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STUDIES ON ISOLATION, PURIFICATION AND STRUCTURE ELUCIDATION OF CHEMICAL CONSTITUENTS FROM METHANOLIC FLOWER EXTRACT OF *BLEPHARIS MOLLUGINIFOLIA* PERS. AND THEIR BIOLOGICAL ACTIVITIES

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Keywords:

Blepharis molluginifolia, Biochanin A, Genistein, Pryoglutamic acid, Arabino gamma lactones, DPPH, antimicrobial, LC-MS, NMR specroscopy

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ABSTRACT: *Blepharis molluginifolia* (Acanthaceae), is a threatened medicinal herb. The methanolic flower extract of *Blepharis molluginifolia* was subjected to Silica Gel Column Chromatography for purification of bioactive compounds and structure elucidation of active compounds were studied. DPPH and antimicrobial activities of two isolated compounds were studied. From the analysed data, the compounds identified were 5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one (BiochaninA) $C_{16}H_{12}O_5$ and 5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen-4-one (Genistein) $C_{15}H_{10}O_5$. These compounds are first time reported in this plant. Another fraction GF1 was subjected to GCMS analysis and obtained 12 compounds (aminoacid, gamma lactone, sugars and fatty acids).As GCMS profile contained majority of sugars antiglycation was done against arbutrin and found >70% inhibition. The present investigation revealed that the methanolic flower extract contained phytoestrogens like Biochanin A and Genistein, the rare aminoacid pyroglutamic acid, gamma lactones, sugars and fatty acids.

INTRODUCTION: From pre-historical times, since the existence of human beings, plants have been used in primary health care. Usage of medicinal plants for phytocompounds has been increasing worldwide day by day. Plants are important for pharmacological research and drug development, not only when bioactive phytocompounds are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds.

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Regulation of their exploitation and exportation is therefore essential to ensure their availability for the future [1]. *Blepharis molluginifolia* belongs to the family Acanthaceae, is a threatened medicinal herb. This plant is used for urinary discharges and also equated with Uttangana [2]. Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. It is necessary to have methods available for efficient separation from plant extracts, which are typically mixtures of thousands of different molecules [3].

The common approach is to set up a fractionation scheme and to screen the fractions for the presence of the desired bioactive properties. Active fractions are sub fractionated and tested, until the molecules responsible for the bioactivity can be identified. Thin-layer chromatography (TLC) and silica gel column chromatography are the simplest and

cheapest methods of detecting plant constituents because the method is easy to run, reproducible and requires little equipment [4, 5]. High performance liquid chromatography can be used for good sensitive detection for purity of bioactive compounds [6, 7]. Modern analytical spectroscopic techniques like Fourier Transform Infrared spectroscopy (FT-IR) Liquid chromatography elctrospray ionization mass spectroscopy, play a important role in identification and characterization of bioactive compounds [8].

MATERIALS AND METHODS:

Chemicals:

Organic solvents like hexane, chloroform, methanol of analytical grade obtained from Merck, India. HPLC grade methanol, acetonitrile were obtained from Ranbaxy fine chemicals limited. 2, 2-Diphenyl-2-picrylhydrazyl (DPPH), precoated silica gel TLC sheets, Arbutin were obtained from Sigma Chemical, USA. Bovine serum albumin (Merck, German), Silica gel (70-230 & 60-120 mesh size) was obtained from Qualigens fine chemicals. All other chemicals and solvents used were of analytical grade.

Collection of plant material:

Fresh flowers of *Blepharis molluginifolia* were collected in the month of November and kept in sterile bags. This plant was collected in Koyathanda of Nallamala forest region, Andhra Pradesh, India. This plant is authenticated by Dr. S.B. Padal, Botany Department, Andhra University, Visakhapatnam and specimen has been deposited in Herbarium of Botany department with voucher number B.D.H-22220.

Preparation of plant extract:

Fresh flowers were collected in the month of November and washed with distilled water and shade dried. The shade dried flowers were blended in mixie to fine powder**.**

Extraction of bioactive constituents:

100gm of *Blepharis molluginifolia* flower powder was extracted with Soxhlet apparatus using solvent methanol nearer to its boiling point until it became colorless. The extracts were then concentrated to dryness using rotary evaporator.

Silica Gel and Thin layer Chromatography of methanolic flower extract: Crude methanolic extract of *Blepharis molluginifolia* was subjected to Silica gel column chromatography to separate into its respective component fractions. Silica gel has been used as stationary phase and varying ratios of n-hexane, chloroform,, methanol and water in increasing polarities. The glass column (80cm in length and 5cm in diameter) was taken, rinsed with hexane and completely dried before use. The column was covered with cotton plug .The column was packed with activated silica gel (70-230 mesh size) slurry with constant tapping. The crude methanolic extract (10g) was dissolved in little amount of methanol and mixed with silica gel (1:2 w/w), loaded onto the column.

The extract was eluted with increasing polarity of solvents. Following ratios were sequentially used n-hexane: chloroform 100:0, 75:25, 50:50 and 25:75; chloroform : methanol 100:0, 75:25, 50:50 and 25:75; methanol and water 100:0, 75:25, 50:50, 25:75 and 0:100 at a flow rate of 10 ml per hour and 10 ml fractions were eluted and collected. Then the fractions subjected to TLC. Aliquots of fractions were spotted on base line of precoated silica gel TLC sheets and allowed for few minutes until dried. Then the TLC plates were kept in TLC chamber saturated with solvent system. TLC plates were kept in iodine chamber to visualize the spots. Fractions with distinct spots and same Rf value were pooled for each solvent system and condensed using rotary evaporator.

High Performance Liquid Chromatography (HPLC):

HPLC (Agilent 1100 series) with LC solution software, PDA Detector (SPD-M 20 A) and Agilent TC C18-G column (4.6 x 250 mm) was used to determine the purity of all the pooled fractions with single spot in TLC analysis. 5 µl of sample was injected using Hamilton syringe (Bonaduz schweiz). Acetonitrile: 0.05%phosphoric acid in water mixture used as mobile phase was filtered using 0.2 micron membrane filter with flow rate of 1 ml/min.

FT-IR Spectroscopy:

The functional groups of isolated antioxidant compound were analyzed using Bruker alpha FT-IR instrument (Software opus 6.5). 1 mg of isolated

bioactive compound was mixed with 100 mg potassium bromide and the mixture was compressed to prepare as small pellet. Then this pellet was analyzed under FT-IR spectrophotometer in the range of 349.053-7800.65cm^{-1} at room temperature.

Liquid Chromatography Electrospray Ionization Mass Spectroscopy (LC-ESI-MS):

The bioactive compound was analyzed by Agilent 1100 series LC-MSD with electro spray ionization (ESI) and quadrupole mass analyzer. Ammonium hydroxide (0.75 M) was used as buffering reagent. The analysis was performed at positive ion mode under at a flow rate 1 ml/min, nebulizer pressure-25 psi, capillary voltage-3 kV, fragmentor voltage-75V and drying gas temperature- 350ºC. Spectrum of bioactive compound was scanned over range of (0-650) m/z.

