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Research Article

Identification of Isolated Flavonoid Glycoside From Methanolic Extract of *Cucumis dipsaceus* Ehrenb. (Fruit)

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ABSTRACT

The present study was objected to investigate isolated flavonoid glycoside from methanolic extract from fruits (*Cucumis dipsaceus* Ehrenb.) as well as identification. Methanolic extract was already screened for the presence of flavonoid glycoside which was strong antioxidant and hepatoprotective agent. It was first time to isolate these by column chromatography with increasing polarity like Petroleum ether, Chloroform, Ethyl acetate and Methanol solvents of different ratios. Each 20ml elute was collected, analyzed with pre coated Merck F_{254} TLC plates to compare similar spots and Rf values for same category of contents. The similar elutes were pooled, concentrated dried by vacuum distillation with rotary apparatus. The chloroform ethyl acetate fractions (sticky dark brown mass) were analyzed by pre coated F_{254} TLC plates and then visualized under visible light, UV 254nm and UV366nm. The developed pre coated TLC plates was further derivatized with 0.5% solution of anisaldehyde - H_2SO_4 acid and again visualized under visible light and UV366nm.TLC, HPTLC and LCMS methods were used to found and estimate polyphenolic compounds. Spectroscopic methods (IR, 1D and 2D NMR and Mass spectrometry) were used to confirm and elucidate the structure. The concluded structure of flavonoid glycosides was quercetin- 3-rutinoside-7-rhamnoside (M Wt: 756.663g/mol) and plaid with literature data.

Keywords: *Cucumis dipsaceus* Ehrenb., Polyphenolic glycosides, HPTLC LCMS and Spectrometric methods.

INTRODUCTION

Natural compounds as phytoconstituents from plant kingdom have notable diversity and special significance[1]. Flavonoids, terpenoids and alkaloids are the secondary metabolites which are at present used as drugs or to avert a variety of diseases[2]. Flavonoids fetch more attention for research and development consideration[3]. These compounds not only able protect the plants from stress and oxidative reaction but also give protection[4-8]. to human being from free radicals cascade due to the pharmacological properties like antioxidants[9-10], hepatoprotective[11], antimicrobial[13,14], antiallergic[15], analgesic and anti- inflammatory[16], anticarcinogenic[17] and anti- obesity activities[18]. Current scenario needs more attention for safety, effectiveness and quality of medicinal products from plants and herbs. Thus, it becomes necessity to identify and quantify all the secondary metabolites to ensure the pharmacological research repeatability and reliability along with maintaining the quality control on pharmacological merits or demerits[19,20].

Cucumis dipsaceus Ehrenb. is also known as hedgehog cucumber and its native availability and distribution is Tanga Region and in northern and western parts of Tanzania and Uganda, Kenya, Africa and Sudan and Southern. Now a day, this herb is also found in Western Ghats of foothills of Maruthamalai of Coimbatore (Tamil Nadu) and Mysore (Karnataka), India. The fruit of this herb was screened for the presence of phytoconstituents saponin (triterpenoids), fatty acids, flavonoids, glucosides and phytosterols etc. The reported pharmacological activities of this fruit extracts were antioxidant, analgesic and anti- inflammatory, hepatoprotective, cytotoxic and antimicrobial. The present work is for identification and structure elucidation of the isolated compounds from methanolic extracts of fruit[21]. To the best of our knowledge, there are no reports on column chromatography and spectroscopic method for isolation and structure elucidation of flavonoids and glycosides. So, in this present work to isolate and identify the polyphenolic glycosides from methanolic extract of *Cucumis dipsaceus* Ehrenb. (Fruit)

MATERIAL AND METHODS

Plant Material and Chemical

Cucumis dipsaceus Ehrenb. Fruits were collected in November- December 2014 from Mysore, (Karnataka, India). Dr. Sunita Garg (Head of Raw material, Herbarium & museum) at National institute of science communication and information resources (NSCAIR CSIR), New Delhi 110067 has identified the sample fruits. A voucher specimen / Reference number NISCAIR/RHMD/Consult/2014/2367-147 was the obtained for these fruit from the same herbarium.

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Perkin Elmer - Spectrum RX-IFTIR was used for sample analyses to identify the functional groups and identification with KBr pellet as internal standard. It has resolution of 1 cm^{-1} and scan range of 4000 cm^{-1} to 250 cm^{-1}.

Liquid Chromatography Mass Spectrometer of Waters Micromass Q-Tof Micro instrument is hybrid quadruple time of flight mass spectrometer equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APcI) sources having mass Range of 4000 amu in quadruple and 20000 amu in ToF was used for identification of methanolic extract. Thermo Scientific TSQ 8000 Gas Chromatograph - Mass Spectrometer was used for analyses of methanolic extract.

HPTLC (CAMAG, Switzerland) WinCATS 4 software was installed with a silica gel 60 F_{254} (Merck, Darmstadt, Germany) TLC plate and by using the solvent system Ethyl acetate: Glacial acetic acid: Formic acid: water (100: 26: 11:11 v/v) for flavonoids detection and Scanner 3 (CAMAG, Switzerland) was as mobile phase in a CAMAG- twin-trough glass chamber used for the analysis methanolic extract.

