GG-C-1/1'α,β), 124.5, 124.6, 124.9, 125.3 (GG-C-1"/1"'α,β), 135.2, 135.4, 135.9, 136.1, 136.4, 136.8, 137.2, 137.3, 138.0, 138.1, 138.4, 139.2 (GG-C-4",3',4"',3,4,4'α,β), 144.1, 144.2, 144.3, 144.5, 145.0, 145.1, 145.9 (GG-C-5",3",3"', 5"'α,β), 147.6, 147.7, 147.8, 147.9 (GG-C-5',5α,β), 157.6 (GG-C-7'β), 157.7 (GG-C-7'α), 158.1 (GG-C-7α), 158.2 (GG-C-7β), 168.0 (GG-C-7"'β), 168.1 (GG-C-7"'α), 169.5 (GG-C-7"α), 169.9 (GG-C-7"β).

3.6. Characterization and identification of 2-galloylpunicalin 7

Yellow amorphous powder (107 mg); R_f-values: 0.31 (BAW), 0.25 (15% AcOH) on PC; deep purple spot in UV-light (360/254 nm), turned to yellow fluorescence in ammonia vapors, $AlCl_3$andNP/PE (360 nm), indigo-red color/KIO_3 and deep blue color/$FeCl_3$; Negative HR-ESI-MS, Figure 10: *m/z* 933.1954 $[M–H]^-$ (*calcd* for $C_{41}H_{26}O_{26}$–H, 933.0634 and MW 934.0712), 466.2309 $[M–2H]^{-2}$; ^{1}H NMR (400 MHz, DMSO-d_6), Figure 11: δ ppm 2.80-4.60 (m, remaining sugar protons of α/β-anomers), 4.70 (br d, J= 9 Hz, Glc-H-2α), 4.83 (t-like, J= 9 Hz, Glc-H-2β, hidden by H-1 β), 4.99 (br s, Glc-H-1α), 6.46 (s, GG-H-4β), 6.68 (br s, G-H-4α,6β), 6.93 (s, GG-H-6α), 6.99 (s, G-H-2/6α,β); ^{13}C NMR (100 MHz, DMSO-d_6), Figure 12: δ ppm 62.3 (Glc-C-6α), 64.3 (Glc- C-β6), 66.1(Glc-C-α3), 69.0 (Glc-C-α5), 72.3 (Glc-C-β5), 74.0 (Glc-C-2/4α), 74.5 (Glc-C-β4), 75.2 (Glc-C-β3), 77.2 (Glc-C-β2),88.8 (Glc-C-α1), 94.0 (Glc-C-β1), 105.6, 108.4 (GG-C-6"α), 109.3 (GG-C-6"β), 109.4, 109.7 (galloyl-C-2/6α,β), 110.3, 111.3, 112.2 (GG-C-6/6'α,β), 112.3 (GG-C-2'β), 113.0 (GG-C-2'α), 113.2, 113.3, 114.5, 114.6 (GG-C-2/2"α,β), 118.2, 118.5 (GG-C-2"'α,β), 119.7, 120.1 (G-C-1α,β), 122.1, 122.2, 122.8, 122.9 (GG-C-1/1'α,β), 124.2, 124.5, 124.9, 125.3 (GG-C-1"/1"'α,β), 135.2, 135.3, 135.7, 135.9, 136.8, 137.2, 137.3, 137.4, 139.0, 140.0, 140.1, 140.7,141.5 (GG-C-4",3',4"',3,4,4'α,β), 138.8 (G-C-4α,β), 144.5, 144.7, 144.8, 145.1 (GG-C-5",3",3"', 5"'α,β), 145.0 (G-C-3/5α,β), 145.9, 146.0 (GG-C-5',5α,β), 157.9 (GG-C-7'β), 158.2 (GG-C-7'α), 158.3 (GG-C-7α), 159.8 (GG-C-7β), 165.2, 166.0 (G-C-7α,β), 168.5 (GG-C-7"'β), 168.6 (GG-C-7"'α), 169.5 (GG-C-7"α), 169.7 (GG-C-7"β).

4. DISCUSSION

4.1. Characterization and Identification of compound 1

A deep purple spot was detected on the 2DPC of compound **1**that showed chromatographic properties of an apigenin-like *C*-glycoside structure. Negative ESI-MS^2 spectrum showed a molecular ion peak at m/z 431.0963 $[M-H]^-$ calculated for 431.09783 and corresponding to the MF $C_{21}H_{20}O_{10}$ and MW of 432.10565 amu. In addition two intrinsic fragment ions of *C*-glycosides were interpreted at *m/z* 341.0663 and 311.0551 describable for $[M–H–90]^-$ and $[M–H–120]^-$, respectively. Thus the MS data was consistent with the previous expected structure. Moreover,^{1}H NMR of 1, exhibited an A_2X_2-spin coupling system of two *ortho* doublets at δ ppm 8.02 and 6.95 (d, J= 8.8) that was indicative to 1,4-disubstituted ring B. Additionally, two aromatic singlets, each of 1H, were observed at 6.77 and 6.39 assignable for H-3 and H-6, respectively. Absence of H-8 signal together with the interpretation of an anomeric proton doublet at 4.69 (d, J= 10 Hz) was an evidence for a 8-*C*-glycoside structure. Further structural confirmation was achieved from ^{13}C NMR spectrum that demonstrated 13 characteristic C-resonances of an apigenin aglycone including 4 key signals at 182.5 (C-4), 161.0 (C-4'), 129.5 (C-2'/6'), 116.5 (C-3'/5') ppm. The attachment of the *C*-glycoside moiety to C-8 was followed from its downfield shift at 105.1 (Δ + ~10 ppm). The type and stereo structure of the sugar moiety was deduced to beβ-*C*-$^4C_1$-glucopyranose according to the δ and J-