NMR Spectroscopy:

Nuclear magnetic resonance (NMR) spectroscopy was performed to determine the structure of isolated bioactive compound. About 25 mg sample which was dissolved in 0.5 ml dmso-d6 which was used. ^{1}H and ^{13}C NMR spectra were recorded on NMR - Jeol / AL-400 MHz NMR instrument using deuterated dimethyl sulfoxide. The region from 0 - 12 ppm for ^{1}H NMR and 0 - 200 ppm for ^{13}C NMR was used for scanning. Trimethylsilane (TMS) was used as internal standard. The chemical shifts (δ) were expressed as parts per million (ppm) and the coupling constants (J) were indicated as hertz (Hz).

Gas Chromatography Mass Spectrometry (**GC-MS**):

The GC-MS analysis of the F1 extract was run on a Hewlett-Packard GC–MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, The fused-silica HP-20 M polyethylene glycol column (50 m x 0.2 mm, 0.2 mm thickness, Hewlett-Packard, was directly coupled to the mass spectrometer. The carrier gas was helium (1 ml/min). The program used was 4 min isothermal at 70°C, then 4°C /min to 180 °C and 10 min isothermal. The injection port temperature was 250°C and the detector temperature was 280°C. Ionization of the sample components was performed in the EI mode (70 eV).

Bioactivity of isolated compounds:

Determination of antioxidant activity: [9]

DPPH assay of all pooled fractions was determined by using Mensor *et al* (2001).

Antimicrobial activity:

Screening of gram positive, gram negative and fungal strains of all pooled fractions were done by modified Kirby-Bauer disc diffusion method [10].

Anti-glycation activity:

The anti-glycation activity was determined as per the method of Matsura *et al*, 2002 [11] with slight modifications. Bovine serum albumin (1mg/ml) and 0.5ml glucose (9%) with 100µl of GF1 and IF1 extract was incubated at 60^{0}C for 24hrs with control and blank. After the incubation, the reaction was stopped by adding 100µl of 100% TCA to 0.5ml of the above mixture and incubated at 4°C for 10min. The samples were centrifuged at 10000rpm for 15min and supernatant was discarded. The pellet was dissolved in 1ml 0.9% saline phosphate alkaline buffer pH 10 and then assayed for anti-glycation activity by borohydride assay. Arbutin was used as a reference standard. The percent inhibition of fructosamines by plant extracts was calculated by the equation

$$\text{Inhibitory activity (\%)} = [(A_o - A_1)/A_o] \times 100$$

Alpha glucosidase inhibition:

The inhibition of *a*-glucosidase activity was determined using the modified published method [12]. One mg of *a*-glucosidase (*Saccharomyces cerevisiae*, Sigma-Aldrich, USA) was dissolved in 100 mL of phosphate buffer (pH 6.8) containing 200 mg of bovine serum albumin. The reaction mixture consisting 10 μL of GF1 and IF1 extracts separately at varying concentrations (0-100 μg/mL) was premixed with 490 μL phosphate buffer pH 6.8 and 250μL of 5mM *p*-nitrophenyl *a*-D-glucopyranoside. After preincubating at 37°C for 5 min, 250μL *a*-glucosidase (0.15unit/mL) was added and incubated at 37°C for 15 min. The reaction was terminated by the addition of 2000 μL Na_2CO_3 200 mM. *a*-glucosidase activity was determined spectrophotometrically at 400 nm on spectrophotometer UV-Vis (Shimadzu 265, Jepang) by measuring the quantity of *p*-nitrophenol released from p-NPG. Acarbose was used as

positive control of *a*-glucosidase inhibitor. The concentration of the extract required to inhibit 50% of *a*-glucosidase activity under the assay conditions was defined as the IC_{50} value.

RESULTS AND DISCUSSION:

Silica gel column chromatography:

Pure bioactive compounds reported to have more significant protective effect against oxidative stress mediated damage [13]. In the present study, methanolic extract of *B.molluginifolia* flower was subjected to silica gel column chromatography for further purification of antioxidant compounds. Totally 500 fractions obtained and pooled to 16 fractions by gradient elution silica gel column chromatography and represented in **Table 1.**

Fractions (109-150) were eluted with 100% chloroform. These fractions showed significant antioxidant and antimicrobial activities. This fractions were pooled with TLC-single spot showed at Rf value of 0.59 and called as EF1. This EF1 fraction was further subjected to silica gel column chromatography. The chloroform extract (EF1) was rechromatographized (silica gel 60-120 mesh size) with hexane and increasing 5% of $CHCl_3$ and with methanol until 100% methanol. Fraction E1eluted with chloroform-methanol (9.5: 0.5), showed one major spot with R_f value 0.5 in solvent system chloroform: methanol (7:3) and gives yellow colour with $AlCl_3$ 2% in methanol. These fractions were collected together, solvent evaporated to give white colour compound (Compound 1).

Fraction T1 eluted with chloroform-methanol (7:3) were found to contain major spot with R_f value 0.3. Fractions were collected together and evaporated to yield yellowish white colour compound (Compound 2). These two compounds were further subjected to HPLC analysis.

TABLE 1: SILICA GEL AND THIN LAYER CHROMATOGRAPHY OF METHANOLIC FLOWER EXTRACT OF *BLEPHARIS MOLLUGINIFOLIA*

Fraction No	Ratio of solvents	Single spot Pooled fractions	TLC Rf Value	DPPH radical Scavenging Activity (%)	Antimicrobial activity		
					Gram +Ve	Gram – Ve	Fungi
1-15	n-hexane100%	-	-	-	-	-	-
16-46	Hexane:chloroform 75:25	BF1	0.58	18.54	+	-	-
47-77	Hexane: chloroform 50:50	CF1	0.32	22.48	+	+	+
78-108	Hexane: chloroform 25:75	DF1	0.45	32.05	+	+	-
109-150	Chloroform100%	EF1	0.59	81.98	++	++	++
151-170	Chloroform: Methanol 75:25	FF1	0.85	47.09	+	+	+
171-201		FF2	0.79	25.86	-	-	-
202-232	Chloroform: Methanol 50:50	GF1	0.81	62.09	++	++	++
233-263		GF2	0.67	51.02	+	+	+
264-294	Chloroform: Methanol 25:75	HF1	0.58	68.22	+	+	+
295-325	Methanol 100%	IF1	0.51	71.59	++	++	++
326-346		IF2	0.71	60.45	+	+	+
347-377	Methanol:water 75:25	JF1	0.9	53.5	-	+	-
378-408	Methanol:water 50:50	KF1	0.76	50.25	+	+	+
409-439		KF2	0.38	62.38	-	-	-
440-470	Methanol:water 25:75	LF1	0.35	50.25	-	-	-
471-500	Water100%	MF1	0.5	34.65	+	+	+

"++" High "+" Traces & "-" Absent

Determination of purity by High Performance Liquid Chromatography: From **Fig. 1,** Analysis of E1 by HPLC showed a sharp peak at 9.813 retention time (RT) and peak area of 98.897%. This indicates that eluted compound has purity of 98.897%. From **Fig. 2**, HPLC analysis of T1 showed peak at 8.053 retention time (RT) and peak area is 99.09%. This confirms the purity of compound 2 as 99.09%.