NMR spectra were recorded on Avance-II Bruker NMR Spectrometer (400 MHz for 1H and 100 MHz for 13C NMR) using DMSO-d6, $CDCl_3$ and D_2O deutrirated solvents for resolution of isolated compounds with 1D NMR and 2D NMR Spectroscopy. The residual solvent peaks were used as internal standards.

ESIMS analysis was performed on Waters Q-Tof Premier Micromass Mass spectrometer.

Isolation of Flavonoid Glycosides by Column Chromatography From Methanolic Extract

Dried powdered (1.0 Kg) of fruits (*Cucumis dipsaceus* Ehrenb.) was made after slicing and drying under shade. The methanolic extract was prepared by using methanol as solvent with the help of soxhlet apparatus at 35 - 38˚C, after n-hexane maceration at room temperature. The methanol filtrate was concentrated and dried with rotary vacuum distillation apparatus.[21] The yield found was 120gm. Methanolic extract (100gm) was dissolved in 50ml methanol and mixed in silica gel powder of mesh 60 – 120, then subjected to column chromatography for isolation of phytoconstituents with chloroform, ethyl acetate, and methanol in different ratios (Petroleum ether100%, Petroleum ether: Chloroform (95:5), Petroleum ether: Chloroform (90:10)…….. Petroleum ether: Chloroform (5:95), Chloroform (100%), Chloroform: Ethyl acetate (95:5)..……. Ethyl acetate (100%), Ethyl acetate: Methanol (95:5) … Methanol 100%). Each 20ml elute was collected Each 20ml elute fraction were collected and then analyzed for phytoconstituents presence by pre coated Merck F_{254} TLC plates to know similar spots and Rf values for same category of isolated phytoconstituents further same elutes were pooled and concentrated by using vacuum distillation rotary apparatus and stored in vacuum desiccators to keep fractions free from moisture. Then Chloroform: Ethyl acetate fraction which was sticky dark brown mass was again dissolved in 20ml methanol. The Chloroform: Ethyl acetate fractions again repeated for Column chromatography for isolation and purity with different ratios of Chloroform (100%), Chloroform: Ethyl acetate (95:5)..……Ethyl acetate (100%) and (CEF_1, CEF_2 and CEF_3) were obtained[22].

Phytochemical Analysis

TLC and HPTLC studies were performed by prescribed methods[23,24].

Thin Layer Chromatography Profile

Chloroform: Ethyl acetate fractions (CEF_1, CEF_2 and CEF_3) were subjected for separation of flavonoids its glycosides by performing TLC fingerprinting. The sample of these fractions were prepared in methanol solvent and then spotted on precoated silica gel G aluminum plates $60F_{254}$ (E Merck Germany) with the glass capillary tubes. The spotted plate with proper handling was placed in mobile phase of solvent system (Ethyl acetate: Formic acid: Glacial Acetic Acid: Water 100:11:11:26 v/v/v/v solvent). The spotted plate after proper separation of chemical constituents was take out from solvent system, and then dried at room temperature. The derivatization was done by using 1% ethanolic aluminum chloride or 0.5% anisaldehyde in H_2SO_4 solution dipping or spraying method. Then plate was dried at dried at room temperature and also heated in hot air oven at 100°C temperature for 10 to 15 minutes. The color of spots were seen in visible and UV light (254nm and 366nm) and Rf values of each spots in were calculated and were tabulated in table 1and figure 1[25].

High performance thin layer chromatography (hptlc) profile

Preparation of sample

The methanolic extract fraction 100mg was dissolved in 1ml methanol (HPTLC grade) and the solution was centrifuged at 3000 rpm for 5 minutes and this test solution was further used for HPTLC estimation.

Developing Solvent System

Ethyl acetate: Formic acid

Glacial Acetic Acid: Water 100:11:11:26 v/v/v/v solvent system was used for developing HPTLC finger print profile of flavonoids and its glycosides[26].

Sample Application

Test solution (2µl) and standard solution (3µl) were filled in Hamilton syringe for 5mm band length on precoated silica gel G aluminum plates $60F_{254}$ (E Merck Germany) of (3x10cm) with the help of Linomat 5 applicator connected to CAMAG HPTLC system installed and programmed with WINCATS software.

Development of Chromatogram

After applying 5mm bands on precoated TLC plate was put into twin trough glass chamber (20x10 cm) and chromatogram was developed in Ethyl acetate: Formic acid: Glacial Acetic Acid: Water 100:11:11:26 v/v/v/v solvent system (mobile phase).

Detection of Spot

The developed chromatogram was dried at room temperature and then placed in CAMAG REPROSTAR 3 a photo documentation chamber and photographs were captured in visible light, UV 254nm and UV 366nm. The densitometer was used to scan the chromatogram at 405 nm after using detecting / derivatizing reagent and drying in hot air oven at 100°C. The finger print data as peak