values of H-1" and 2" in ^{1}H NMR and all typical δ values in ^{13}C NMR spectrum, particularly C-5", C-3" and C-1" that were interpreted at 82.3, 79.0, 73.9, respectively. Thus compound **1** was established as 8-β-*C*-$^{4}C_{1}$-glucopyranosylapigenin (Vitexin). It is worth mentioning that some signals were assigned in ^{1}H (H-2/6, 1 and 2) and ^{13}C NMR (C-5" and C-9) spectra for the presence of its positional isomer at C-6, i.e. isovitexin, see experimental data given above.

4.2. Characterization and identification of compounds 2 and 3

This Sample revealed chromatographic properties for a mixture of two *C*-glycosyls, based on an apigenin and luteolin aglycone moities. ^{1}H NMR spectrum (Figure 1) demonstrated two singlets each of one proton, highly deshieded at 13.60 and 13.56, corresponding to the two H-bonded OH-5. In addition, it exhibitedd an A2X2-splitting pattern of two *ortho* doublets at δ 7.93 and 6.94 (J= 8.8), each integrated for 2H, assignable for H-2′/6' andH-3'/5', respectively, of a 4-hydroxy B-ring in anapigenin aglycone. Simultaneously, an ABM-spin coupling system of three proton types was indicated at 7.41 (m, 2H) and 6.91 (d, J= 8.8), interpretable to H-2′/6' and H-5' of 3,4-dihydroxy B-ring in the minor metabolite. Similarly, both of the major and minor metabolites exhibited a pair of singlets, each of 1H, assigned at 6.78 (H-3_{Isovit}), 6.67 (H-3_{Isoor}), 6.53 (H-8_{Isovit}) and 6.45 (H-8_{Isoor}) to conclude 6-C-glycosidation structures due to the absence of the two H-6 resonances. Furthermore, both of the two

