SUPPLEMENTARY MATERIAL

Isolation and characterization of flavonoids from the roots of medicinal plant *Tadehagi triquetrum* (L.) H.Ohashi

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Abstract:

Three flavonoid compounds were isolated from the roots of medicinal plant *Tadehagi triquetrum* (L.) H.Ohashi, also known as *Desmodium triquetrum* (Fabaceae). On the basis of the chemical and spectral analysis, the compounds were identified as baicalein (Flavone), naringin and neohesperidin (Flavonone). To the best of our knowledge and based on the literature survey all three compounds were first time reported from this medicinal plant.

Keywords: *Tadehagi triquetrum* (L.) H.Ohashi, medicinal plant, flavonoids, spectral analysis

Experimental

General

IR spectra were recorded using Shimadzu FT-IR 8400S (KBr Discs) on an FT-IR Spectrophotometer (ν_{max} in cm^{-1}). ^{1}H NMR spectra were recorded on a Bruker Advance II (400 MHz) Instruments in DMSO with TMS as an internal standard (chemical shift in δ, ppm). ^{13}C NMR spectra were recorded on a Bruker Advance II (400 MHz) instruments in DMSO with TMS as an internal standard (chemical shift in δ, ppm). GC-MS spectra were recorded (EI-TOF) on a Thermo trace 1300GC coupled with Thermo TSQ800 Triple Quadrupole MS. TLC was performed with silica gel GF_{254}. All the solvents were analytical reagent grade.

Procurement and authentication of Plant material

The Trefle Gros, (*Tadehagi triquetrum*), is a species of flowering plant in the legume family Fabaceae, in related to Scientific classification the plant mainly belongs to Kingdom- Plantae, unranked- Angiosperms, unranked- Eudicots, unranked- Rosids, Order-Fabales, Family Fabaceae, Subfamily- Faboideae, Genus- *Tadehagi*, Species-*T. triquetrum*, Binomial name-*Tadehagi triquetrum* (L.) H. Ohashi.

Synonyms: *Desmodium triquetrum* (L.) DC. *Desmodium triquetrum subsp. genuinum Prain Desmodium triquetrum subsp. Triquetrum Hedysarum triquetrum L*.

Tadehagi triquetrum (L.) H.Ohashi (Fabaceae) roots were collected from the Aminabad, Lucknow, Uttar Pradesh, India and the plant were authenticated by Dr. A.K.S Rawat and Dr. Alok Lahri respectively from Ethanopharmacognosy & Pharmacology division from National Botanical Research Institute under Council of Scientific and Industrial Research (NBRI-CSIR) Lucknow, Uttar Pradesh, India. A voucher specimen (Ref. no: NBRI/CIF/492/2015) was deposited in the herbarium for future reference of the Dept. of Pharmacognosy & Phytopharmacy, JSS College of Pharmacy, Rocklands, Ooty, Tamil Nadu, India.

Extraction

The plant root (1kg) material was oven dried at 40˚C for 10 days and ground into course powder, extracted with hydro-alcohol (50:50) and filtered twice using a muslin cloth. It was further filtered through Whatman (No 1) filter paper. Distillation process was done with the help of distillation assembly and then undergone evaporation. The extract was concentrated using a Buchi rotatory evaporator R124 and air dried under a stream of cold air. The percentage yield of extract was calculated (w/w) and was found to be 43.62 g (4.3 %). Crude extracts were kept at 4˚C in the dark until analysis (Harborne 1998).

Phytochemical screening

The prepared extracts and fractions were subjected to a preliminary qualitative phytochemical screening by the following methods based, on the standard protocol (Harborne 1998).

Fractionation

The crude hydro-alcoholic extract (20.60 g) was partitioned by using separating funnel (liquid-liquid) with n-hexane, chloroform, ethyl acetate and *n*-butanol, yield of *n*-hexane (5.8 g), chloroform (12.5 g), ethyl acetate (8.4 g) and *n*-butanol soluble (6.5 g) fractions respectively. The % of yield of the crude extract was more in chloroform fraction when compare to the % yield from the other fractions. This was further taken up for fractionation.

Isolation

The chloroform clear soluble fraction (12.5 g) was subjected to column chromatography (2 Mm, 30 cm length with silica gel (60-120 mesh, Merck) eluted with (ethyl acetate: n-hexane, 90:10) solvent system. The repeated chromatography gave three major fractions (Fraction-1, 0.178 g (R_f 0.51); Fraction-2, 0.210 g (R_f 0.69); Fraction-3, 0.98 g (R_f 0.89) (Lall *et al*. 2017)

Fraction-1

Fraction-1 obtained from column chromatography, was further purified by preparative TLC over silica gel GF 254 using ethyl acetate: n-hexane (3:2) as the developing solvent. It was obtained as yellow crystals; m.p. 190-192 ^{0}C (Hossain MA, Rahman SM. 2015); FT-IR (KBr, cm^{-1}): 3410, 3392, 3356 (-OH), 3031.23 (-CH aromatic), 1616.40 (C=C), 1717.67 (>C=O), 1103.32 (C-O-C); ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 12.72 (s, 1H, -OH), 8.90 (s, 1H, -OH), 8.09-7.61 (m, 5H, -CH aromatic), 6.95 (s, 1H, -CH aromatic), 6.67 (s, 1H, -CH aromatic); ^{13}C NMR (DMSO-d_6, δ ppm): 182.08 (>C=O), 162.83 (C=C), 153.58, 149.78, 146.90, 131.76, 130.88, 129.28, 129.04, 126.22, 104.40, 104.24, 93.98 (-CH aromatic); GC-MS (EI-TOF) m/z calculated for $C_{15}H_{10}O_5$: 270.24. Found: m/z 267.2 ($M^{+\cdot}$-3), 220.2, 152.1, 95.1 (base peak), 77.1. It was characterized as baicalein.

Fraction-2

Fraction-2 obtained from column chromatography was further purified by preparative TLC over silica gel GF_{254} using ethyl acetate: n-hexane (3:2) as the developing solvent. It was crystallized from methanol as reddish brown powder; m.p. 180-190 ^{0}C (Hossain MA, Rahman SM. 2015); FT-IR (KBr, cm^{-1}): 3410, 3357 (-OH), 2931.90 (-CH aromatic), 1647.26 (C=C), 1683.91 (>C=O), 1205.55, 1178.55 (C-O-C); ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 12.07 (s, 1H, -OH phenolic), 9.66 (s, 1H, -OH phenolic), 7.35-6.08 (m, 6H, -CH aromatic), 5.51-4.53 (m, 13H, -CH cyclic), 3.72-2.51 (m, 6H, -OH alicyclic), 2.50 (d, 2H, $-CH_2OH$), 1.17-1.15 (s, 3H, $-CH_3$); ^{13}C NMR (DMSO-d_6, δ ppm): 197.34 (>C=O), 164.80, 162.89,

162.71, 157.81, 157.77, 128.56, 128.49, 128.45, 115.17, 103.25, 100.39, 97.32 (-CH aromatic), 95.10, 78.79, 78.60, 77.06, 76.79, 76.01, 71.75, 70.40, 70.33, 69.49, 68.25, 60.36 (-CH alicyclic), 42.03 (-CH_2), 41.94 (-CH_3); GC-MS (EI-TOF) m/z calculated for $C_{27}H_{32}O_{14}$: 580.53. Found: m/z 575.90 ($M^{+\cdot}$-4), 270.1, 198.3, 168.0, 140.1, 77.10. It was characterized as naringin.