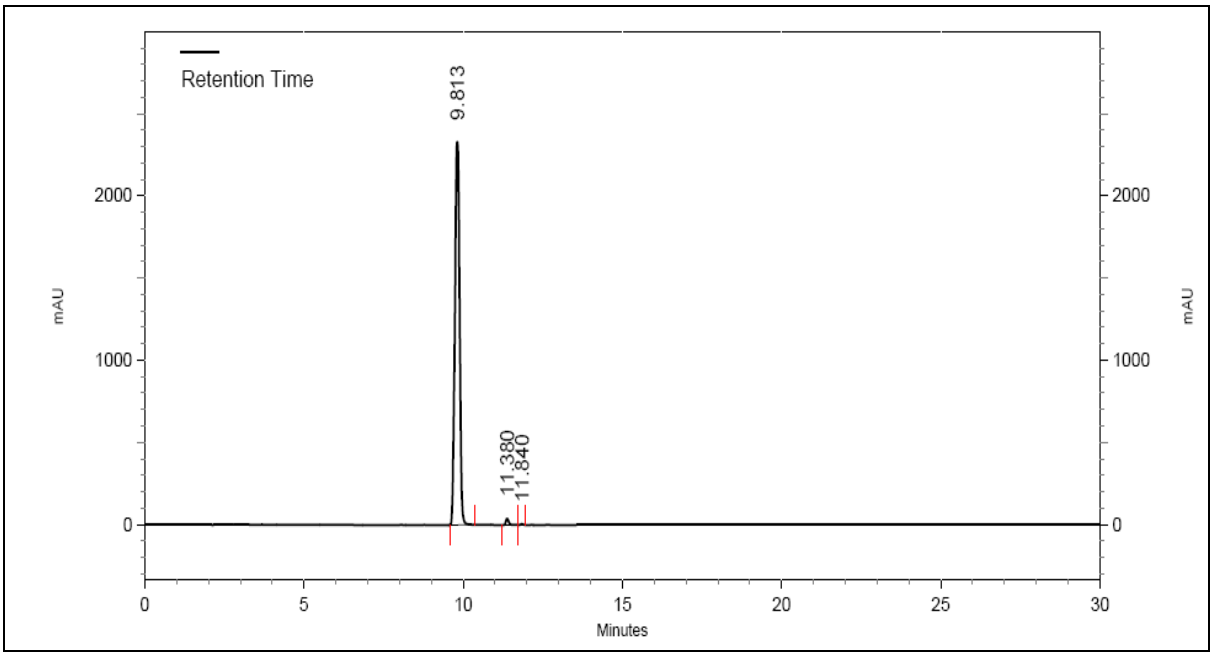

FIG. 1: HPLC OF ISOLATED COMPOUND 1

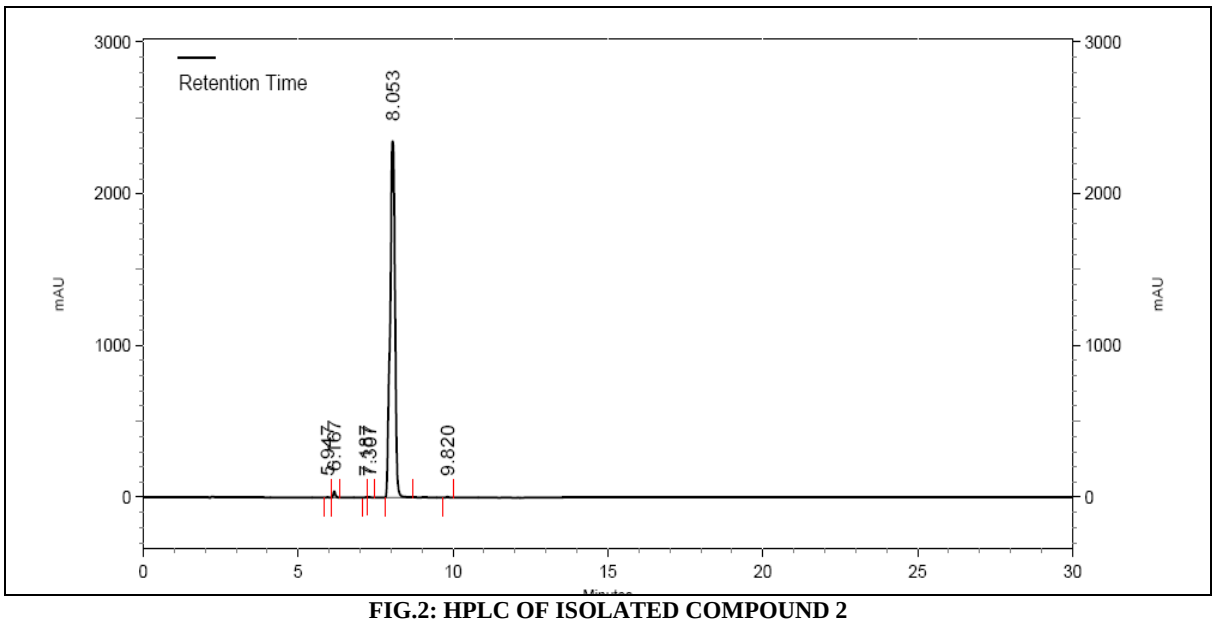

FIG.2: HPLC OF ISOLATED COMPOUND 2

Fourier Transform Infrared Spectroscopy:

The FTIR absorption spectra of isolated compounds were shown in **Fig. 3** and **4**. Compound 1 exhibited absorptions at 3387, 3309, 3074, 2985, 1652, 1566, 1513, 1438, 1359 cm-^{1}. The characteristic peaks at 3387, 3309 cm^{-1} and peak at 3074^{-1} cm suggests the presence of hydroxyl group (free phenolic 0H) and aromatic ring. A strong absorption band at 1652 cm^{-1} indicates the presence of carbonyl group(C=O) and aliphatic ($CH_{3)}$ stretching at 2985 cm^{-1} respectively. Further peaks at 1566, 1513, 1438 cm^{-1} suggests the presence of (C=C) of aromatic ring and peak at 1359 cm^{-1} indicates characteristic CH_3 bending.