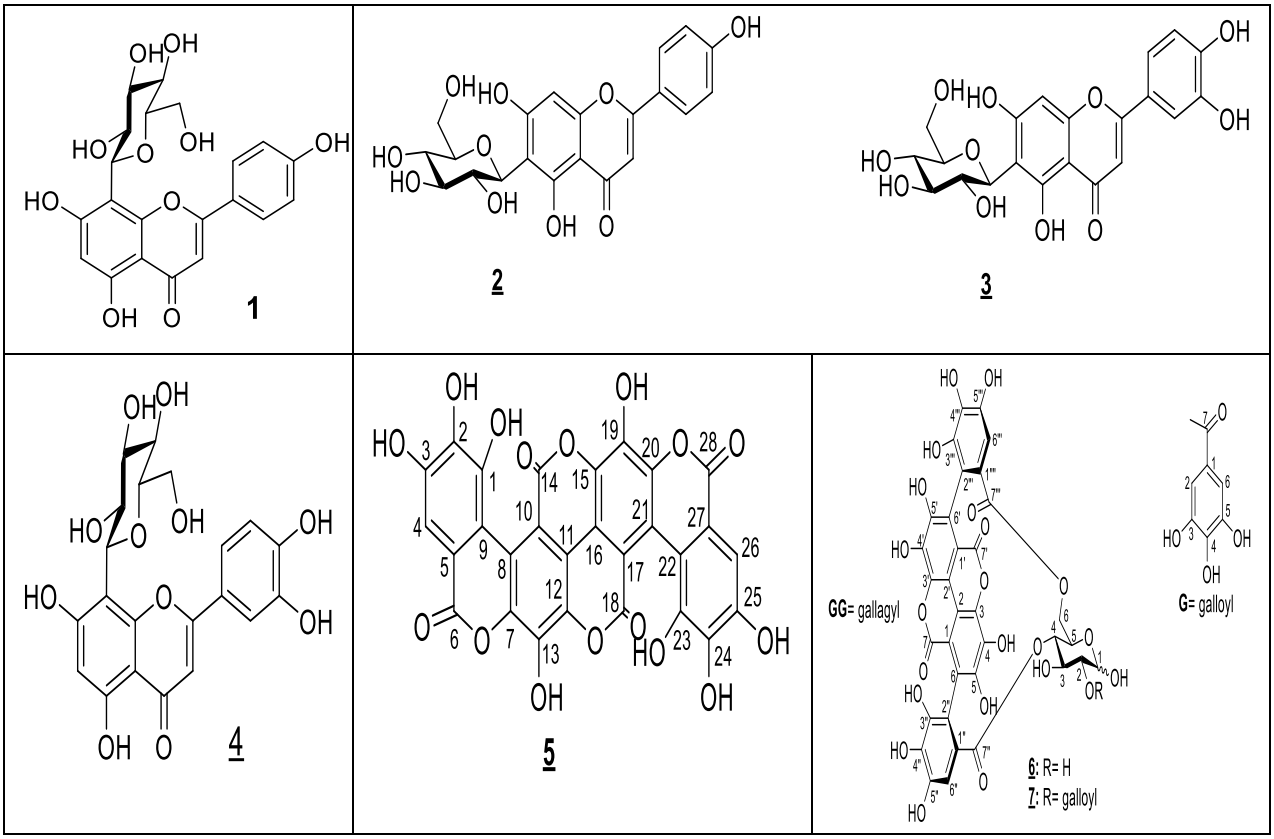

Figure 1. Schematic diagram of compounds 1,2,3,4, 5, 6 and 7

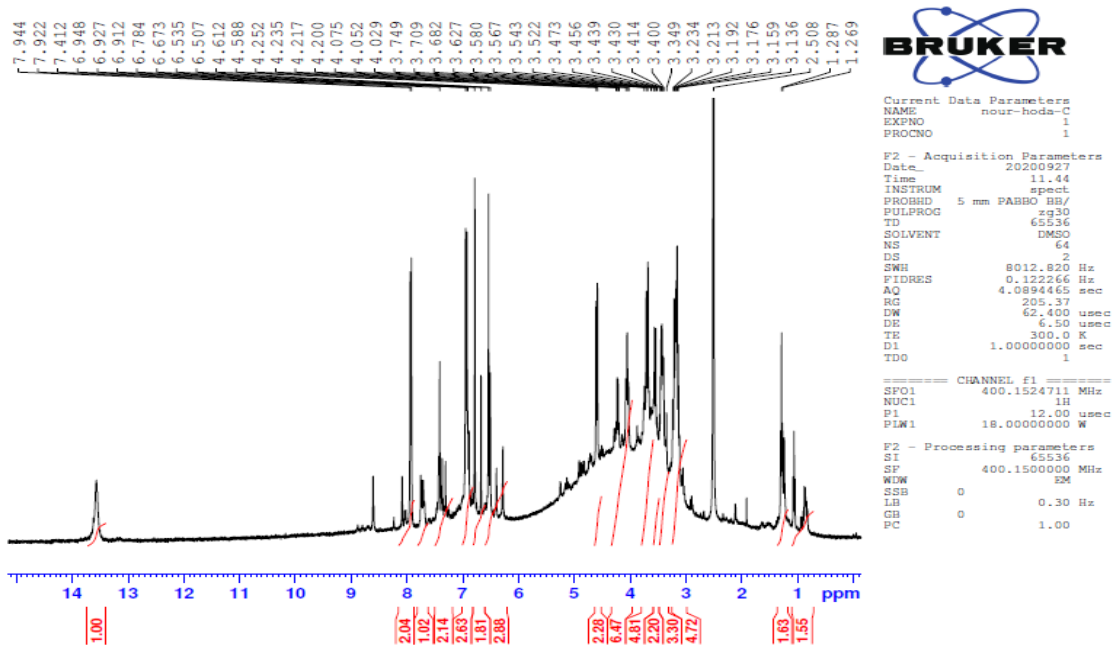

Figure 2. ^{1}H NMR spectrum of compounds **2** and **3** in DMSO-d_6

glycosyls were identified as glucosyls and recommended to adopt a β-*C*-$^{4}C_{1}$-pyranose stereostructure according to the splitting pattern, δ and J-values of H-1" and H-2" at 4.60 (d, J= 9.6), 4.05 (t-like, J= 9.2 Hz). Accordingly, the two metabolites were primarily identified as 6-β-*C*-$^{4}C_{1}$-glucopyranosyl derivatives of apigenin (major) and luteolin (minor), respectively. This was further reinforced by ^{13}C NMR spectrum that showed clearly 19 C-resonances of the major analogue along with some very weak peaks for the minor one (Figure 2). The position of glycosidation and stereostructures of both compounds were proved also from the intrinsic downfield shift of both C-6 resonances and the typical δ-values of the sugar moiety. Based on the comparison of the above discussed data with the literature[21,22] and mentioned for the previous compound, the major metabolite was established as 6-β-*C*-$^{4}C_{1}$-glucopyranosylapigenin (Isovitexin), while the minor one was identified as 6-β-*C*-$^{4}C_{1}$-glucopyranosylluteolin (Isoorientin).

4.3. Characterization and Identification of compound 4

Metabolite **4** exhibited a chromatographic behaviour of a luteolin *C*-glycoside like structure. This evidence was further recommended from ^{1}H NMR that revealed an ABM-spin coupling system for three different types of protons at δ ppm 7.51 (br d, J= 8) and 7.47 (br s) and 6.87 (d, J= 8.4) that was interpretable to H-6', H-2', and H-5' of 3,4-disubstituted ring B. Furthermore, two aromatic singlets, each of 1H, were observed at 6.62 and 6.27 assignable for H-3 and H-6, respectively. The assignment of H-1" at 4.71 (d, J= 9.6) together with the absence of H-8 resonance was an evidence for the attachment of a β-*C*-$^{4}C_{1}$-glycoside moiety to C-8 of 5,7,3',4'-tetrahydroxyflavone (luteolin). Finally, the investigated metabolite afforded more or less the same R_f-values of an authentic sample for orientin in S1 and S2 solvent systems. Accordingly metabolite **4** was identified as 8-β-*C*-$^{4}C_{1}$-glucopyranosylluteolin (Orientin).