Fraction-3

The isolated fraction -3 was purified by preparative over silica gel GF using benzene: acetone: ethyl acetate (7:5:1) as the developing solvent. It was crystallized from methanol as brownish orange colour needles; m.p. 182-194 ^{0}C; FT-IR (KBr, cm^{-1}): 3506, 3410 (-OH), 3100.67 (-CH aromatic), 1623.15 (C=C), 1734.06 (>C=O), 1204.59, 1193.98 (C-O-C); ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 12.05 (s, 1H, -OH phenolic), 9.15 (s, 1H, -OH phenolic), 6.95-6.08 (m, 5H, -CH aromatic), 5.53-4.52 (m, 13H, -CH cyclic), 3.77-3.71 (s, 3H, -OCH_3), 3.69-2.51 (m, 6H, -OH alicyclic), 2.49 (d, 2H, -CH_2OH), 1.17-1.15 (s, 3H, -CH_3); ^{13}C NMR (DMSO-d_6, δ ppm): 197.02 (>C=O), 164.80, 162.88, 162.55, 147.94, 146.42, 130.81, 117.80, 114.07, 111.90, 103.29, 100.35, 97.32 (-CH aromatic), 95.11, 78.39, 77.08, 76.84, 76.00, 71.75, 70.40, 70.33, 69.50, 68.25, 60.36, 55.61 (-CH alicyclic), 42.10 (-CH_2), 40.02, 39.40 (-CH_3); GC-MS (EI-TOF) m/z calculated for $C_{28}H_{34}O_{15}$: 610.56. Found: m/z 574.2, 255.1, 152.1, 95.1 (base peak), 88.1, 77.1. It was characterized as neohesperidin (Agarwal PK.1989, Hossain MA, Rahman SM. 2015).

The detailed methodology for GCMS study with all technical specifications are as follows

GCMS Analysis was performed using Shimadzu GCMS-QP 2010 equipped Thermo Trace 1300GC coupled with Thermo TSQ 800 Triple Quadrupole MS. For GC - THERMO TRACE 1300 GC For MS - THERMO TSQ 8000Software used: XCalibur 2.2SP1 with Foundation 2.0SP1 Column:TG 5MS (30m X 0.25mm, 0.25µm)Injector: S/SL (Split/ Splitless) Injection volume: 1.0µLInjector temp: 250˚CMS transfer line temp:280˚C, Ion source temp: 230˚CMass Range: 50-600Scan time: 0.5 sec, Desired scans per peak: 6Minimum baseline peak width: 3sec, Carrier Flow: 1ml/min, Oven Program: Initial Temp: 60˚C ; Hold time: 2.0 min, Final Temp: 280˚C ; Hold Time:10.0 min Temperature Rate: 10˚C/min, Detector: MS TSQ 8000Library used: NIST 2.0, Carrier gas used was helium.

Toxicity studies of hydro-alcoholic root extract of *Tadehagi triquetrum* (L.) H. Ohashi

Animal husbandry

Specific-pathogen-free female Swiss albino mice (20-28 g) were obtained from the animal house, JSS College of Pharmacy, Ootacamund, India, and were housed under standard environmental conditions (12-h dark-light cycle, 22-28°C, 60-70% relative humidity). The animals were housed in stainless steel cages and had free access of standard laboratory feed (M/S Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The experimental protocol was approved by the institutional animal ethics committee (IAEC) constituted in accordance with the rules. The experiments were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), Chennai, India (Approval no. JSSCP/IAEC/OT/P.COG/04/2018-19).

Acute toxicity study of Hydroalcoholic extract of Tadehagi triquetrum **(**L.) H.Ohashi

The procedure was followed according to the OECD guidelines 423 (Acute toxic class method). Adult female Swiss albino of weight (22-28g) was used for acute toxicity study. The dose level was selected from one from the defined doses (5, 50, 300, 2000 mg/kg body weight) orally (p.o.) and the results allow a substance to be ranked and classified according to the globally harmonized system (GHS). The mice were observed for mortality, body weight changes, urine color analysis. Observations recorded twice daily for 14 days also did not reveal any drug-related observable changes.

DPPH and ABTS Assay for determining *in-vitro* antioxidant potential of isolated compounds

DPPH ASSAY

The 2, 2 diphenyl-1- picrylhydrazyl (DPPH) test were carried out in 96 well microtitre plate and add 200µl of DPPH solution, 10µl of each of the test sample or the standard solution was added separately in wells of the micro titre plate. The plates were incubated at 37 ^{0}C for 20 minutes and the absorbance of each well was measured at 490 nm, using ELISA plate reader (Bio-Rad) against the corresponding test and standard blanks. The IC_{50} (Inhibitory Concentration) is the concentration of the sample required to scavenge 50% of DPPH free radicals was calculated after getting the absorbance. The test was carried out in triplicate and the antioxidant activity was calculated as by the formula is given below

Activity (%) = Ac-At/Ac×100

Where: At was the absorbance of sample and Ac was the absorbance of DMSO DPPH solution.

ABTS ASSAY

This assay was carried out based on the ability of different substances to scavenge 2, 2'-azino–bis (ethylbenzthiazoline-6 sulfonic acid ($ABTS^{+}$) radical cation. The radical cation was prepared by mixing 0.2 ml of various concentrations of the sample or standards, 1 ml of distilled DMSO and 0.16 ml of ABTS solution were added to make a final volume of 1.36 ml. The Absorbance was measured, after 20 min at 734 nm using ELISA plate reader (Biorad). Blank solution was taken without ABTS.The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following

$$E = (Ac\text{-}At)/Ac) \times 100$$

Where At and Ac are the respective absorbance of tested samples and $ABTS^{+}$, was expressed as µmol. (Shalaby and Shanab 2013, Tang *et al*. 2014)

Position	Ring	Type of proton	Type of signal	No of Protons	δ H (ppm)
3	C	Ethylenic	S	1	6.95
5	A	OH	S	1	12.72
6	A	OH	S	1	8.90
7	A	OH	S	1	10.65
8	A	Aromatic	S	1	6.67
2' 3' 4' 5'6'	B	Aromatic	M	5	8.09-7.61

Table S1: ^{1}H NMR data of baicalein

Position	Ring	Type of carbon	No of carbon	δ C (ppm)
C5, C6, C7, C8, C9, C10	A	Aromatic	6	146.90, 149.97, 93.98, 153.58, 104.40
C1' C2' C3' C4' C5'C6'	B	Aromatic	6	126.22,129.04, 129.28, 130.88, 131.76
C2	C	Ethylenic	1	162.83
C3	C	Ethylenic	1	104.24
C4	C	Carbonyl	1	182.08

Table S2: ^{13}C NMR data of baicalein

Position	Ring	Type of proton	Type of signal	No of Protons	δ H (ppm)
4’	B	OH	s	1	12.07
5	A	OH	s	1	9.66
5,8/2’3’5’6’	A/B	Aromatic	m	6	7.35-6.08
2,3/1’’2’’3’’4’’5’’/ 1’’’2’’’3’’’4’’’5’’’	C/D/E	Oxymethine, Aliphatic methylene/Glycone	m	13	5.512-4.53
3’’4’’6’’/2’’’3’’’4’’’	D/E	Glycone-OH	m	6	3.72-2.51
6’’	D	Hydroxymethyl	d	2	2.50
6’’’	E	Methyl	s	3	1.17-1.15