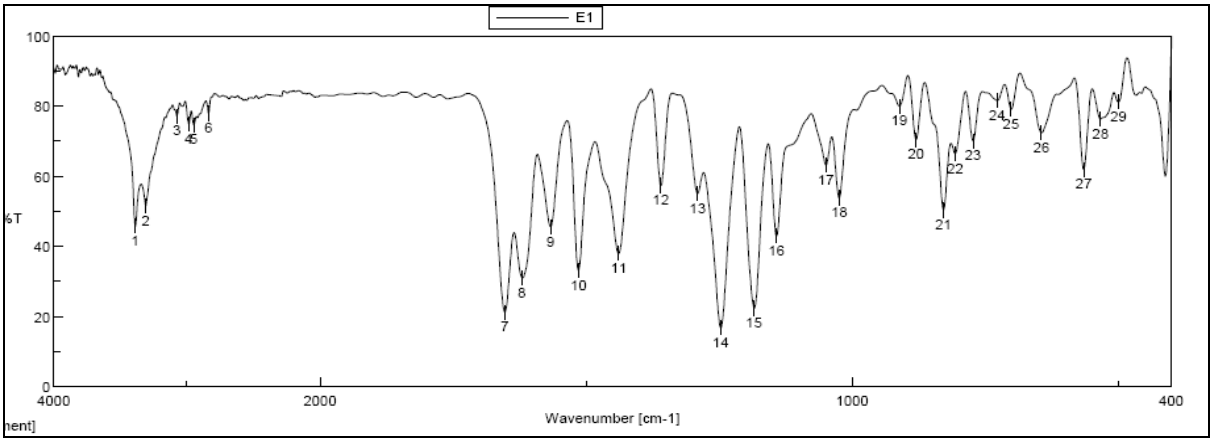

FIG.3: FT-IR SPECTRUM OF ISOLATED COMPOUND 1

From IR spectrum Compound 2 showed characteristic signal at 3411.5^{-1}cm indicated the presence of hydroxyl group (OH). A strong absorption band at 1651^{-1}cm and 3130^{-1}cm indicates the presence of carbonyl group(C=O) and aromatic hydrogen respectively. Further peaks at 1567, 1515 cm^{-1} suggests C=C of aromatic ring.

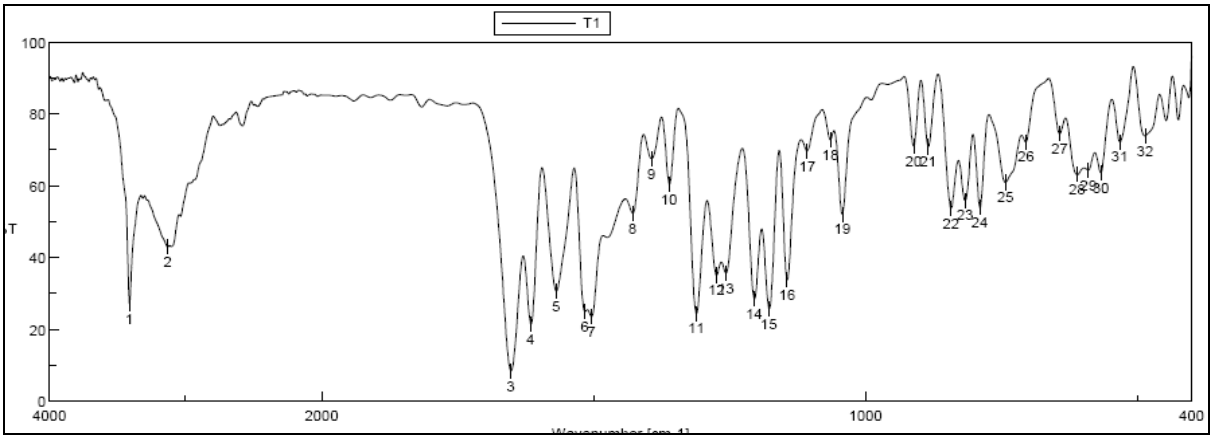

FIG. 4: FT-IR SPECTRUM OF ISOLATED COMPOUND 2

Mass Spectrum:

The mass spectrum of compound 1 and compound 2 were represented in **Fig. 5** and **6.** The results of mass spectrum of Compound 1&2 at positive ion mode and ESI spectrum showed major peak (M^+H^+) at 284.9(m/z) and 270.9(m/z) respectively. So the molecular weight of obtained compounds were found to be 284.9 and 270.9 daltons respectively.

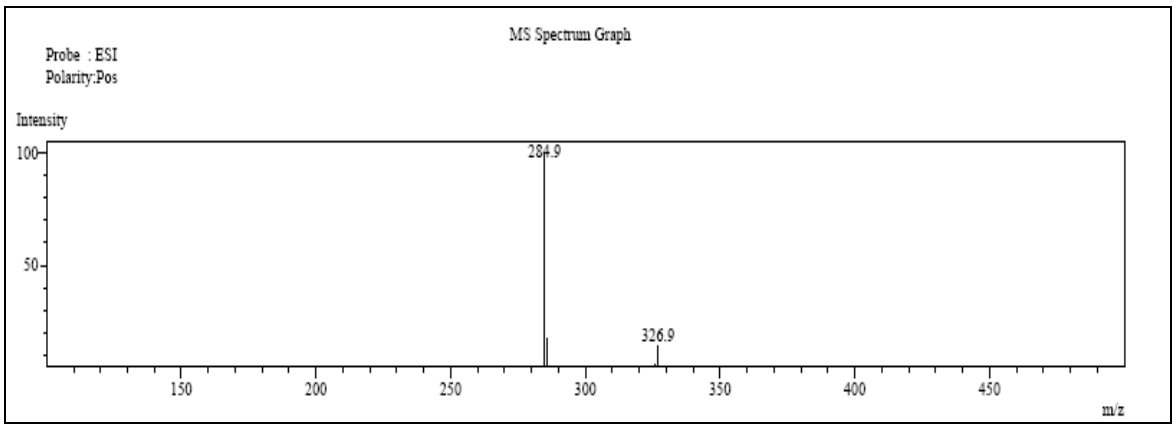

FIG. 5: LC- -MS SPECTRUM OF ISOLATED COMPOUND 1

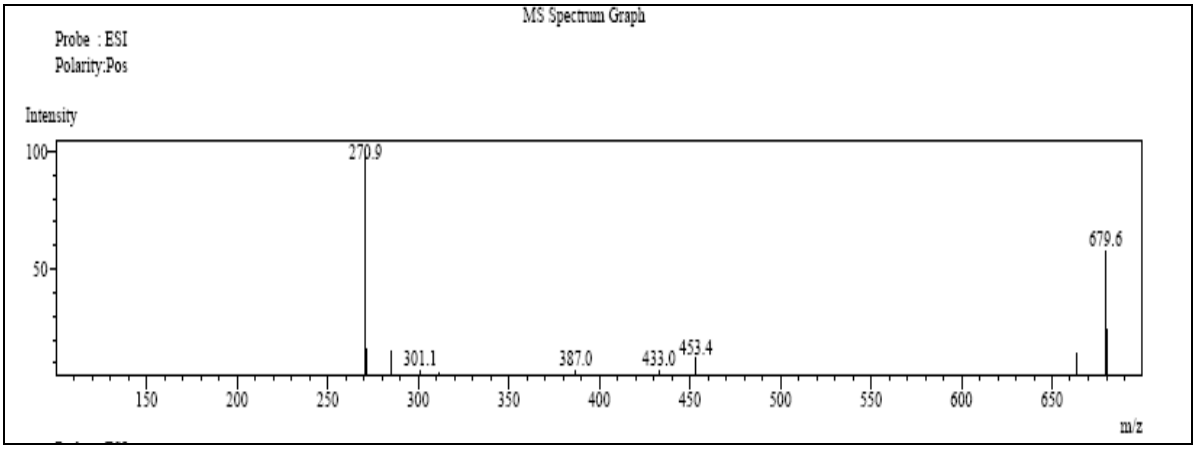

FIG. 6: LC-ESI-MS SPECTRUM OF ISOLATED COMPOUND 2

NMR Analysis:

The ^{1}H-NMR spectrum of compound 1 was shown in **Fig. 7**. Spectral analysis showed a signal δ 8.37ppm, (1H, s) of proton at C2, characteristic of isoflavones. The ^{1}HNMR resonances with two coupled doublets (*J* =2.3Hz) at δ 6.40 and 6.23 ppm, showed 2 meta related H6 & H8 of ring A proton of isoflavone. ^{1}H-NMR signal δ3.71ppm (3H, s) exhibits a methoxy group. The spectral signals at δ 7.02 ppm (2H, dd, *J* =6.6, H3′ & H5′) and δ 7.52 ppm (2H, dd, *J* =6.6, H2′ & H6′) indicated the presence of the methoxy group at C4′ in ring B. This observations suggested that the characteristics of unsubstituted benzene ring and two singlet signals at δ12.92ppm and δ10.90ppm suggesting hydroxyl group at H5 and H7 positions.

The ^{13}C-NMR spectrum of compound 1 was represented in **Fig. 8**. The spectral signals show 14carbons for fifteen skeletal carbon atoms, characteristic of flavonoids. Compound 1 shows one carbonyl group with downfield shift at δ180.06ppm, one carbon signal at δ 55.11ppm, seven methane carbon on five signals at 154.1ppm(C-2),130.13ppm(C-2′,C-6′), 113.60ppm (C-3′,C-5′), 99.02(C-6) and 93.67ppm (C-8). Further seven quaternary carbon signals at δ 164.32ppm (C-7), 162.02ppm (C-5), 159.2ppm(C-4′),157.58ppm (C-9), 121.94 (C-3) and 104.47ppm (C-10). Based on NMR spectral analysis, when compared with published literature, the isolated compound 1 was identified to be 5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one(named C1) [14].