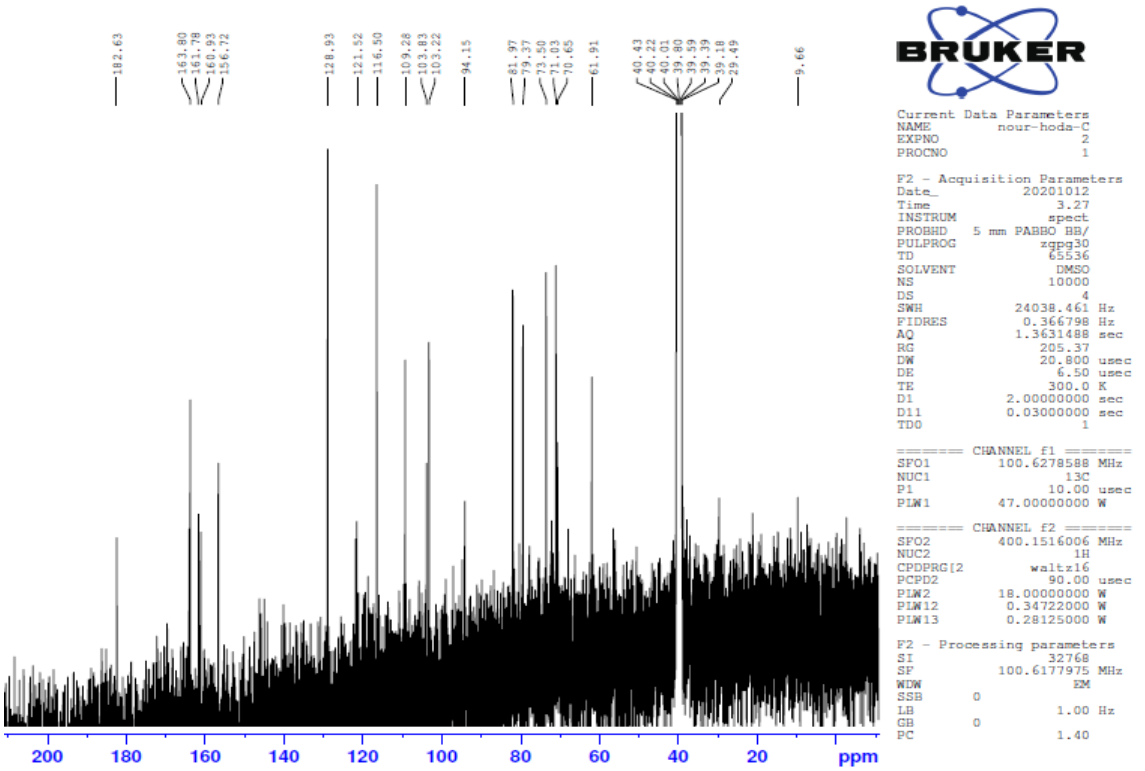

Figure 3. ^{13}C NMR spectrum of compounds **2** and **3** in DMSO-d_6

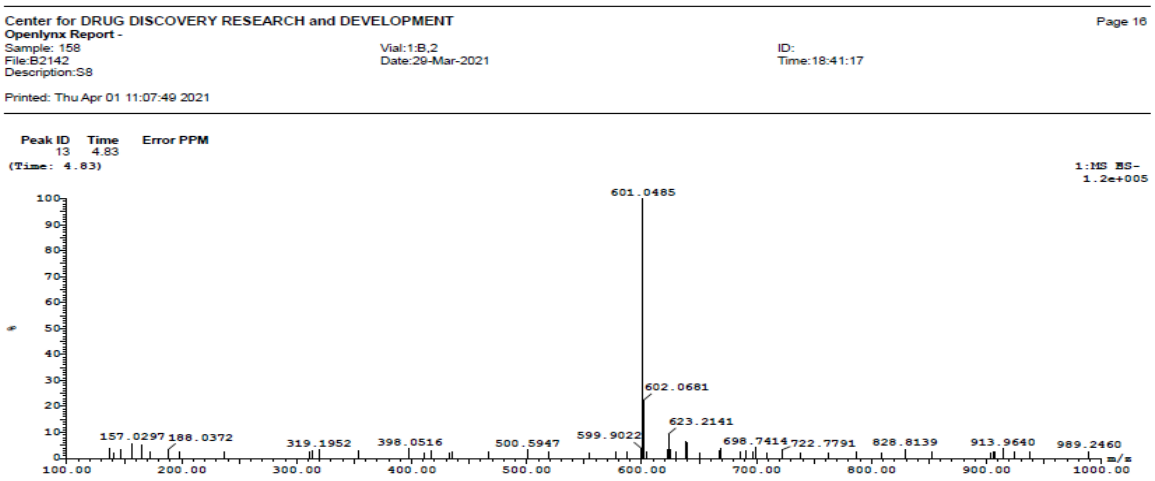

Figure 4. Negative HRESI-MS spectrum of compound **5**

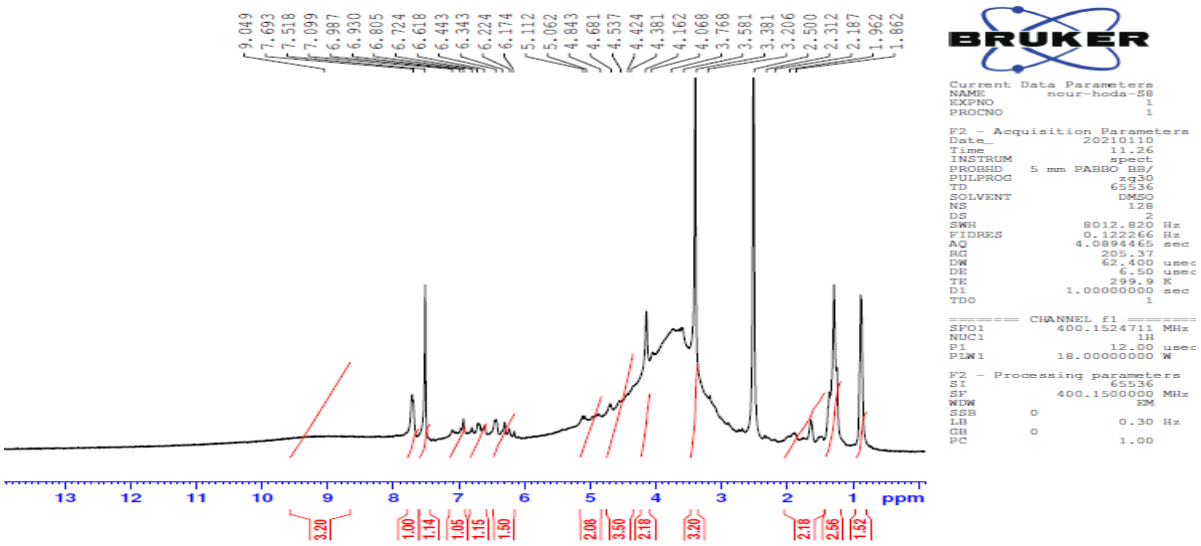

Figure 5. ^{1}H NMR spectrum of compound **5** in DMSO-d_6

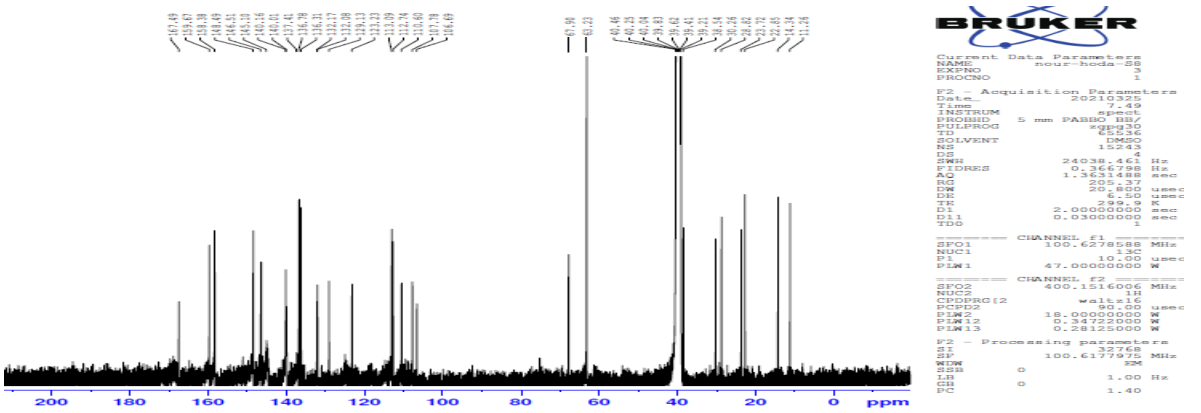

Figure 6. ^{13}C NMR spectrum of compound **5** in DMSO-d_6

4.4. Characterization and identification of compound 5

Based on the chromatographic characters (R_f-values, fluorescence in UV-light and its changes in different spray reagents), the structure of **5** was expected to be an ellagic acid derivative. Its negative HRESI-MS spectrum (Figure 3) demonstrated a molecular ion peak at *m/z* 601.0485 $[M–H]^-$ that was calculated for 600.98907 amu, corresponding to the MF $C_{28}H_{10}O_{16}$ of an ellagic acid connected to two gallic acid residues through oxidative coupling. ^{1}H NMR spectrum (Figure 4) showed a singlet at δ ppm 7.52 that was describable for two equivalent aromatic protons (H-4/26) in a symmetric ellagic acid-like structure. Further confirmation was obtained from the ^{13}C NMR spectrum (Figure 5), which exhibited only fourteen pairs of ^{13}C resonances to recommend, with the MF and ^{1}H NMR data a symmetric bis-dilactonized phenolic acid structure. For simplification they were grouped in four types, i.e. the first included the most downfield two carbonyl-lactone carbons at δ ppm 159.7 and 158.4. The second group showed six pairs of oxygen-bearing carbons in the range of 148.5–136.3. The third one displayed five equivalent pairs of quaternary carbons at 123.2–107.8 ppm together with the most upfield last one that assigned at 106.7 for the protonated equivalent pair of carbons(C-10/17). Both ^{1}H and ^{13}C NMR data together with HRESI-MS results were in complete accordance with the previously published data of gallagic acid bilactone (terminalin)[23].