Table S3: ^{1}HNMR data of naringin

Position	Ring	Type of carbon	No of carbon	δ C (ppm)
C5, C6, C7, C8, C9, C10	A	Aromatic	6	162.89, 96.21, 162.71, 103.25, 164.80, 115.17
C1’ C2’ C3’ C4’C5’C6’	B	Aromatic	6	164.67, 128.56, 157.81,162.84, 157.77, 128.49
C2	C	Oxymethine	1	78.79
C3	C	Aliphatic methylene	1	42.03
C4	C	Carbonyl	1	197.20
C1’’C2’’C3’’C4’’C5’’ C6’’	D	Glycone	6	97.18, 70.43,95.05, 76.01, 76.79, 60.36
C1’’’C2’’’C3’’’C4’’’ C5’’’C6’’’	E	Glycone	6	97.32, 71.75, 95.10, 76.14, 77.06, 18.01

Table S4: ^{13}CNMR data of naringin

Position	Ring	Type of proton	Type of signal	No of Protons	δ H (ppm)
4’	B	OH	s	1	12.05
5	A	OH	s	1	9.15
2’3’6’/6, 8	B/A	Aromatic	m	5	6.95-6.08
2,3/1’’2’’3’’4’’5’’/ 1’’’2’’’3’’’4’’’5’’’	B/D/E	Oxymethine, Aliphatic methylene/ Glycone	m	13	5.532-4.52
3’’4’’6’’/2’’’3’’’4’’’	D/E	Glycone-OH	m	6	3.69-2.51
6’’	D	Hydroxymethyl	d	2	2.49
6’’’	E	Methyl	s	3	1.17-1.15
4’	B	Methoxy	s	3	3.77-3.71

Table S5: ^{1}HNMR data of neohesperidin

Position	Ring	Type of carbon	No of carbon	δ C (ppm)
C5, C6, C7, C8, C9, C10	A	Aromatic	6	162.88, 96.22, 162.55, 103.29, 164.80, 114.07
C1’ C2’ C3’ C4’C5’C6’	B	Aromatic	6	130.81, 111.90, 147.94, 146.42,117.80,
C2	C	Oxymethine	1	78.39
C3	C	Aliphatic methylene	1	42.01
C4	C	Carbonyl	1	197.02
C1’’C2’’C3’’C4’’C5’’C6’’	D	Glycone	6	97.32, 70.40,95.11, 76.0, 77.08, 60.36
C1’’’C2’’’C3’’’C4’’’C5’’’C6’’’	E	Glycone	6	97.22, 71.75, 97.32, 76.84, 78.39, 18.02
C3’	B	Methoxy	1	55.61

Table S6: ^{13}CNMR data of neohesperidin

Sr.No	Sample Description	DPPH ASSAY IC_{50} Value	ABTS ASSAY IC_{50} Value
1	Baicalein	8.80± 1.82	11 ± 1.70
2	Naringin	68.25 ± 1.70	57.25 ± 1.85
3	Neohesperidin	26± 2.00	64 ± 2.70
4	Ascorbic acid	3.5 ± 1.14	12.35 ± 1.14

Table S7: *In-vitro* antioxidant activity of the isolated compound by using DPPH and ABTS assay method.

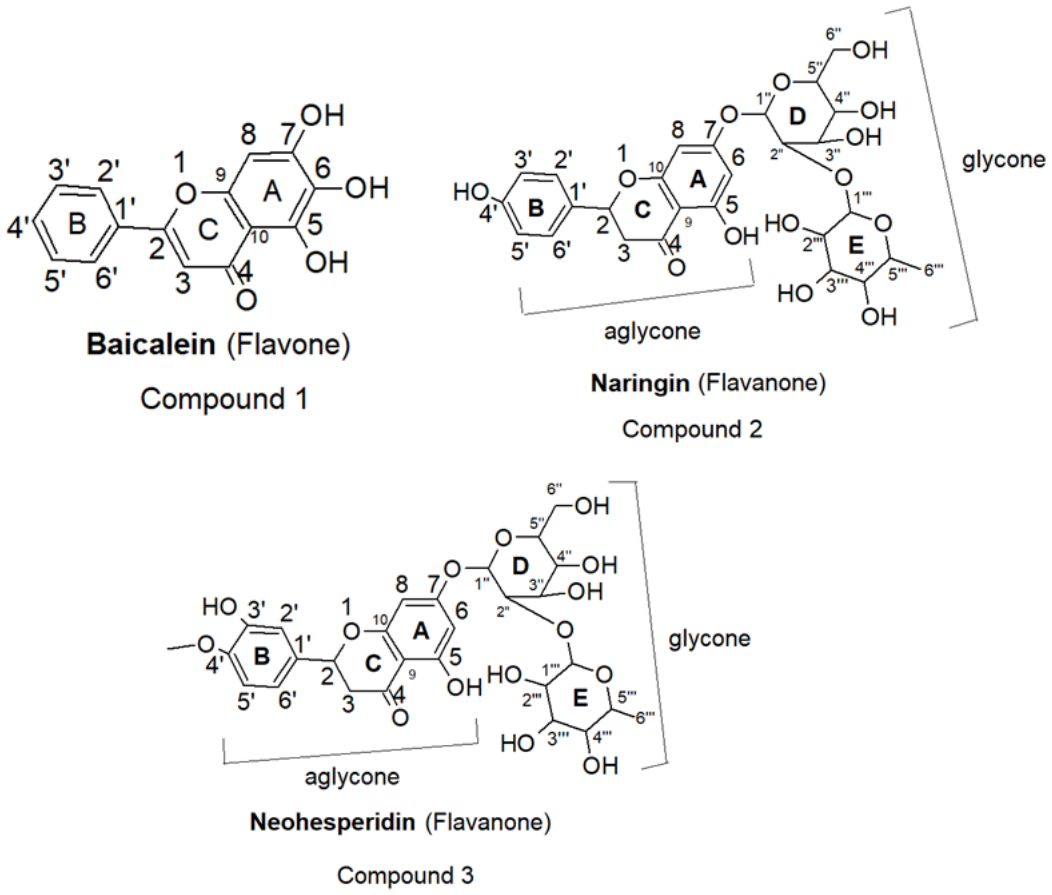

Figure S1. The chemical structures of the isolated flavonoids from the roots of *Tadehagi triquetrum* (L.) H.Ohashi.