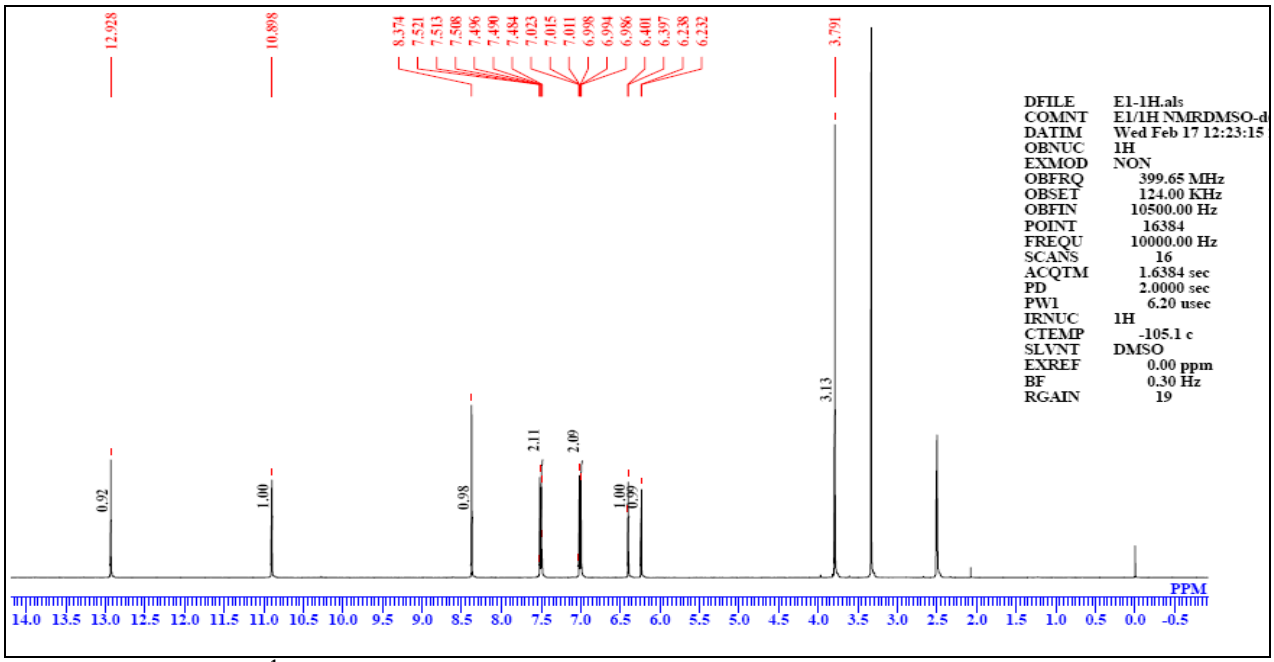

FIG. 7: ^{1}H NMR SPECTRUM (399.65MHz,DMSO-d_6) OF ISOLATED COMPOUND 1

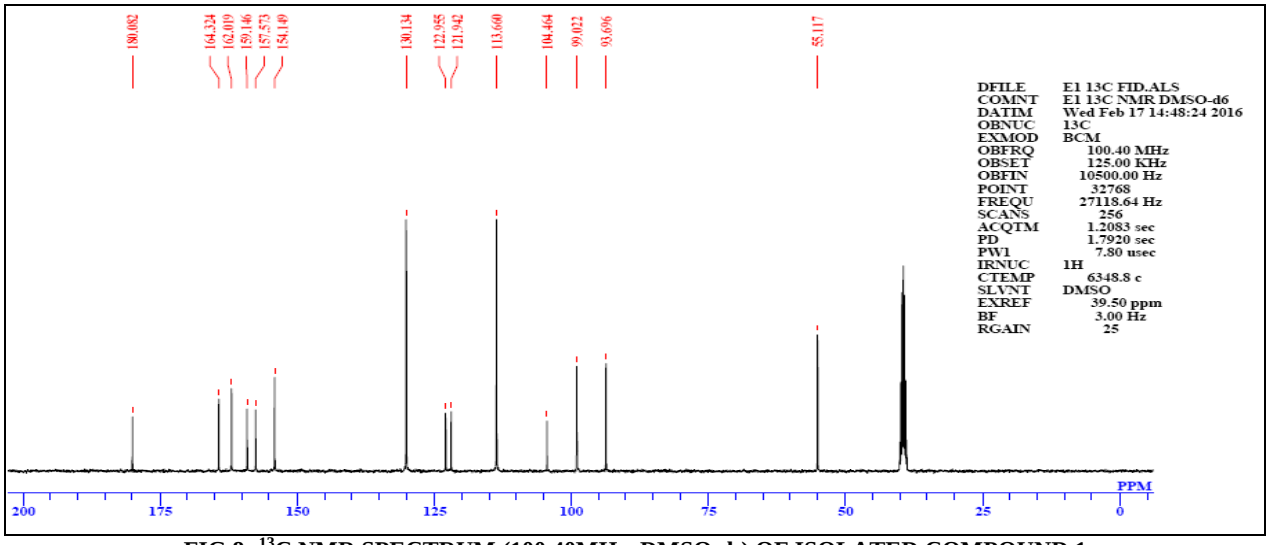

FIG.8: ^{13}C NMR SPECTRUM (100.40MHz, DMSO-d_6) OF ISOLATED COMPOUND 1

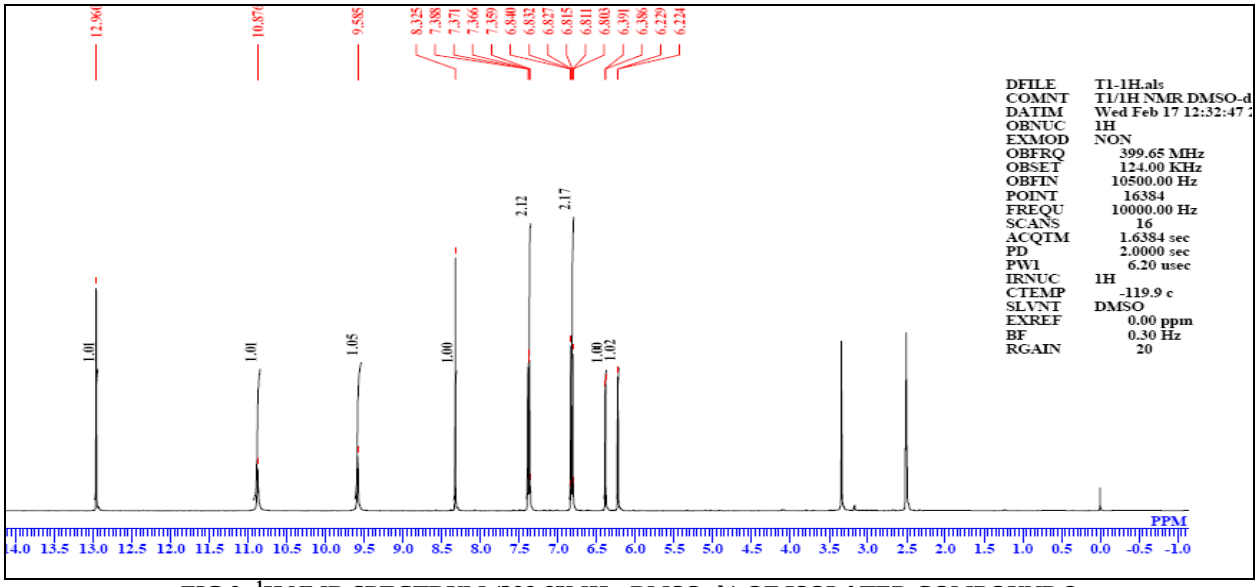

FIG.9: ^{1}H NMR SPECTRUM (399.65MHz, DMSO-d_6) OF ISOLATED COMPOUND 2

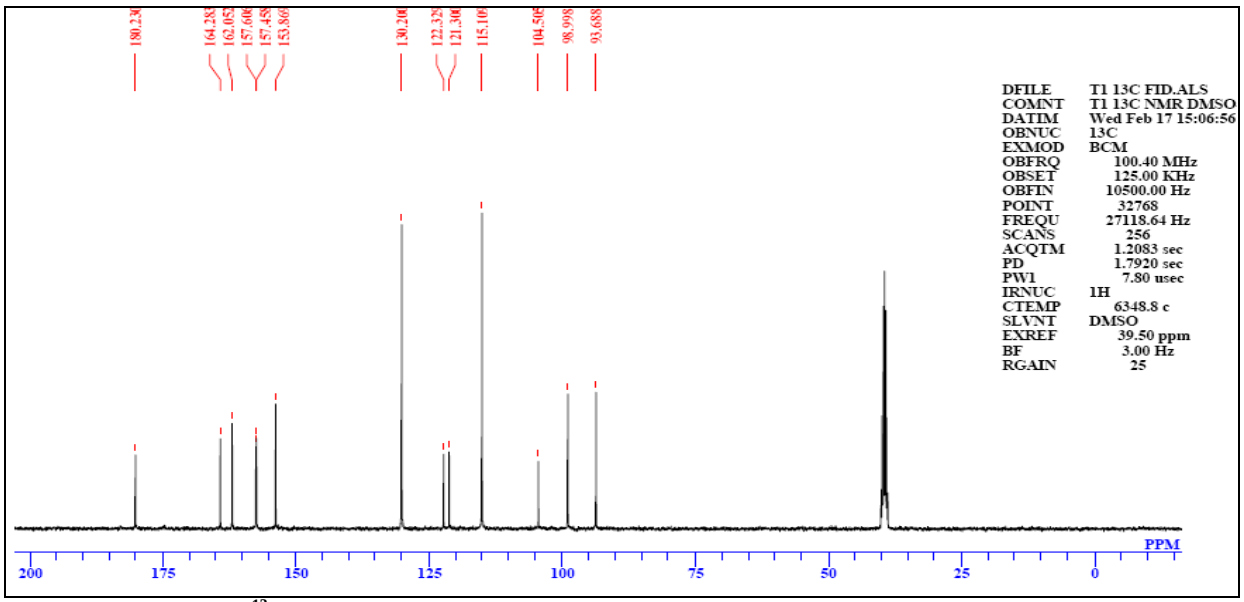

FIG.10: ^{13}C NMR SPECTRUM (100.40MHz, DMSO-d_6) OF ISOLATED COMPOUND 2

The ^{1}H-NMR spectrum of compound 2 was shown in **Fig. 9**. The ^{1}HNMR includes four signals of aromatic protons at δ 6.22 (1H, d, *J* =2.10Hz) and 6.39 ppm (1H, d, *J* =2.10Hz) for H6 & H8 in the ring A and δ 7·38(2H, d, *J* =8.70Hz) for H2′ & H6′ positions and doublet signal at 6.83ppm (2H, d, *J* =8.70Hz) H3′ & H5′ positions in the B ring. The spectrum shows aromatic region containing characteristic resonance for H-2 of isoflavone at δ 7.92 ppm(1H,s).

The ^{13}C-NMR spectrum of compound 1 was represented in **Fig. 10.** The spectral signals show 13carbons for fifteen skeletal carbon atoms, characteristic of flavonoids. Compound 1 shows one carbonyl group(C-4) with downfield shift at δ180.23ppm, seven methane carbon signals at 153.8 ppm(C-2),130.20 ppm(C-2′,C-6′), 115.10 ppm(C-3′,C-5′), 98.99(C-6) and 93.69ppm (C-8). Further seven quaternary carbon signals at δ 164.28ppm (C-7), 162.05ppm (C-5), 157.6ppm(C-4′),157.4ppm (C-9), 122.32 (C-3) and 104.50 ppm(C-10). Depending upon the spectral data analysis and when compared with published literature, compound 2 was identified to be 5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen – 4 - one(named C2) [15]. The obtained structures of compounds were shown in **Fig. 11** and **12** using Chemdraw ultra 12.0. The molecular formula of C1 was determined as $C_{16}H_{12}O_5$ and C2 was $C_{15}H_{10}O_5$. The purified compounds C1 had similar structure with Biochanin A and C2 had similar structure with Genistein.