4.5. Characterization and identification of (α/*β*)-punicalin 6 and 2-*O*-galloyl-(α/*β*)-punicalin 7

Both compounds **6** and **7** gave the chromatographic behaviour of ellagitannins containing bilactone structure because of the characteristic R_f-values, deep purple fluorescence in UV-light that changes in ammonia vapours, $AlCl_3$ or NP/PE (360 nm) into yellow. Moreover, their spots changed into deep blue color with $FeCl_3$ and indigo-red color on spraying with saturated aqueous solution of KIO_3 specific for ellagitannins. On their complete acid hydrolysis, both gave **D**-glucose in aqueous phase, while gallagic acid bilactone (**5**) was detected in the organic phase together with gallic acid in case of **7** only (CoPC). This was in complete accordance with their negative HRESI-MS data (Figures 6 & 9), that showed two molecular ion peaks ($[M–H]^-$) at *m/z* 781.1450 and 933.1954, *calcd.* for $C_{34}H_{22}O_{22}$–H (781.0525) and $C_{41}H_{26}O_{26}$–H (933.0634), corresponding to the accurate MWs of 782.0503 (gallagylglucose, **6**) and 934.0712 (galloyl-gallagylglucose, **7**). Also the double charged ions ($[M–2H]^{-2}$) were recorded at 390.1761 and 466.2309 for **6** and **7**, respectively. Further confirmative structural features, particularly for the substitution positions of galloyl and gallagyl moieties; configuration and conformation of the glucose cores, were concluded from ^1H and ^{13}C NMR spectra (Figures 7,8,10&11) and their comparison with the corresponding data in literature[24–31]. The fact for the occurrence of both **6** and **7** in the form of (α/*β*)–anomeric mixture was achieved mainly from the full duplication of all ^1H and ^{13}C-resonances to prove a free anomeric-OH[30]. The anomeric protons (H-1) were recorded at δ ppm 4.04 (*β*) and 4.70 (α) in case of compound **6**, while they were relatively downfield located at 4.83 and 4.99 in case of **7** to conclude the acylation of adjacent OH-2 with the galloyl ester. The corresponding anomeric carbons were assigned at δ ppm 91.0 (C-1α) and 96.7 (C-1β) in ^{13}C NMR of **6**, while they were typically upfield shifted to 88.8 and 94.0, respectively, in **7** due to the galloylation of OH-2. Similarly, the typical downfield observation of H-4 and H-6 resonances and corresponding carbon signals i.e C-4 (73.6, 74.0; 74.0, 74.5 ppm) and C-6 (64.2, 64.6; 63.2, 64.3 ppm) in both compounds, were confirmative evidence for the acylation of OH-4 and OH-6 with the gallagyl moiety in both compounds[24,25]. In the aromatic region, four singlets were assigned at 6.46 (H-6‴β), 6.73 (H-6‴α), 6.76 (H-6‴β) and 6.92 (H-6‴α) that were indicative to the two gallagyl protons in both anomers of **6**. Similar duplication was observed in the ^1H NMR spectrum of **7** in addition to an extra singlet at 6.99 ppm, describing for H-2/6α,β of a galloyl-ester at C-2. The presence of a gallagyl moiety in each of **6** and **7** was further documented from the duplication of its 28 resonances (4 C=O, 12 C-O, 10 quaternary, 2 CH), especially those of the two lactone carbonyls at 157.6 (GG-C-7'β), 157.7 (GG-C-7'α), 158.1 (GG-C-7α) and 158.2 (GG-C-7β), and the two ester carbonyls at 168.0 (GG-C-7‴β), 168.1 (GG-C-7‴α), 169.5 (GG-C-7‴α) and 169.9 (GG-C-7‴β) in case of **6**. Similar duplication was recorded for lactone and ester carbonyls in the structure of **7** together with extra two carbonyls ester at 165.2 and 166.0 typical for the ester carbonyl of galloyl group at C-2 in both of α- and β-anomers. Depending on the splitting pattern, δ- and J-values of ^1H and ^{13}C-resonances of glucose core, its stereostructure was established as (α/*β*)-$^4C_1$-glucopyranose in both compounds. All other ^1H and ^{13}C-resonances of glucose core, gallagyl and galloyl moieties were assigned by the aid of their matching with the previous published data [24–31].