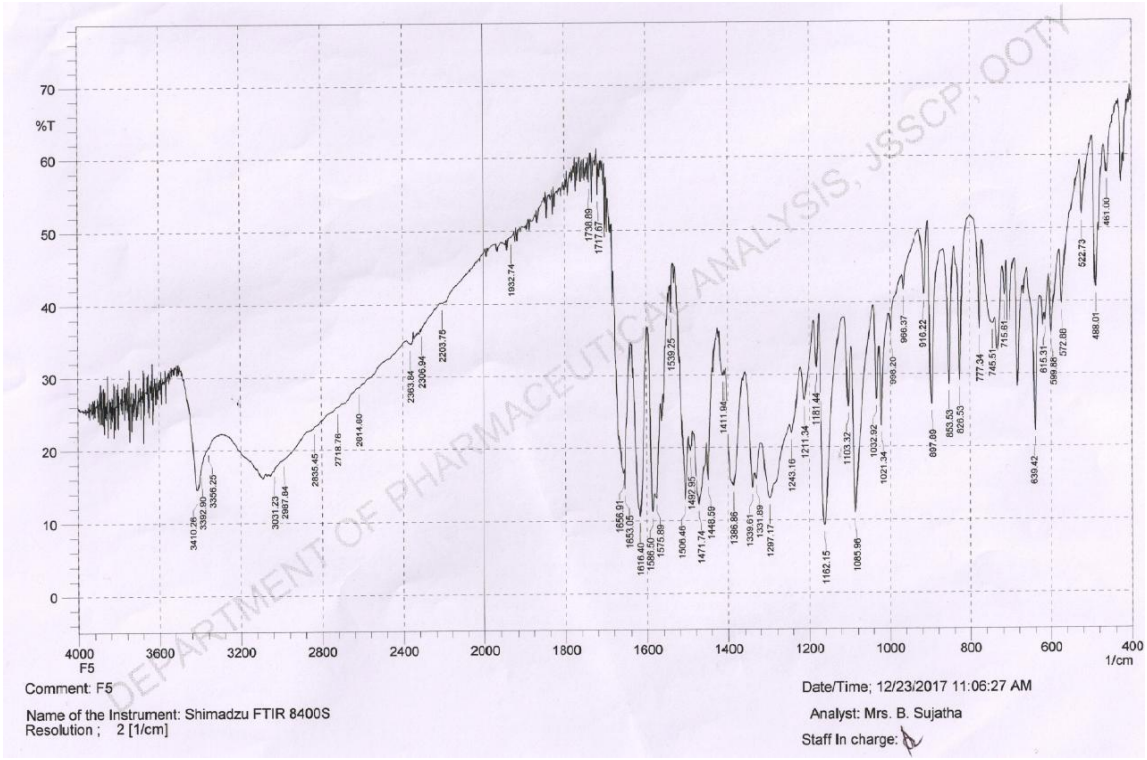

Figure S2. The FT-IR spectrum of isolated compound baicalein (1).

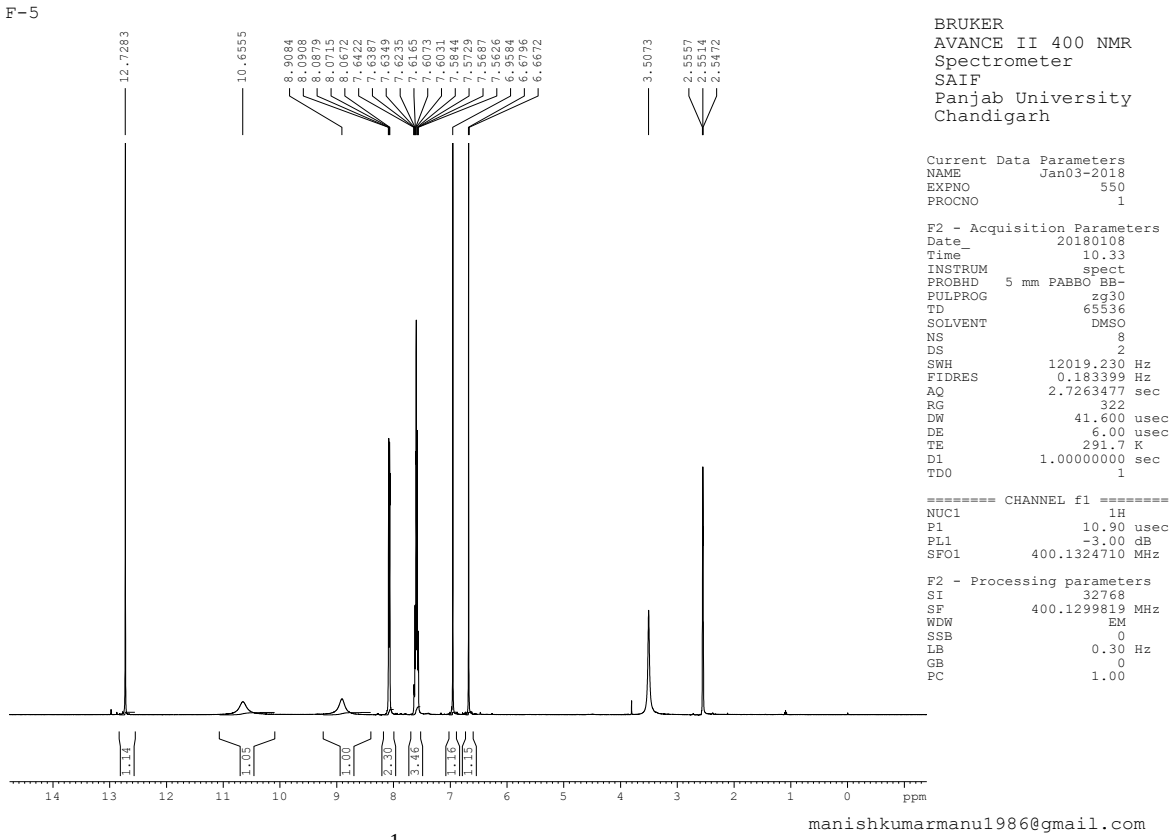

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Figure S3. The ^{1}H NMR spectrum of isolated compound baicalein (1).

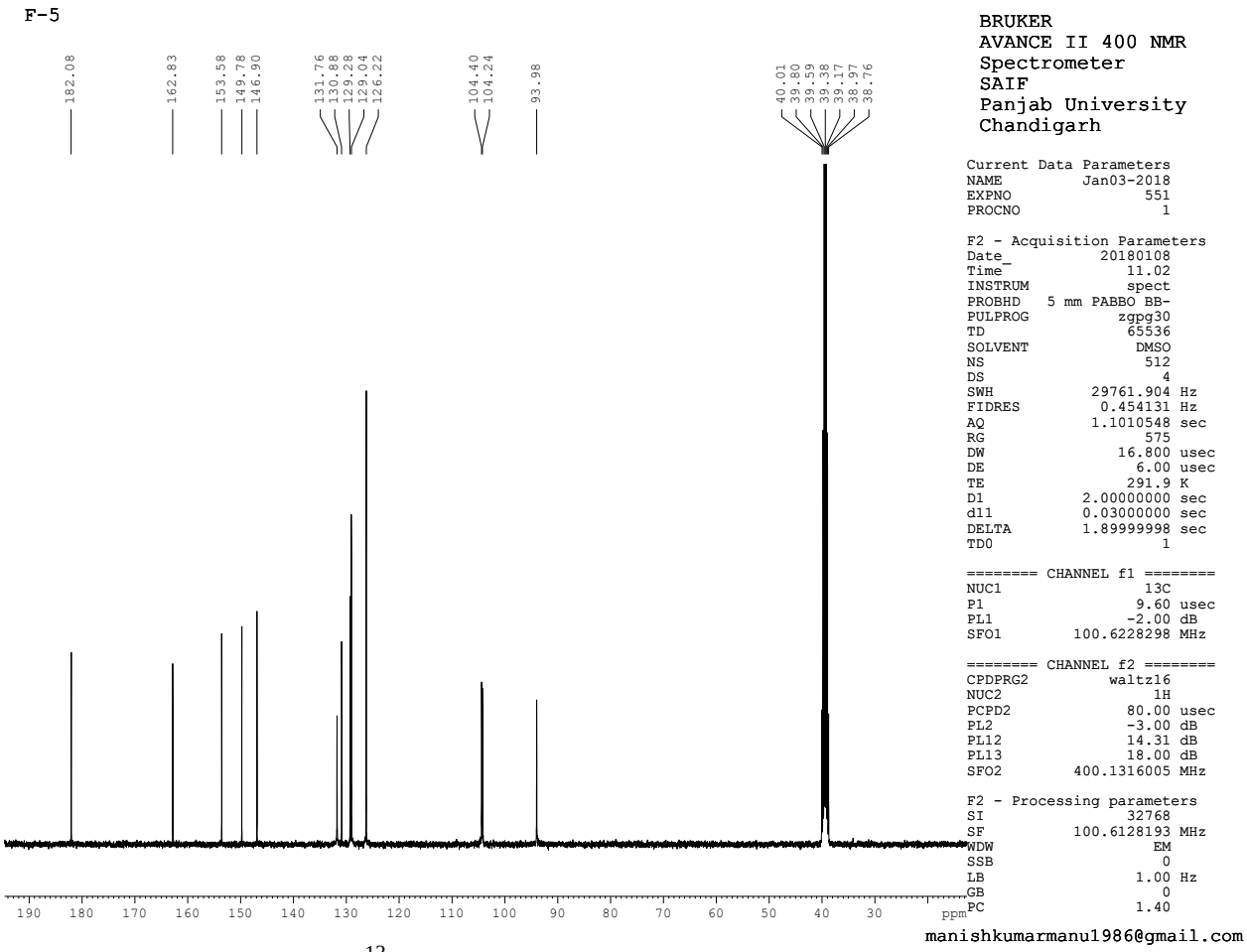

Figure S4. The ^{13}C NMR spectrum of isolated compound baicalein (1).