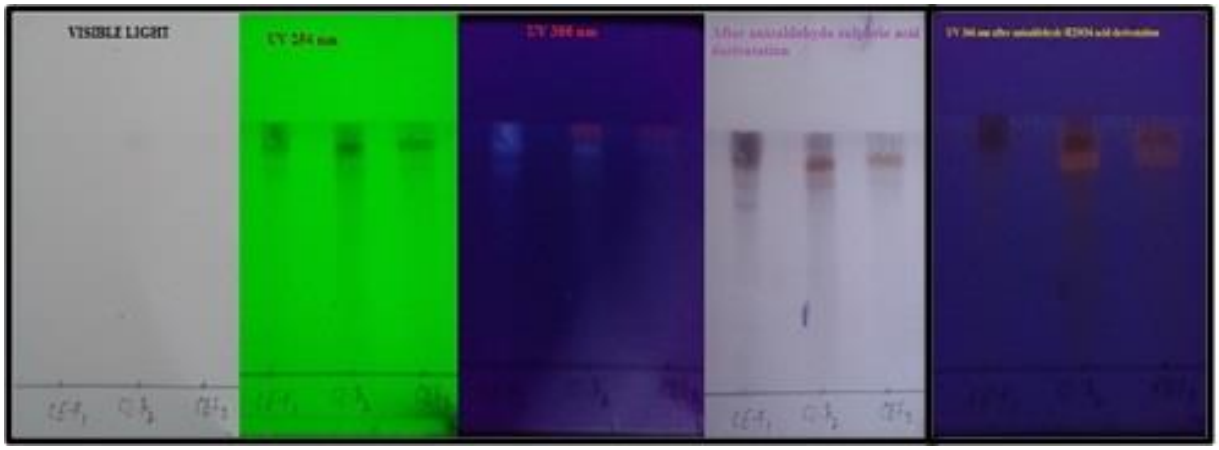


Figure 1: TLC of Chloroform: Ethyl acetate fractions (CEF_1, CEF_2 and CEF_3) showing flavonoids, phenolic acids and glycosides.

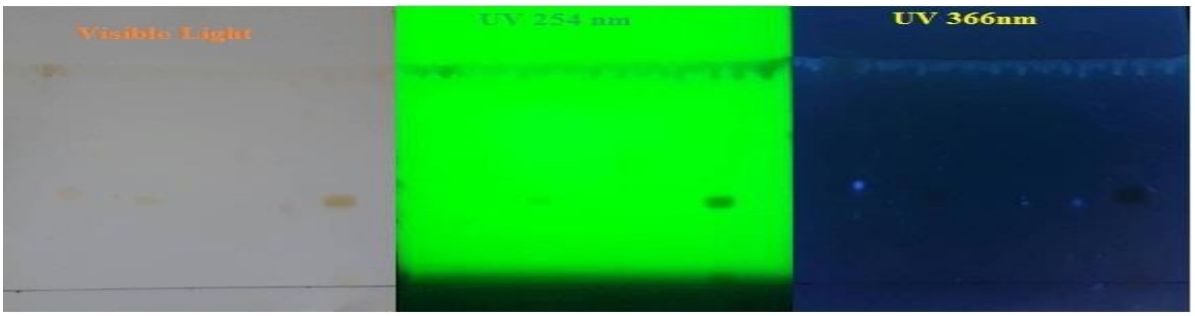


Figure 2: HPTLC of Methanolic extract for phenolic acids, flavonoids and glycosides.

High performance thin layer chromatography profile:

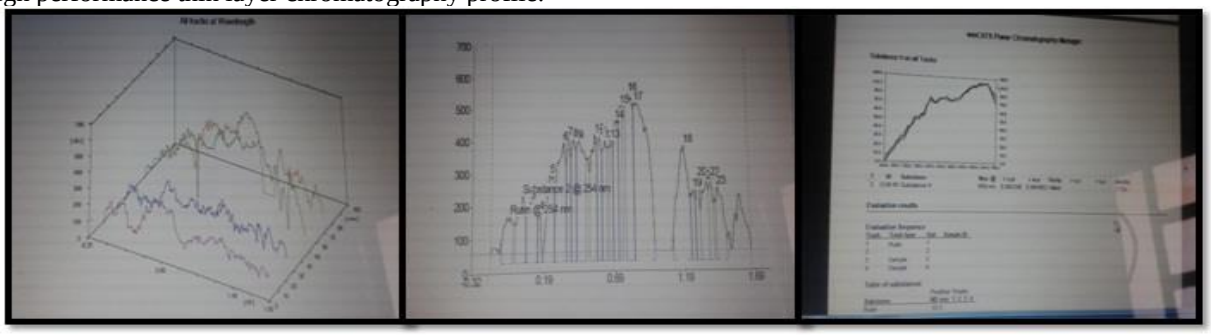


Figure 3: Quantification of CDME by HPTLC for phenolic acids, flavonoids and glycosides.

number with area and Rf values of were recorded by WINCATS software (1.3.4) and tabulated in table 2 and figure 2 and 3[26, 27].

Liquid Chromatography Mass Spectroscopy Profile

The recrystallized chloroform ethyl acetate fraction (10mg CEF_2) dissolved in 10ml methanol (HPLC grade) and used for polyphenolic compounds (phenolic and flavonoids) separation. The injection of 10µl volume was filled and injected to the Waters 2795 HPLC (Waters Micromass Q-Tof Micro) which having flow rates from 0.05- 5.0 ml/Min and configured with quaternary pumping and this equipment was auto sampler for next injection refilled with capacity 100 µl volume. This Instrument is hybrid of electrospray ionization (ESI) with atmospheric pressure chemical ionization (APCI) sources (mass Range of 4000 amu in quadruple) and 20000 amu in ToF with quadrupole time of flight mass spectrometer. The acidified water (0.5% Acetic acid, v/v) and acetonitrile was used mobile phase A and B respectively. The programmed gradient mobile phase system was done as 0 min, 0% B; 10 min, 20% B; 20 min, 50% B; 25 min, 75% B; 30 min, 100% B; 32 min, 0% B; and finally, the re- equilibration step was done as initial conditions for 8 min. 0.8 ml/ min flow rate was settled for all gradient mobile phase. This effluent was splitted when introduced to mass spectrometer using T-type splitter (split = 1:3) from the HPLC column and temperature was maintained at 25°C. Thus, the flow rate 0.2% was reached to the ESI-Q-TOF-MS detector. Due to maintained temperature Q-TOF provide calibration curve which provided accurate masses values for complete run. This method not needed any type of dual spray requirement for internal mass calibration and all vales were recorded in table 5[28].