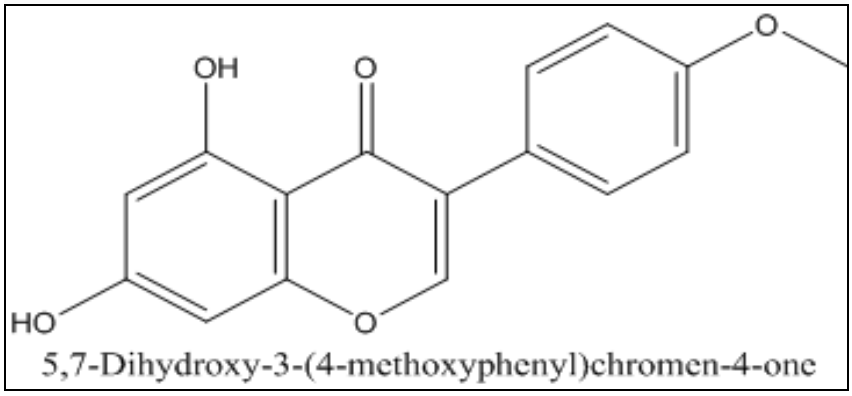

FIG. 11: STRUCTURE OF COMPOUND 1

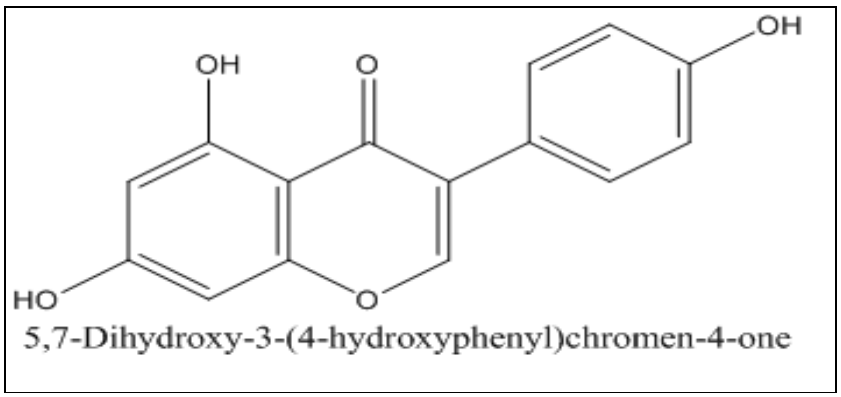

FIG. 12: STRUCTURE OF COMPOUND 2

Gas chromatography mass spectroscopy analysis (GC-MS):

The fractions obtained from column chromatography (202-232) named as GF1 was subjected to Gas chromatography mass spectroscopy analysis. The peaks in the chromatogram were integrated and were compared with the database of spectrum of known components stored in the GC-MS library (NIST) were represented in the table. The GCMS profile showed aminoacid, gamma lactone, sugars and fatty acids.

TABLE 2: GCMS PROFILE OF FRACTION GF1 OF *B.MOLLUGINIFOLIA*

Peak	RT	Compound	MW	Molecular formula	Peak area(%)
1	18.467	2-pyrrolidone-5- carboxylic acid	129.114	$C_5H_7NO_3$	3.20
2	21.425	D-Arabino hexonoic acid	164.26	$C_7H_{12}0_4$	9.21
3	21.639	1-Cyclohexene-1-carboxylic acid	174.15	$C7H_{10}O_5$	20.4
4	21.788	D-Fructose	180.16	$C_6H_{12}O_6$	15.38
5	22.765	D-Mannopyranose	180.156	$C_6H_{12}O_6$	11.56
6	23.225	D-Mannitol	182.17	$C_6H_{14}O_6$	3.63
7	23.555	Glucopyranose	180.16	$C_6H_{12}O$	9.65
8	23.606	Beta-D-Galactofuranose	180.156	$C_6H_{12}O$	8.08
9	23.781	Beta-l-Idofuranuronic acid	180.156	$C_9H_{12}O_6$	3.80
10	24.072	Hexadecanoic acid	256.42	$C_{16}H_{32}O_2$	4.58
11	24.662	Inositol	180.16	$C_6H_{12}O_6$	6.95
12	25.963	Octadecanoic acid	284.47	$C_{18}H_{36}O_2$	4.02

The DPPH activity of C1 & C2 of 25µg(1mg/ml conc) were determined and compared with ascorbic acid (100µg) and Butyalated Hydroxy Toulene (100µg) standards and was represented in **Fig. 13**. DPPH activity of C1 was equal to ascorbic standard & C2 showed more activity than standard

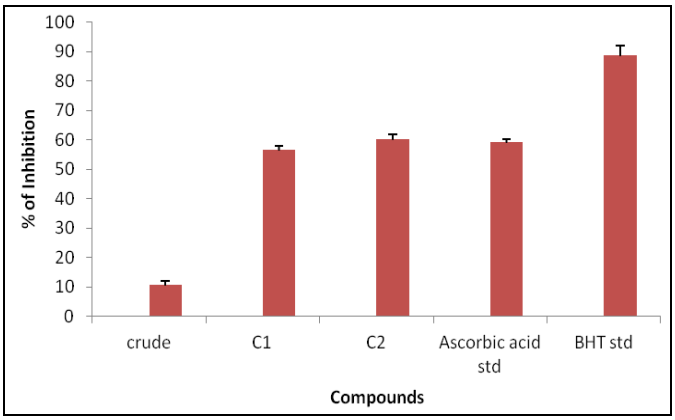

FIG.13: DPPH ACTIVITY OF C1 & C2

Each value represents mean ±SD of three independent experiments. The values are significant at p <0.05. The antimicrobial activity of C1 & C2 were compared with Streptomycin standard against tested microorganisms by disc diffusion method and were represented in **Fig. 14 & 15** respectively**.**

The antimicrobial activity (2mg/ml) C1 & C2 showed zone of inhibition in the range of 17-27mm and 13-24mm against tested bacterial and fungal strains respectively. C1 & C2 showed highest antimicrobial activity against *S.typhi* when compared with streptomycin standard. C1 & C2 showed significant antifungal activity against *P.notatum.* These two compounds showed similar zone of inhibition when compared to the standard.

The minimum inhibitory concentration (MIC) of tested strains was showed in MIC ranged from 62.5µg/ml- 250µg/ml for tested strains.