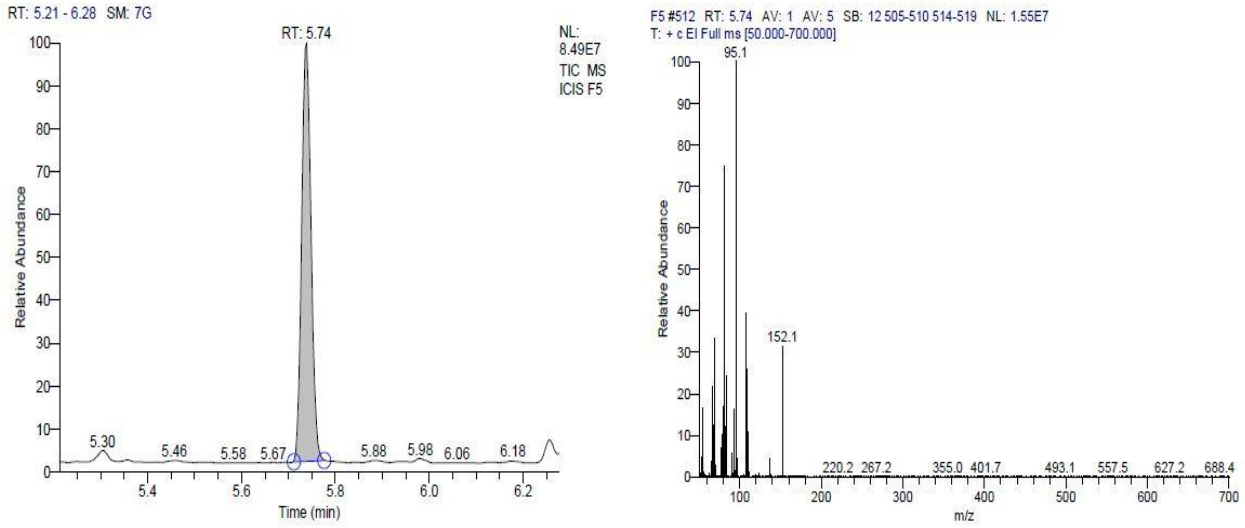

Figure S5. The GC-MS spectrum of the isolated compound baicalein (1).

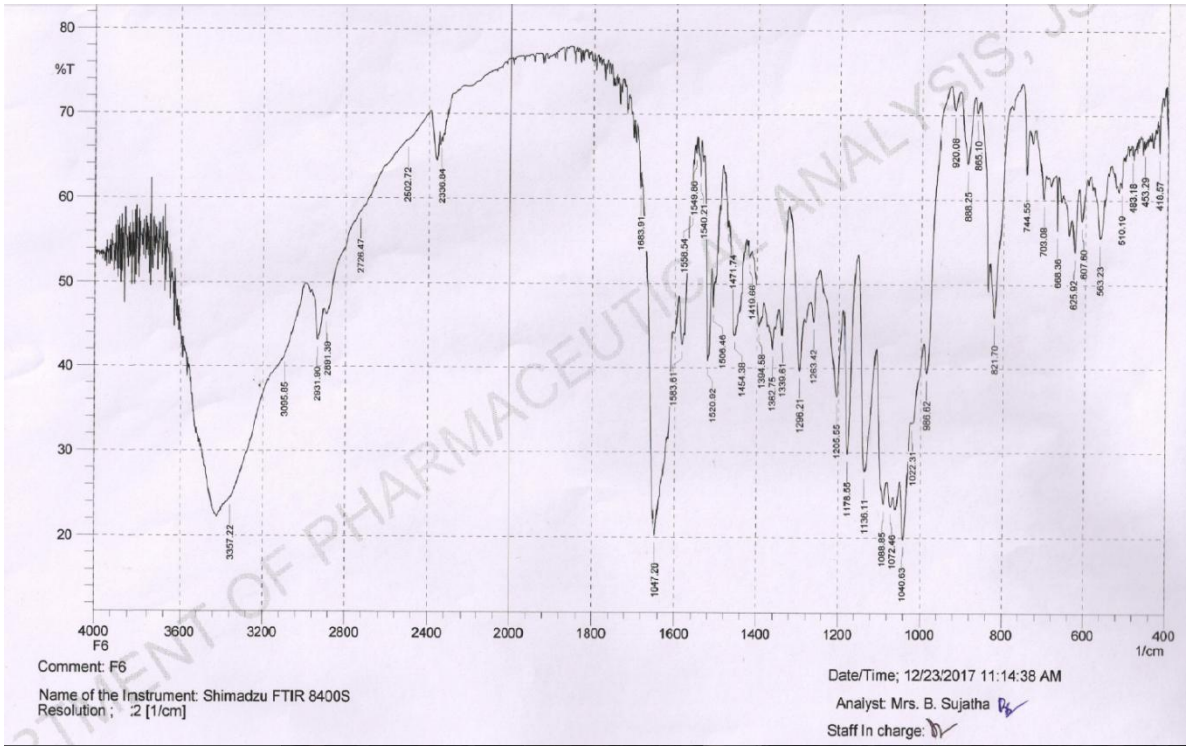

Figure S6. The FT-IR spectrum of isolated compound naringin (2).