Spectrometric methods profile

Table 1: Thin Layer Chromatography fingerprinting of CEF_1, CEF_2 and CEF_3 (Chloroform – Ethyl acetate fraction of methanolic extract).

S No	Fraction (Rf values)	Before Derivatization			After Derivaztion with 0.5% Anisaldehyde – H_2SO_4	
		Visible Light	UV 254nm	UV 366nm	Visible Light	UV 366nm
1	CEF_1(0.70, 0.88, 0.90)	No Color	Green blue	Whitish blue	Light violet, Light dark violet, dark brown color, indigo violet	Brownish black
2	CEF_2(0.35, 0.76, 0.80, 0.88)	(light yellow, Fluorescent yellow, violet pink, indigo)	All spots were Green blue	Blue, blue black, Whitish blue, dark pinkish red	Light violet, Light brown, dark brown, Indigo violet.	Black, Light brown, Fluorescent yellowish red, coffee brown.
3	CEF_3 (0.35, 0.76, 0.80, 0.88)	Only 0.76 light yellow	All spots were Green blue	Blue, blue black, Whitish blue, dark pinkish red	Light violet, Light brown, Light violet.	Black, Fluorescent yellowish red, coffee brown

Table 2: Identification of CDME for phenolic, flavonoids and its glucosides with Gallic acid, quercetin and rutin using HPTLC.

Rf values of CDME	0.14	0.17	0.26	0.34	0.37	0.41	0.48	0.56	0.59	0.63	0.68	0.71	0.75	0.83	0.97
% Area of AUC	3.23	0.77	3.93	6.65	3.19	3.79	4.84	3.61	3.75	4.12	2.82	4.12	3.66	8.39	15.77

Table 3: Fingerprint region of IR Spectroscopy for flavonoid glycoside.

S No.	Functional Groups	Frequency of functional group (cm^{-1})
1	-OH (Hydroxyl)	3422,3298
2	HC=CH (aromatic)	2957, 2894
3	>C=O (Carbonyl)	1666,1615, 1517, 1103,
4	C – O	1459, 1432
5	C=C	947, 820

Fourier transform infar red spectroscopic technique

The methanolic extract was mixed in dried KBr powder and pellet was made by hydraulic press and then IR spectrum was obtained as % transmittance of energy vs frequency (cm^{-1}) and tabulated in table 2.

1D *Nuclear Magnetic Resonance*

The methanolic extract ^1H NMR and ^{13}C NMR δ values (ppm) were tabulated in table 3.

2D *Nuclear Magnetic Resonance Between* ^1H – ^1H COSY AND ^1H – ^{13}C COSY

The observations was tabulated in table 4[29].

RESULTS

The present study effort was tilting towards the phytochemical screening, isolation and structure elucidation of compounds from methanolic extract of *Cucumis dipsaceus* Ehrenb. (Fruit) using TLC and HPTLC, LCMS, IR, NMR and Mass Spectroscopy.

Thin layer chromatography profile

Spectroscopic methods

IR, 1D and 2D NMR and LCMS spectra were used to elucidate the structures of isolated polyphenolic compounds.

Isolation and Characterization Of Chloroform Ethyl Acetate Fraction (Cef)

The structure was elucidated by using spectroscopic methods interpretation. UV spectra of flavonoid glycoside was took in MeOH (a), then by addition of NaOMe (b), $AlCl_3$ (c), $AlCl_3$/HCl (d) NaOAc (e) and NaOAc/H_3BO_3 (f).[34] Quercetin- 3-rutinoside-7-rhamnoside (M Wt: 756.663g/mol), Quercetin (aglycone): $C_{15}H_{10}O_7$, pale yellow needles (50mg) yellow florescence under visible light, Rf = 0.34, 0.60 and 0.88 (Ethyl acetate: Formic acid: Glacial Acetic acid: Water, 100:11:11:26) M. Wt 303 ESI-MS m/z 303$[M+H]^+$, UV λ_{max}, nm) (a) 255, 268, 370; (b) 247, 321; (c) 270, 360, 440; (d) 258,400; (e) 254, 276, 375 (f)272, 388.1H NMR (DMSO, 400 MHz, δ = ppm): 6.18 (1H, d, J = 2.0 Hz, H-6), 6.41(1H, d, J = 2.0 Hz, H-8), 6.89 (1H, d, J = 8.0 Hz, H-5'), 7.6 (1H, dd, J = 2.0 Hz, H-6'), 7.7 (1H, d, J = 2.0 Hz, H-2'), 13C NMR (DMSO, 125 Hz) C_2: 146.76, C_3 : 135.69, C_4 : 175.79, C_5 : 160.68, C_6 : 98.14, C_7 : 163.83, C_8 : 93.3, C_9 : 156.09, C_{10} : 102.97, $C_{1'}$: 121.91, $C_{2'}$: 115.02, $C_{3'}$: 145.01, $C_{4'}$: 147.66,, $C_{5'}$: 115.56, $C_{6'}$: 119.93. ^1H and ^{13}C NMR (DMSO) Sugar Glucose: ^1H NMR: OH_1 = 6.225 m (J = 4.68 Hz), H_1 = 4.862 t (J = 4.250 Hz), OH_4 = 4.806 d (J = 5.54 Hz), OH_3 = 4.722 d (J = 4.88 Hz), OH_2 = 4.532 d (J = 6.468 Hz), OH_6 = 4.407 t (J = 5.96

