SUPPLEMENTARY MATERIAL

Phytochemical profiling, antioxidant, enzyme inhibition and cytotoxic potential of *Bougainvillea glabra* flowers

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Abstract

In this study, phytochemical composition, antioxidant, enzyme inhibition and cytotoxic activities of methanol and dichloromethane (DCM) extracts of *Bougainvillea glabra (B. glabra)* flowers were investigated. Methanol extract was found to have higher total bioactive contents and UHPLC-MS analysis of methanol extract revealed the presence of well-known phenolic and flavonoid compounds. Antioxidant activities were performed by radical scavenging (DPPH and ABTS), reducing power (FRAP and CUPRAC), phosphomolybdenum (TAC) and metal chelating assays. From our result, we observed that methanol extract had many antioxidant compounds. The DCM extract exhibited higher cholinesterases and α-glucosidase enzyme inhibition, while methanol extract showed significant urease inhibition. Both extracts exhibited strong to moderate cytotoxicity against MCF-7, MDA-MB-231, CaSki, DU-145 and SW-480 cancer cells with IC_{50} values ranging from 88.49 to 304.7 µg/mL. The findings showed the *B. glabra* to possess considerable antioxidant, enzyme inhibition and cytotoxic potentials and therefore has potential to discover novel bioactive molecules.

Keywords: *Bougainvillea glabra*, Enzyme inhibition, Phytochemical, Antioxidant, Cytotoxicity.

Experimental

Plant material and extraction

B. glabra flowers were collected in March 2016, from District Muzaffar Garh (Punjab), Pakistan, identified by Dr. Abdul Munsif, Department of Botany, S.E. College, Bahawalpur. In addition, a voucher specimen number (BG-AP-01-16-111) was also deposited in the herbarium of Department of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan. Shade dried flowers powder (500 g) was subjected to maceration (72 hrs) successively using DCM (2 L) and methanol (2 L) solvents at room temperature with occasional shaking for 24 hrs. Pooled extracts were concentrated by Rotavapor-R20 at 35 ºC. These extracts were abbreviated as; BGF-M (*B. glabra* flower methanol), BGF-D (*B. glabra* flower DCM)

1. Phytochemical analysis

1.1. Total bioactive components

Total phenolics were determined by employing standard Folin-Ciocalteu method (Kahkonen et al. 1999) using gallic acid as standard and the results were expressed as mg GAE/g (gallic acid equivalents). Total flavonoid content assay was done by Aluminium chloride colorimetric method (Chew et al. 2009). Quercetin was used as standard and the results were expressed as mg QE/g (quercetin equivalent).

1.2. UHPLC-MS analysis

UHPLC Accurate-Mass Q-TOF (Agilent 1290 Infinity LC system coupled to Agilent 6520) mass spectrometer with dual ESI source was used. Column specifications were: XDB-C18 Agilent Zorbax Eclipse, narrow-bore 2.1 x 150 mm, 3.5 micron (P/N: 930990-902). The temperature of column was maintained at 25^{o}C, while auto-sampler temperature was 4 °C. The following two mobile phases used were: A (0.1% formic acid in water), B (0.1% formic acid in acetonitrile) at flow rate of 0.5 mL/min. Injection volume was 1.0 µL. Run time was 25 min and post-run time was 5 min. MS analysis full scan was carried out over a range of *m/z* 100-1000 employing electrospray ion source in the negative ionization mode. Flow rate for nitrogen as nebulizing and drying gas was 25 and 600 L/hour, respectively with drying

gas temperature of 350 ^{o}C. The fragmentation voltage was optimized to 125 V. Capillary voltage for analysis was 3500 V. Data processing was done using Agilent Mass Hunter Qualitative Analysis. Identification of compounds was done from Search Database: METLIN_AM_PCDL-N- 170502.cdb, with parameters as: Match tolerance: 5 ppm, Negative Ions:-H.

2. Biological evaluation

2.1. Antioxidant assay

2.1.1. DPPH radical scavenging capacity (RSC)

In this method, 1 mL of plant extract of different concentrations (1000-15.625 µg/mL) was added to 2 ml of DPPH solution (0.059 mg/mL methanol). Absorbance was measured at 517 nm after 30 min incubation, (Miliauskas et al. 2004).

Data was expressed as:

$$RSC\ (\%) = 100 - \{(abs_c - abs_s) / abs_c\}$$

Abs_s = absorbance of sample, Abs_c = absorbance of control.