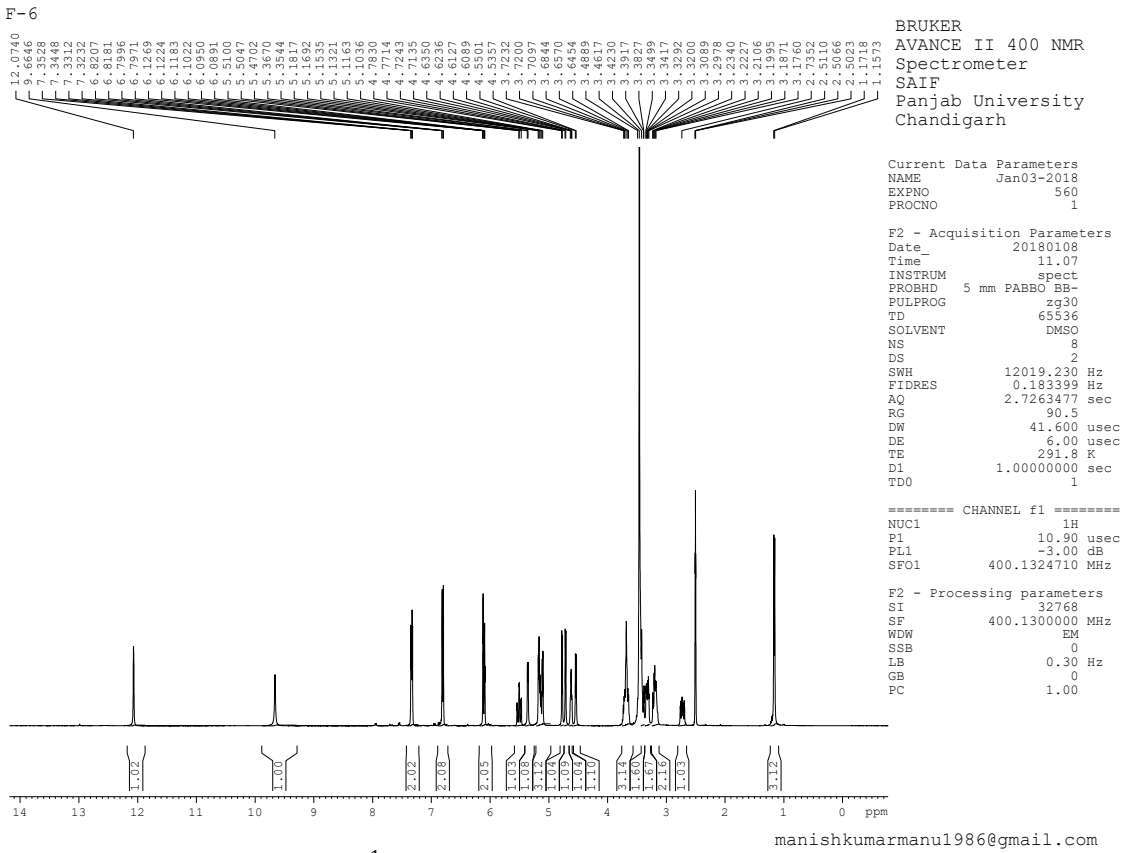

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Figure S7. The ^{1}H NMR spectrum of isolated compound naringin (2).

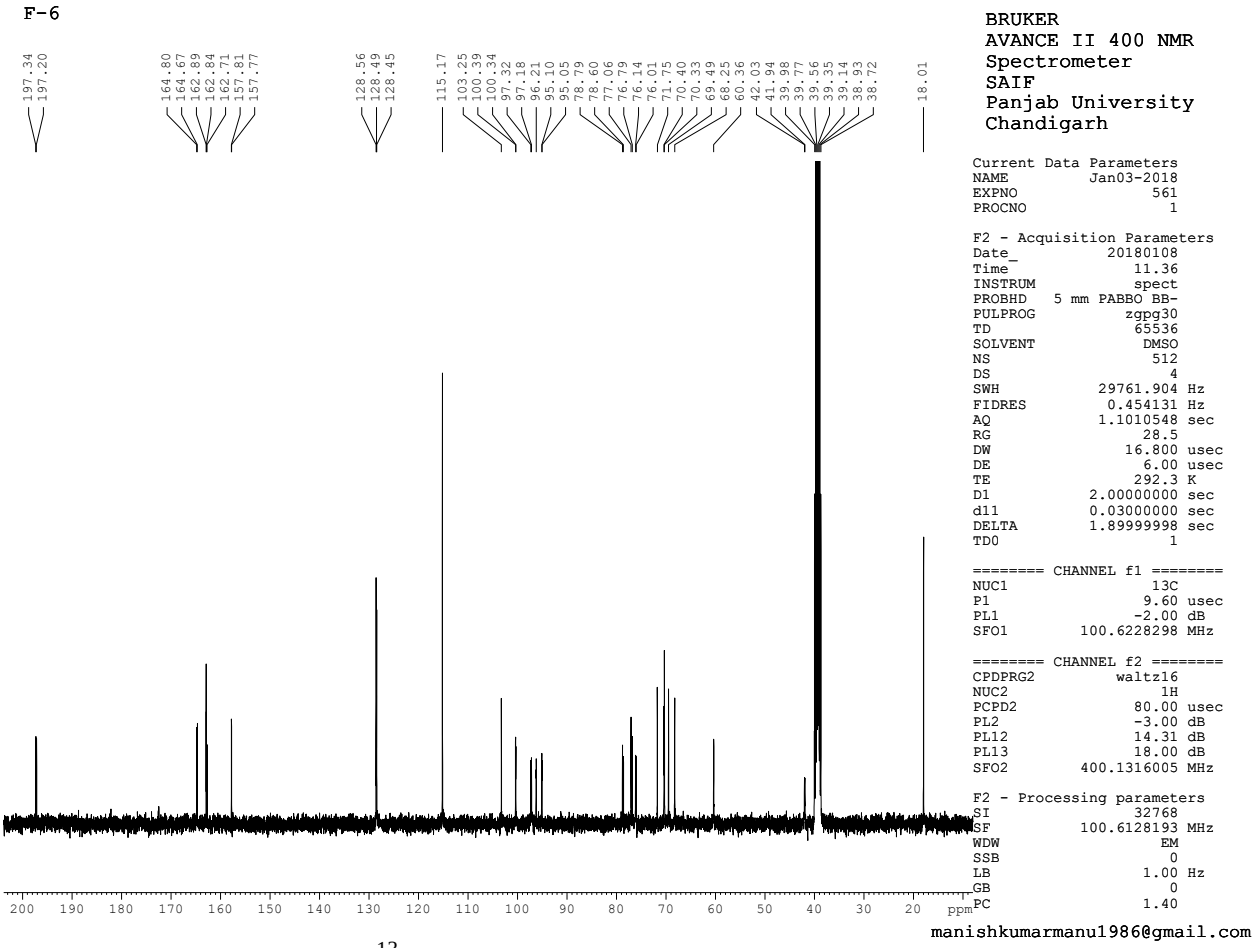

Figure S8. The ^{13}C NMR spectrum of isolated compound naringin (2).

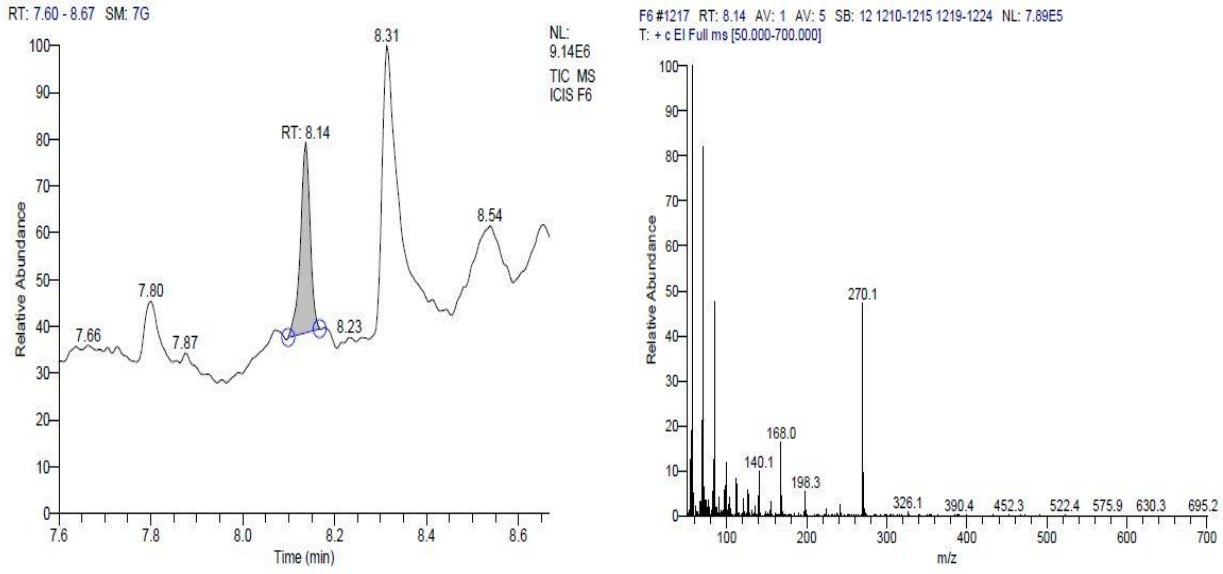

Figure S9. The GC-MS spectrum of isolated compound naringin (2).