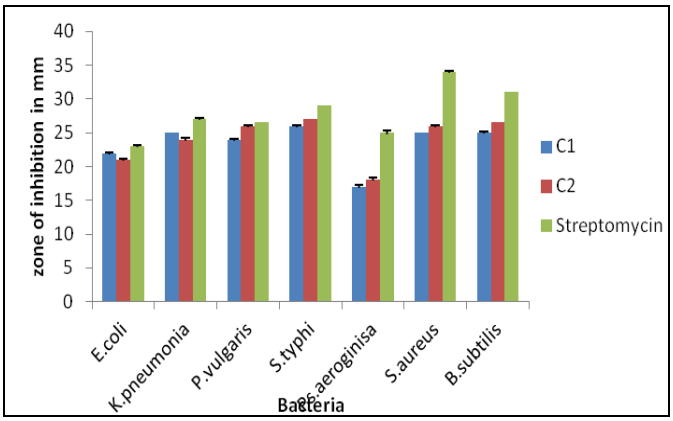

FIG. 14: ANTIBACTERIAL ACTIVITY OF C1 & C2

Each value represents mean ±SD of three independent experiments. The values are significant at p <0.05

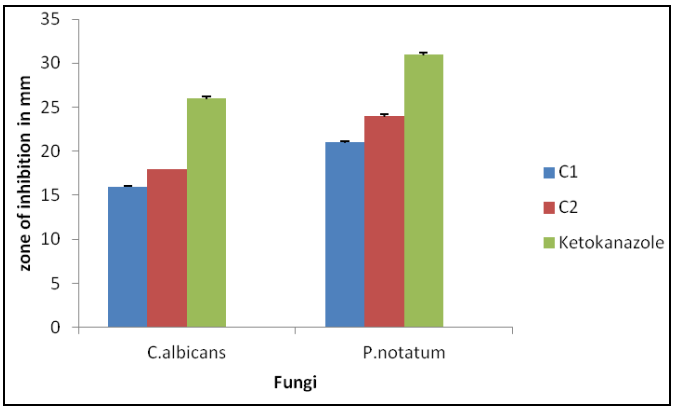

FIG. 15: ANTIFUNGAL ACTIVITY OF C1 & C2

Each value represents mean ±SD of three independent experiments. The values are significant at p <0.05

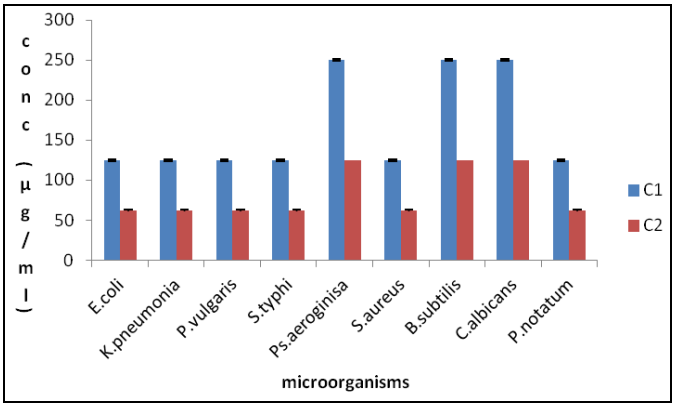

FIG. 16: MINIMUM INHIBITORY CONCENTRATION OF C1 & C2 AGAINST BACTERIAL AND FUNGAL STRAINS

Each value represents mean ±SD of three independent experiments. The values are significant at p <0.05

Antiglycation:

The antiglycation activity of fractions GF1 & IF1 were evaluated for the inhibition of advanced glycation endproducts (AGEs) formation and represented in the figure. The results indicated that GF1 fraction exhibited potential antiglycation activity (>70% inhibition) compared with arbutrin. *α*-Glucosidase inhibitors can be used as a new class of antidiabetic drug. By competitively inhibiting glycosidase activity, these inhibitors help to prevent the fast breakdown of sugars and thereby control the blood sugar level. The inhibitory effect of the selected fractions GF1 & IF1 were represented in the Table. The IC_{50} values showed that GF1 fraction (45.65µg/mL± 1.98) had statistically higher antiglycation activity than IF1 (16.91 µg/mL ± 1.060).

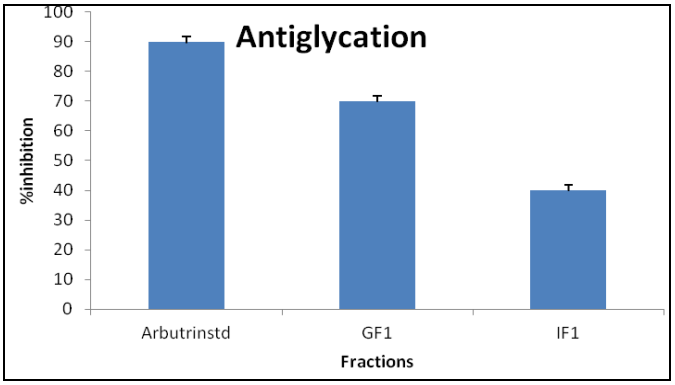

FIG. 17: ANTIGLYCATION OF FRACTIONS GF1 AND IF1 OF *B.MOLLUGINIFOLIA*

Each value represents mean ±SD of three independent experiments. The values are significant at p <0.05

TABLE 3: *IN-VITRO* α-GLUCOSIDASE INHIBITION OF FRACTIONS GF1 AND IF1 OF *B.MOLLUGINIFOLIA*

S. No.	Sample	IC_{50} (µg/ml)
1.	Acarbose	120.35 ± 3.57
2.	GF1 Extract	45.64 ± 1.98
3.	IF1 Extract	16.91 ± 1.06

CONCLUSION: In the present study, methanolic flower extract of *B.molluginifolia* was subjected silica gel column chromatography for purification of bioactive compounds. The purity of obtained fractions were analysed by TLC & HPLC. To the obtained fractions DPPH radical scavenging activity and antimicrobial activities were done. The highest activity shown fraction was subjected to further fractionation to yield two compounds. The structure of compound was elucidated by FT-IR, LC-ESI-MS and NMR spectroscopic techniques. From analysed data the compounds were 5,7-Dihydroxy-3-(4-methoxyphenyl) chromen - 4 - one (Biochanin A) $C_{16}H_{12}O_5$ and 5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen – 4 - one (Genistein) $C_{15}H_{10}O_5$. No one reported these compounds in this plant.

The antioxidant and antimicrobial activities of C1 & C2 were higher when compared with crude extracts and showed significant fold increase.

The fraction GF1 was subjected to GCMS analysis and obtained 12 compounds (aminoacid, gamma lactone, sugars and fatty acids). attributed to the presence of these phytoconstituents. The n-hexadecanoic acid (synonym: palmitic acid) and 9, 12- octadecadienoic acid (linoleic acid) were also reported in *Benincasa hispida* and *Carissa congesta* plant extracts [16]. Similarly, these phytocompounds were identified in various plants such as *Allium nigrum*, *Kielmeyera coriacea*, *Cyrtocarpa procera*, *Labisia pumila* and *Rosa indica* [17, 18, 19, 20, 21]. As GCMS profile contained majority of sugars antiglycation was done against arbutrin and found >70% inhibition. *In-vitro* α-glucosidase inhibition of Fractions GF1 and IF1 of *B.molluginifolia* showed highest IC_{50} values. GF1 fraction showed 45.65µg/mL± 1.98 IC_{50} against Acarbose.

The results of the present investigation was similar to the ethnobotanical usage of the studied plants which possess several phytoconstituents with

biological activity. Based on the present investigation, it is concluded that *B.molluginifolia* has potential source of bioactive compounds with great pharmaceutical value. The study can be extended, *in vivo* evaluation of bioactive compounds in novel drug discovery.

CONFLICT OF INTEREST: The authors declare there is no conflict of interest.

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