Table 4: 1H, 13C NMR, 1H-1H COSY,1H-13C COSY and LCMS data of Quercetin- 3-rutinoside-7-rhamnoside.

1H NMR	2.49-2.51(1H), 3,37 (12H) 3.85(1H), 6.18(1H), 6.4(1H), 6.87(1H), 7.5(1H), 7.67(1H), 9.35(1H), 10.78(1H),12.49(1H.)
13C NMR	93.3, 98.14, 102.97, 115.02, 115.56, 119.93, 121.91, 135.69, 145.01,146.76, 147.66, 156.09, 160.68,163.83, 175.79.
LCMS	60,74,101,102,103,168,179,274,303,316,463,481,482,507,551,633,634,635,701,702,758.

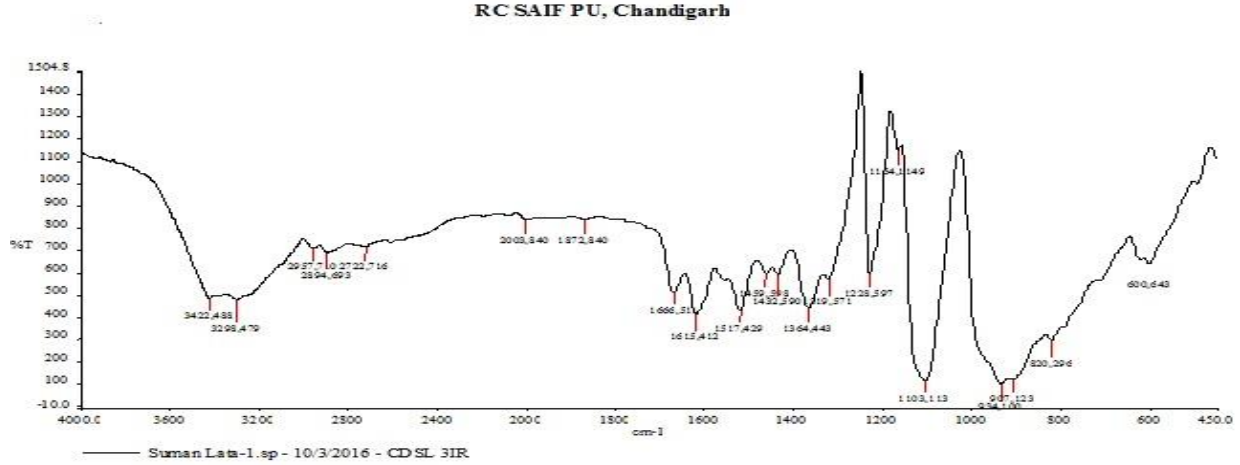
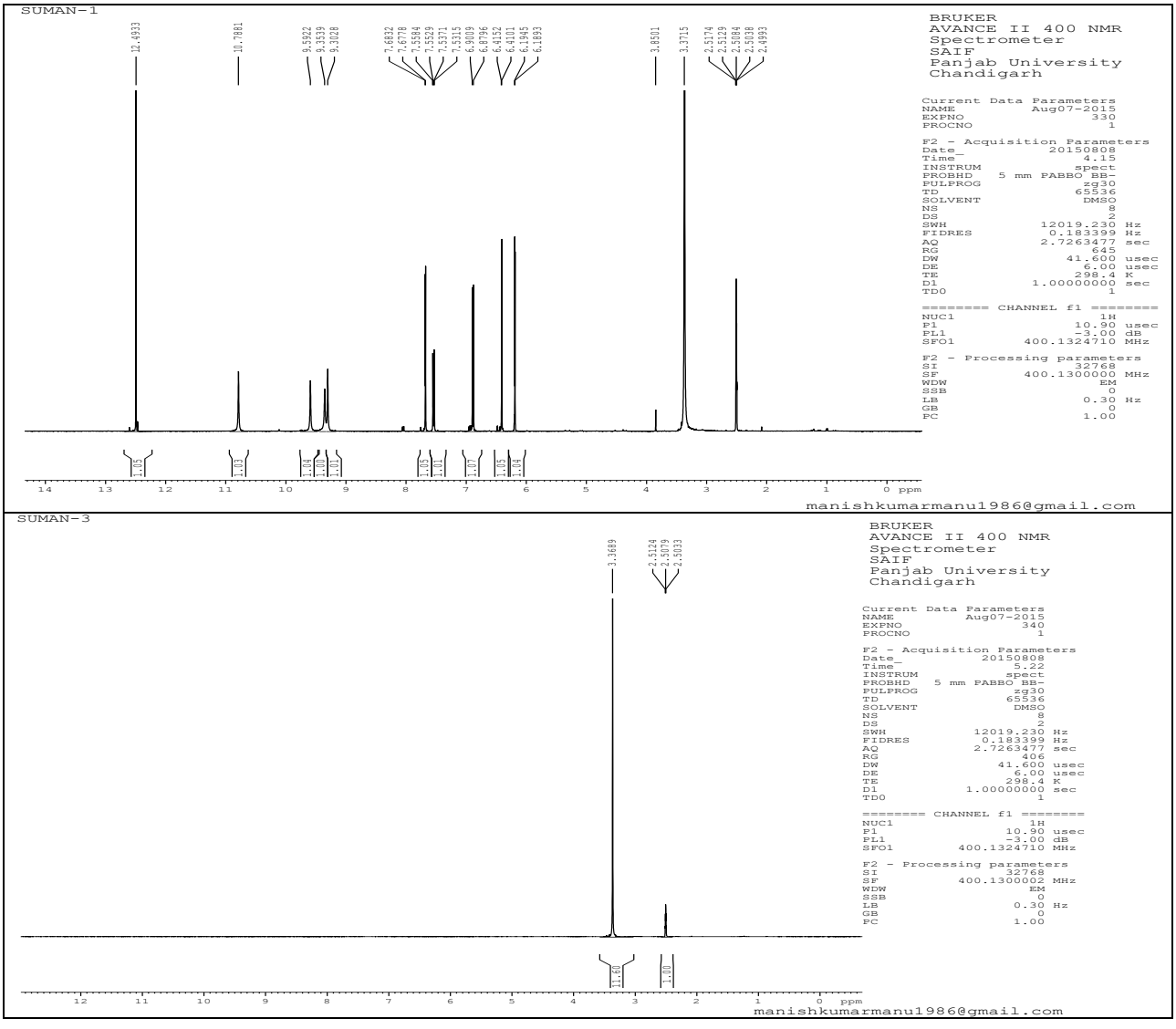
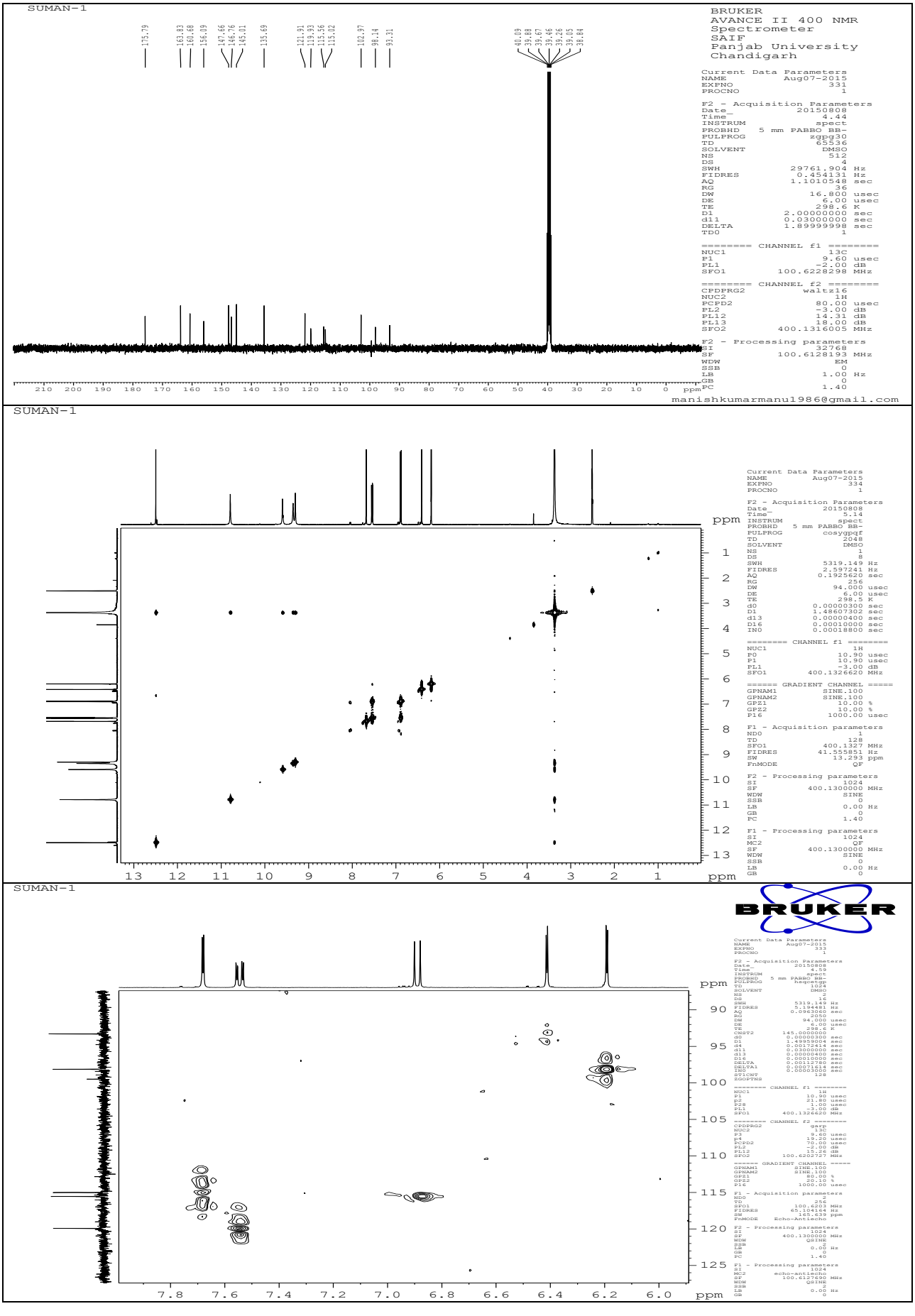


Figure 4: Fourier Transform Infra Red profile.