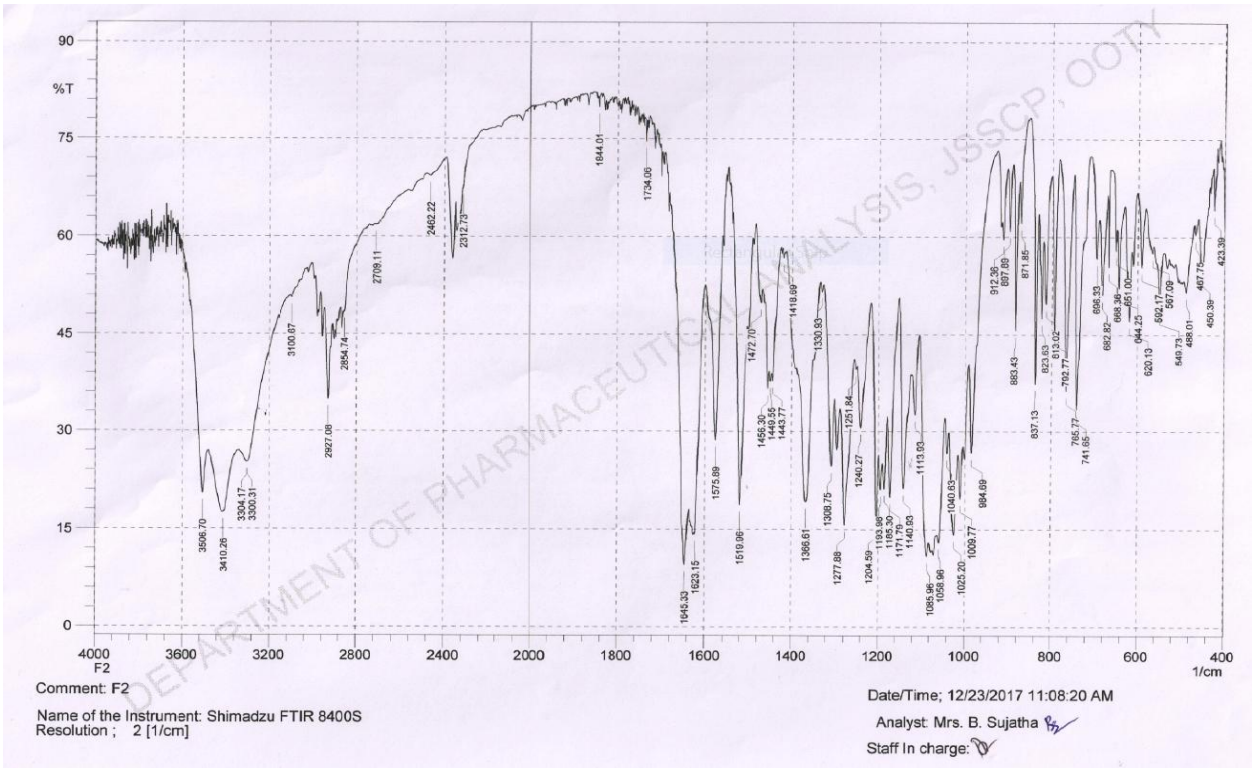

Figure S10. The FT-IR spectrum of isolated compound neohesperidin (3).

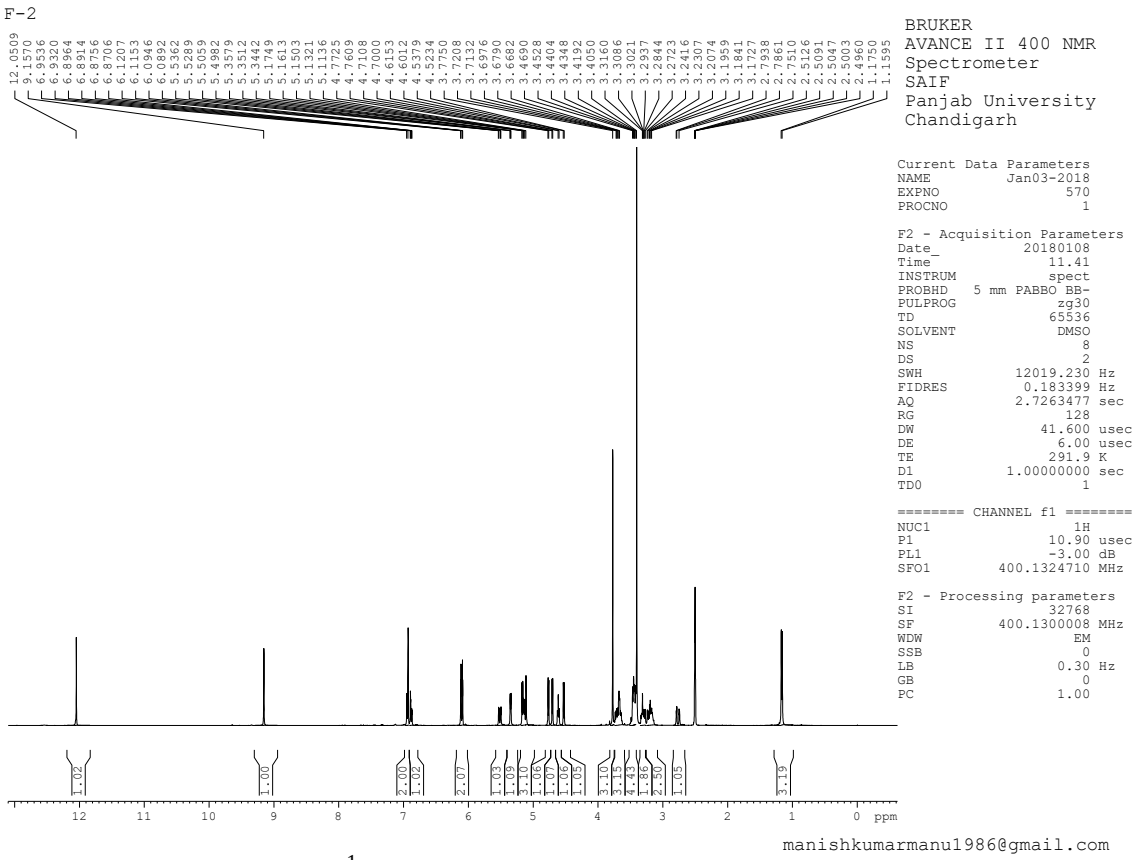

Figure S11. The ^{1}H NMR spectrum of isolated compound neohesperidin (3).