Ascorbic acid (AA) was used as control with IC_{50} 0.00387 mg/mL Therefore, free radical scavenging (FRS) activity was also expressed as equivalent of ascorbic acid (AAEAC) using the following equation (Chan et al. 2010).

$$AAEAC = IC_{50}\ (Ascorbic\ acid) / IC_{50}\ (sample) \times 10^5$$

2.1.2. ABTS (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity

$ABTS^+$ radical cation was formed by a reaction between 7 mM ABTS solution and 2.45 mM potassium persulfate, and allowing the mixture to stand in darkness at room temperature. Firstly, ABTS solution was diluted in methanol until the absorbance reached the value of 0.700 0.02 at 734 nm. The test solution (1 mL), mixed with ABTS solution (2 mL), was mixed and the absorbance was recorded at 734 nm after 30 min of incubation at room temperature. The results were expressed as milligrams of trolox equivalents per gram of dry extract (TEs/g extract) (Grochowski et al. 2017).

2.1.3. Ferric reducing power method

Plant sample (1000 µg/mL) was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v), incubated for 20 min at 50 ^{o}C. After 20 min, trichloroacetic acid (2.5 mL, 10% w/v) was added. The contents were divided into two

halves; equal volume of water was added in one half of 2.5mL and then 0.5 mL of $FeCl_3$ solution (0.1% w/v) was added. The contents were incubated for 30 min at 25 ^oC and the absorbance was measured at 700 nm (Chan et al. 2010). The results were expressed as mg GAE/g.

2.1.4. Cupric ion reducing (CUPRAC) method

Extract solutions (0.5 mL) were added to reaction mixture [$CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM), NH_4Ac buffer (1 mL, 1 M, pH 7.0)] and the absorbance was recorded at 450 nm after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared and analysed according to this procedure. Milligrams of trolox equivalents per gram of dry extract (TEs/g extract) were the measurement unit (Grochowski et al. 2017).

2.1.5. Phosphomolybdenum assay (TAC)

Total antioxidant capacity (TAC) was determined by phosphomolybdenum method (Prieto et al. 1999). Briefly, the extract solution (0.3mL, 1 mg/mL) was mixed with 3 mL of molybdate reagent solution, incubated for 90 min at 95 ^oC for 90 min and the absorbance of the solution was measured at 695 nm against blank. TAC was expressed as equivalent of gallic acid (mg GAE/g) (Prieto et al. 1999).

2.1.6. Metal chelating activity on ferrous ions

Extract solution (2.0 mL) was added to $FeCl_2$ (0.05 mL, 2 mM), and the reaction was started using 0.2 mL of 5 mM ferrozine. Similarly, a blank sample for each sample (prepared in the same manner but without ferrozine) was prepared, and all the absorbances were recorded after 10 min of incubation (room temperature) at 562 nm. Milligrams of EDTA equivalents per gram of dry extract (EDTAEs/g extract) (Grochowski et al. 2017).

2.2. Enzyme inhibitory activities

2.2.1. Cholinesterase inhibition activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity were determined spectrophotometrically according to the method Ellman *et al* (Ellman et al. 1961). Total reaction mixture in 96-well plate was 100 µL containing 60 µL of phosphate buffer (50 mM, pH 7.7), 10 µL plant sample of 10 mg/mL stock solution. Then 10 µL enzyme (0.005 units AChE or 0.5 units BChE) was added. The reaction mixture was mixed, incubated at 37 ^oC for 10 min and its absorbance was taken at 405 nm using Synergy HT, Biotek, USA 96-well plate reader followed by addition of 10 µL of 0.5 mM substrate (acetylthiocholine iodide for

AChE and butyrylthiocholine chloride for BChE) and 10 µL of 0.5 mM DTNB was added to the above reaction mixture to initiate the reaction and incubated again at 37 ºC for 30 min. Absorbance was again measured at 405 nm. Eserine was used as a control.

The inhibition (%) was calculated as

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100$$

EZ–Fit Enzyme kinetics software was used to calculate IC_{50} values (Perrella Scientific Inc. Amherst, USA).

2.2.2. Urease inhibition activity

The total assay mixture of 85 µL in 96 well plates contained phosphate buffer (50 mM, pH 7.0) 10 µL sample and jackbean urease enzyme (25 µL of 0.14 units). Contents were incubated at 37 °C for 5 min. After incubation, 40 µL of urea substrate (20 mM) was added and incubation continued for further 10 min. Then, 115 µL of freshly prepared phenol hypochloride reagent was added in each well and further incubated for 10 min at 37 °C for colour development. Absorbance was measured at 625 nm (Weatherburn 1967). Kojic acid was used as a control. The inhibition (%) and IC_{50} results were determined as given above for cholinesterases.

2.2.3. α-Glucosidase inhibition activity

100 µL reaction mixture in 96-well plate contained 70 µL of phosphate buffer (50 mM, pH 6.8), 10 µL plant sample (0.5 mM) and 10 µL of baker's yeast enzyme (0.057 units). The reaction mixture was mixed, incubated for 37 °C for 10 min and its absorbance was taken at 400 nm. Reaction was initiated by adding 10 µL of substrate, *p*-nitrophenyl-D-glucopyranoside (0.5 mM) (Chapdelaine et al. 1978). Incubation was continued for further 30 min and after-read. Acarbose was used as a control. The inhibition (%) and IC_{50} values were calculated as given above for cholinesterases.