SUMAN-1
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
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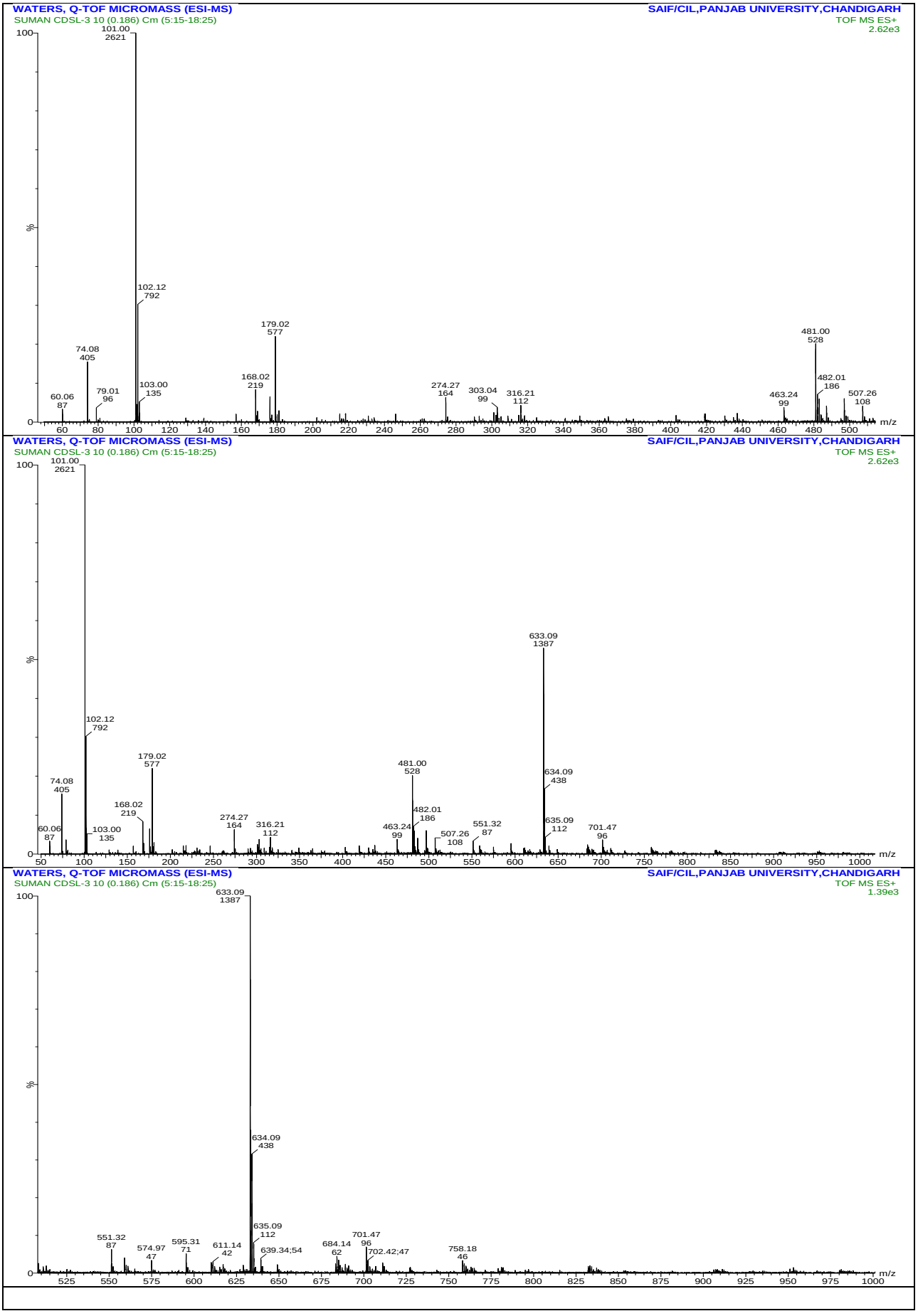
WATERS, Q-TOF MICROMASS (ESI-MS)
SUMAN CDSL-3 10 (0.186) Cm (5:15-18:25)
SAIF/CIL,PANJAB UNIVERSITY,CHANDIGARH
TOF MS ES+
2.62e3
101.00 2621
102.12 792
179.02 577
74.08 405
481.00 528
168.02 219
103.00 135
482.01 186
274.27 164
303.04 99
316.21 112
463.24 99
507.26 108
79.01 96
60.06 87
m/z
WATERS, Q-TOF MICROMASS (ESI-MS)
SUMAN CDSL-3 10 (0.186) Cm (5:15-18:25)
SAIF/CIL,PANJAB UNIVERSITY,CHANDIGARH
TOF MS ES+
2.62e3
101.00 2621
633.09 1387
102.12 792
179.02 577
481.00 528
74.08 405
634.09 438
168.02 219
482.01 186
274.27 164
316.21 112
463.24 99
551.32 87
507.26 108
635.09 112
701.47 96
103.00 135
60.06 87
m/z
WATERS, Q-TOF MICROMASS (ESI-MS)
SUMAN CDSL-3 10 (0.186) Cm (5:15-18:25)
SAIF/CIL,PANJAB UNIVERSITY,CHANDIGARH
TOF MS ES+
1.39e3
633.09 1387
634.09 438
635.09 112
551.32 87
595.31 71
574.97 47
611.14 42
639.34;54
684.14 62
701.47 96
702.42;47
758.18 46
m/z