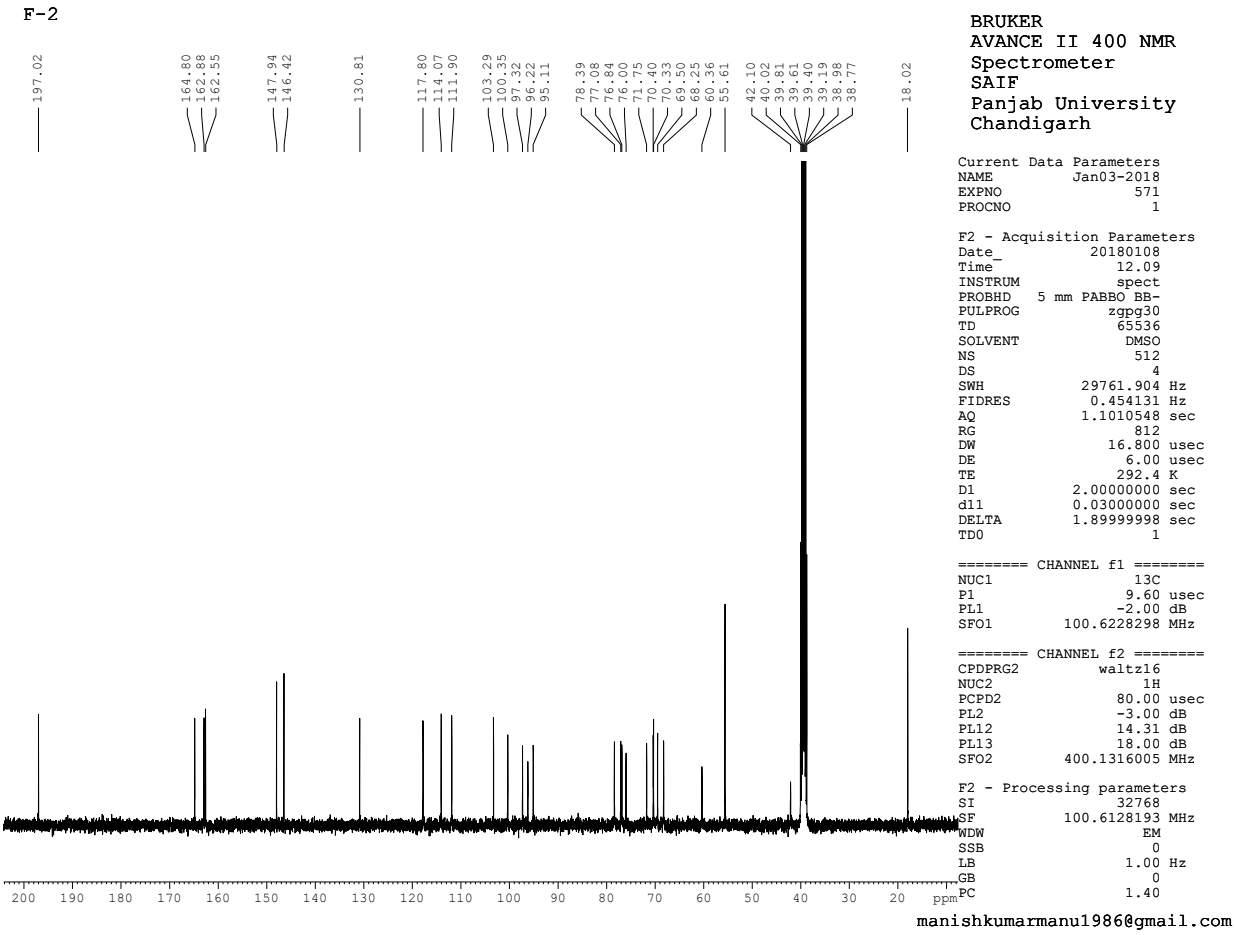

Figure S12. The ^{13}C NMR spectrum of isolated compound neohesperidin (3).

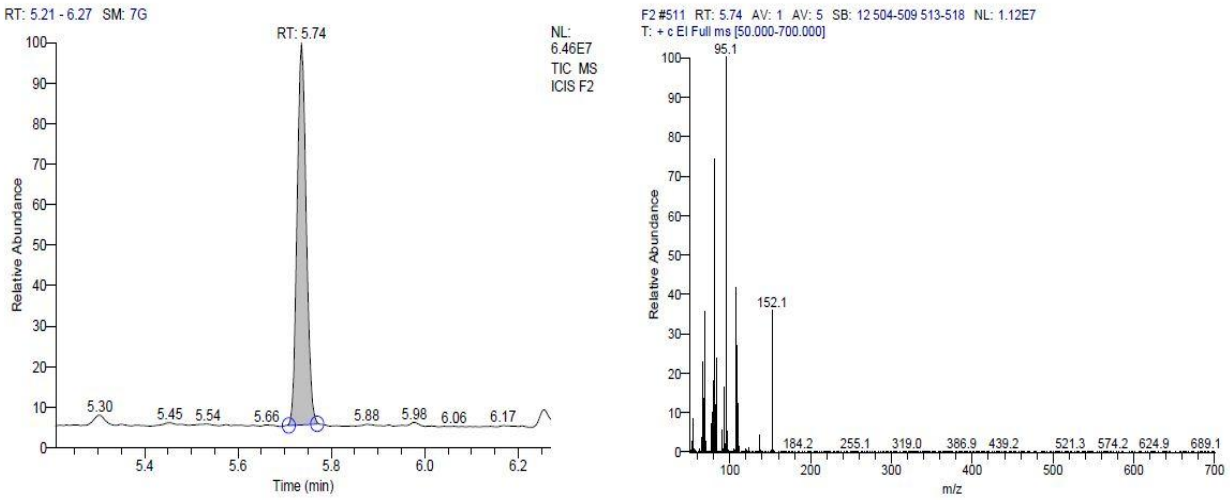

Figure S13. The GC-MS spectrum of isolated compound neohesperidin (3).

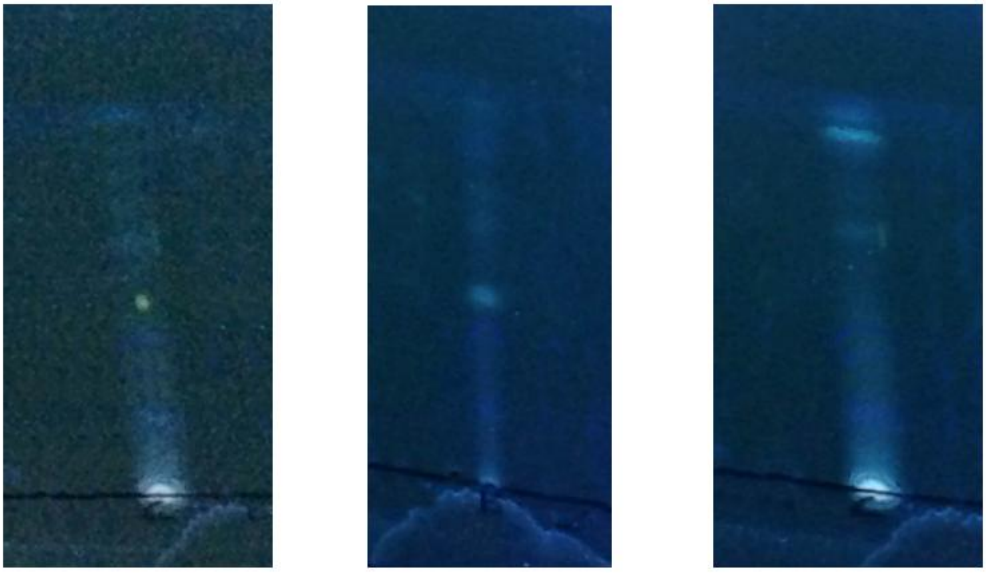

Figure S14: The TLC Images of isolated compounds from *Tadehagi triquetrum* **(**L.) H.Ohashi

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