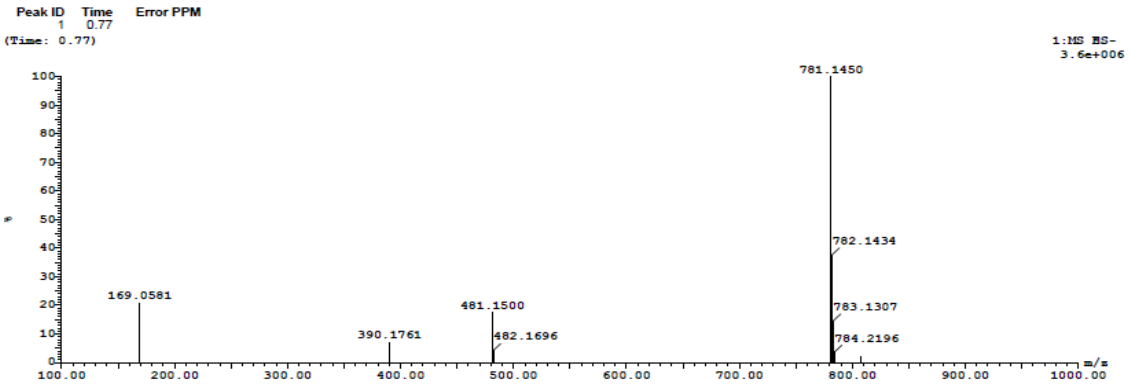

Figure 7. Negative HRESI-MS spectrum of compound **6**

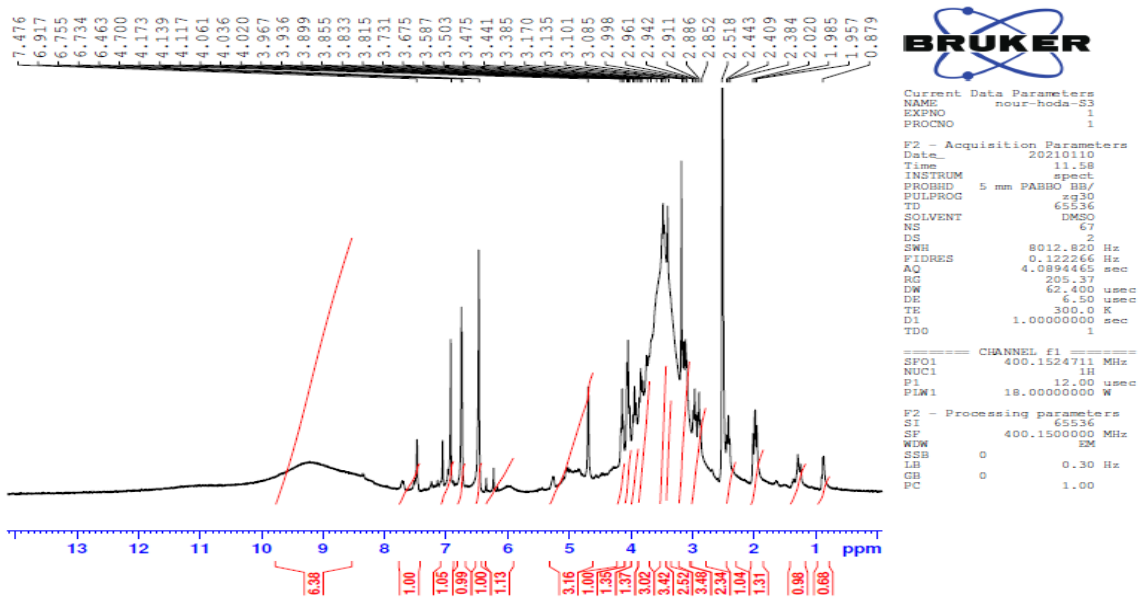

Figure 8. ^{1}H NMR spectrum of compound **6** in DMSO-d_6

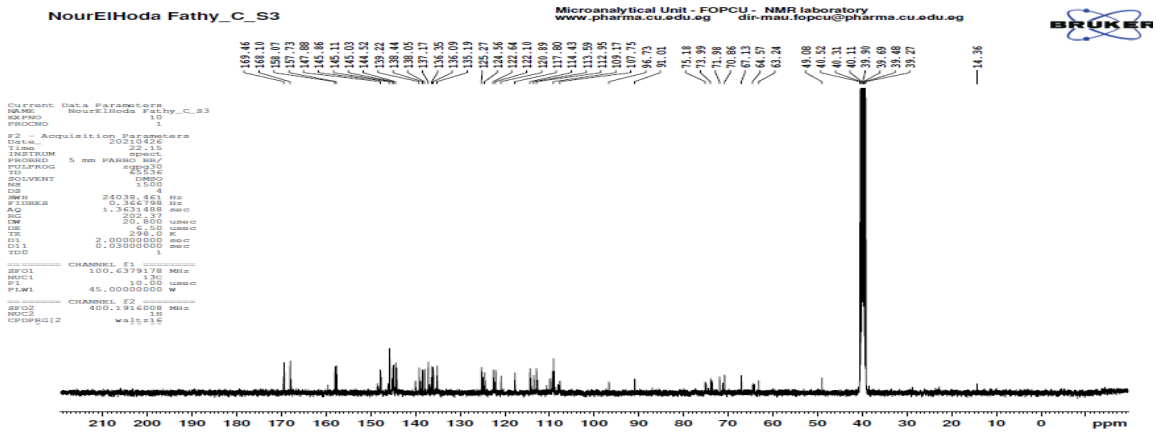

Figure 9. ^{13}C NMR spectrum of compound **6** in DMSO-d_6

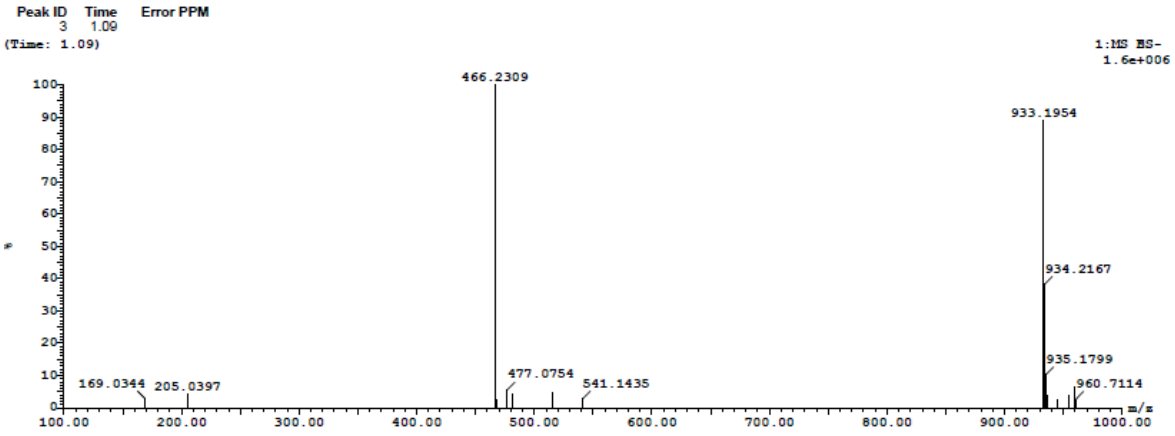

Figure 10. Negative HRESI-MS spectrum of compound **7**

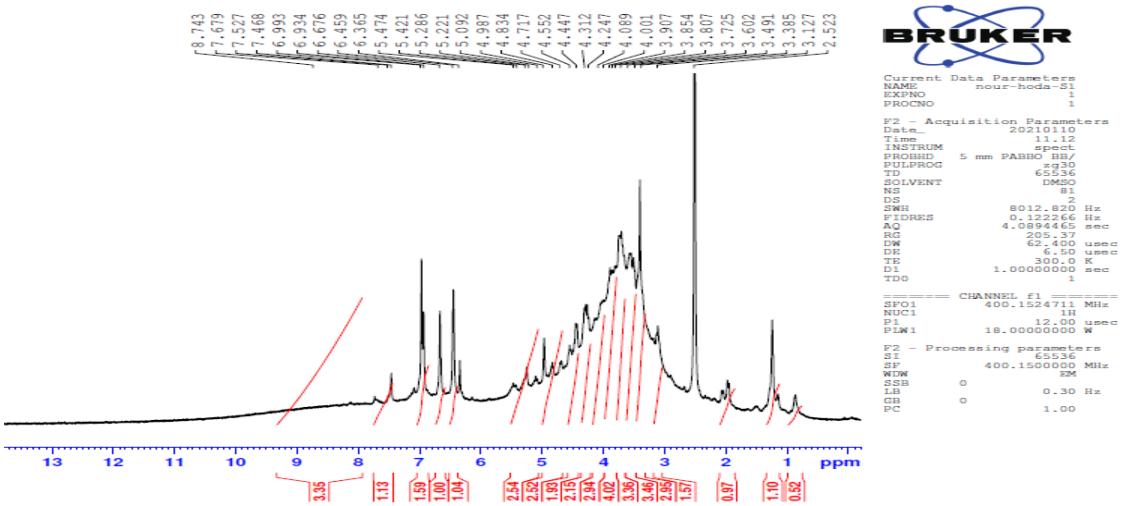

Figure 11. [1]H NMR spectrum of compound **7** in DMSO-d_6

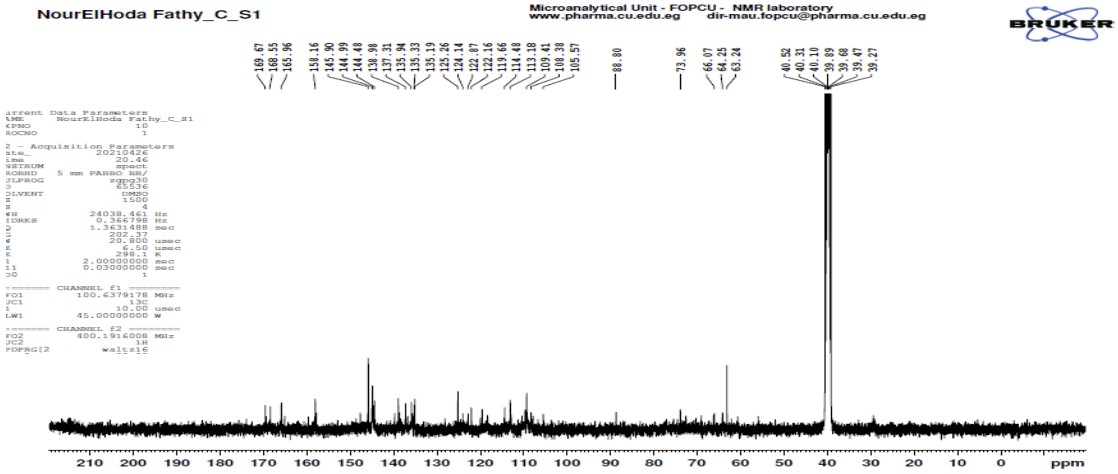

Figure 12. [13]C NMR spectrum of compound **7** in DMSO-d_6

Therefore, **6** was identified as 4,6-*O*-gallagyl-(α/*β*)-$^4C_1$-glucopyranose (punicalin) and **7** as 2-*O*-galloyl-).4,6-*O*-gallagyl-(α/ *β*)-$^4C_1$-glucopyranose (2-*O*-galloyl-(α/ *β*)-punicalin 4,6-*O*-gallagyl-(α/ *β*)-$^4C_1$-glucopyranose (2-*O*-galloyl-(α/*β*)-punicalin

5. CONCLUSIONS

In this study, the methanol –soluble fraction of *A. latifolia* leaves was subjected on a polyamide column followed by series of columns chromatography to provide four C- glycoside flavonoid pure compounds known as; vitexin **1**, isovitexin **2**, isoorientin **3**, and orientin **4**, which were isolated from this plant for the first time. Moreover, the precipitated part of the methanol extract of *A.latifolia* stem was subjected to a series of sephadex LH-20 columns to produce three pure tannins compounds known as; terminalin **5**, punicalin **6**, and 2-*o*-galloylpunicalin **7**, which were isolated and identified for the first time from the plant.

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Author contribution: The authors contribute equally

Authors consent of publication: Authors declared that you have the right to publish this article after acceptance.

List of Abbreviations: **^{1}H NMR**: Proton Nuclear Magnetic Resonance; **^{13}C NMR:** ^{13}C Nuclear Magnetic Resonance; **2D-PC**: 2-Dimensional Paper Chromatography; **HRESI-MS**: High Resolution Electrospray Ionization-Mass Spectrometry; **δ**: Chemical shift; **PC**: Paper Chromatography; **UTI:** Urinary Tract Infection; **DMSO-d_6**: Dimethyl Sulfoxide deuterated-6; **NP/PE**: Naturstoff/ Polyethylene glycol; **R_f** Retention or Retardation factor; **m**: mutiplet; **d**: doublet; **s**: singlet; **t**: triplet; **MF:** Molecular Formula; **MW**: Molecular Weight.

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