Hz), ^{13}C NMR: C_1 = δ 92.13, C_2 = δ 72.97, C_3 = δ 72.23, C_4 = δ 71.87, C_5 = δ 70.43, C_6 = δ 61.06. Rhmnose: 1H NMR: δ 1.28 (3H, d, J = 6.4 Hz), δ 3.07- 3.22 (2H, (3.18 dd; J 10.2, 3.5 Hz), δ 3.13 (dd, J = 10.3, 10.2 Hz) δ 4.00 (^{1}H, dq, J = 10.3, 6.4 Hz), δ 4.77 (^{1}H, d, J = 2.9 Hz). ^{13}C NMR: δ 73.3, δ72.9, δ 6.94, δ 73.2, δ 18.3, δ 94.3.

DISCUSSION

The methanolic extract of fruits (*Cucumis dipsaceus* Ehrenb.) was already screened for presence of flavonoid glycoside by Shinoda's test; quantify the presence of phenolic and flavonoids contents, in vitro antioxidant activity. It's in vitro and in vivo hepatoprotective activity was evaluated. Then flavonoid glycoside was isolate by column chromatography and characterized by using UV Spetrophotometer which showed the presence of double bond conjugation of aromatic rings (Chromophores - CH=CH-). Band I and II was analyzed by 254 and 366nm λ_{max} (200 – 400nm range). TLC and HPTLC showed the spot and Rf values of flavonoid glycoside and this was further quantified with comparing marker standard compounds like rutin and Quercetin. The Infra red spectroscopy showed that presence of free hydroxyl groups with frequency 3422 and 3298 cm^{-1}. Aromatic ring was confirmed due to the presence of frequency 3422 and 3298 cm^{-1}. frequency 1459, 1432 cm^{-1} occurred due to the bonding between C – O which may due to presence of attachment of hydroxyl group on aromatic ring and also due to carbonyl moiety as part 4H- chromen – 4-one of flavonoids structure. 947, 820 cm^{-1}bending frequencies showed due to double bonds of aromatic ring of flavonoids structure. So, IR investigation predicted the flavonoids structure. The ^{1}H NMR spectrum exposed the existence of a hydrogen bonded to hydroxyl groups and gave signal with C=O (Cs - OH) at δ 12.48. A singlet at δ 10.77 was revealed the C_3-OH proton. A singlet at δ 9.58 showed resonating pattern because of C_7 – OH proton. A broad singlet at δ 9.36 was integrated for two protons at C_3 and C_4 as hydroxyl groups. δ 6.18 and 6.40 showed two singlets due to protons (H-6 and H-8) present on phenyl (aromatic ring A) of flavonoidal nucleus at C_6 and C_8, Two doublets at δ 6.88 and δ 7.67 were recognized due to H5' and H6' protons at aromatic ring B[30- 33]. A peak at δ 7.67 due to $C_{2'}$ proton was showed a singlet. The flavonoid glycoside (Quercetin- 3-rutinoside-7-rhamnoside) has molecular weight was 756.663g/mol by Electron Spray Ionization Mass Spectrometry (ESIMS) and was rectified through fragmentation pattern 179, 303, 463, 481 611, 758 which was also matched with literature data[33- 35].

CONCLUSION

All the results and discussion were an explanation to the confirmation of flavonoid glycoside as quercetin- 3-rutinoside-7-rhamnoside with molecular weight was 756.663 g/mol. This bioactive contituent was first time isolated from methanolic extract of fruit of *Cucumis dipsaceus* (Ehrenb.) and also provides protection for hepatic damage or hepatitis.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

There is no interest of conflict.

ABBREVIATION AND ACRONYM

TLC = Thin Liquid Chromatography, HPTLC = High Pressure / High Performance Thin Liquid Chromatography, LCMS = Liquid Chromatography Mass Spectrometer, FTIR = Fourier Transform Infar red, NMR = Nuclear Magnetic Resonance, UV – VIS = Ultra Violet – Visible, ^{1}H NMR = Proton Nuclear Magnetic Resonance, ^{13}C NMR = ^{13}Carbon Nuclear Magnetic Resonance, DEPT = Distortionless Enhancement by Polarization Transfer (45, 90, 135), APT = Attached Proton Test, 1H – 1H COSY = Proton – Proton Correlation Spectroscopy, 1H – 13C COSY = Proton – Carbon Correlation Spectroscopy, HETCOR = Heteronuclear Chemical Shift Correlation, HMBC = Heteronuclear Multiple Bond Correlation, HMQC = Heteronuclear Multiple – quantum correlation, Chloroform: Ethyl acetate fractions = CEF.

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