2.3. Cytotoxicity assays

Cell lines and culture medium

MDA-MB-231, MCF-7 (breast cancer), CaSki (cervical cancer), DU-145 (prostate cancer) and SW-480 (colon cancer) cell lines were used for cytotoxicity studies. The breast cancer cell lines were maintained in DMEM culture medium while RPMI-1640 media was used for CaSki, DU-145 and SW-480 cells. Both media were supplemented with 10% FBS (foetal

bovine serum) and 1% P/S (penicillin-streptomycin) environment. Cells were cultured at 37 ^{o}C in a humidified atmosphere in 5% CO_2 incubator.

MTT assay

Cells were maintained in their respective media, seeded in 96 well plate and kept for overnight incubation at 37 °C. Cells with >80% confluency were tested with plant extracts at a concentration range of (500-15.625 µg/mL). After 48 h incubation, the medium was aspirated by adding MTT solution (5 mg/mL) and incubated again for 4 h. After 4 h, wells were solubilized with 100 µL DMSO per well and absorbance was recorded at a primary wavelength (570 nm) and reference wavelength (670 nm) using microplate reader Infinite@Pro-200 Tecan, Switzerland, (Nemudzivhadi and Masoko 2014). Each plate contained the sample, negative control and blank. DMSO was used as a negative control. The percentage cell viability and IC_{50} were calculated as:

$$\text{Cell viability (\%)} = Abs_s - Abs_c \times 100.$$

Abs_s = absorbance of sample, Abs_c = absorbance of control.

Statistical data analysis

All the experiments were repeated three times and analysis was done in triplicates. The obtained results were expressed as mean value and standard deviation (mean±SD). One-way analysis of variance (ANOVA) was used to calculate the differences, followed by Tukey's significant difference post hoc test ($p < 0.05$). Graph Pad Prism software (San Diego, CA, USA, Version 6.03) was used to calculate $IC_{50.}$ SPSS v22.0 software was used to carry out all experimental analysis.

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Figure captions

Figure S1. LC-MS chromatogram for methanol extract of *B. glabra* flower.

Figure S2. *In vitro* enzyme inhibition of *B. glabra* flower extracts against AChE, BChE, α-glucosidase and urease expressed as percent inhibition as compared with control.

Figure. S3. Cytotoxicity of methanol and DCM extracts of *B. glabra* flower.

The graphs shows cytotoxic effects as **(A)** MCF-7 (BGF-M), **(B)** MCF-7 (BGF-D), **(C)** MDA-MB-231 (BGF-M), **(D)** MDA-MB-231 (BGF-D), **(E)** CaSki (BGF-M), **(F)** CaSki (BGF-D), **(G)** DU-145 (BGF-M), **(H)** DU-145 (BGF-D), **(I)** SW-480 (BGF-M) and **(J)** SW-480 (BGF-D) **** indicates significant difference when compared with untreated (control) cells (p value < 0.05)

Tables and Figures

Table S1. Total bioactive contents of methanol and DCM extracts of *B. glabra* flower.

Samples	Extraction yield (%)	Total phenolic content (mg GAE/g)	Total Flavonoid content (mg QE/g)
BGF-M	12%	26.04±1.04 [a]	20.86±1.09 [a]
BGF-D	14%	13.64±2.01 [b]	12.65±0.30 [b]

BGFM = *B. glabra* flower methanol extract. BGFD = *B. glabra* flower DCM extract. Data from three repetitions, with mean ± standard deviation; means with different superscript letters in the same column are significantly ($p < 0.05$) different.
GAE: gallic acid equivalent; QE: quercetin equivalent;

Table S2. LC-MS Spectral analysis of methanol extract of *B. glabra* flower

ID.	Possible Compound name	Class of Compound	Mol. formula	Mol. mass	T_R (min)	Base Peak (m/z)
1	Oenanthoside A	Phenolic glycoside	C16 H20 O8	340.11	0.71	339.10
2	Egonol gentiobioside	Flavonoid	C31 H38 O15	650.21	0.93	649.21
3	N-Carboxyethyl-γ-aminobutyric acid	Amino acids	C7 H13 N O4	175.08	1.09	174.07
4	N-(1-Deoxy-1-fructosyl) phenylalanine	Amino acids	C15 H21 N O7	327.13	2.11	326.12
5	Lucuminic acid	Glycoside	C19 H26 O12	446.14	7.14	445.13
6	Viscumneoside III	Flavonoid	C27 H32 O15	596.17	7.82	595.16
7	Isorhamnetin 3-rhamnosyl-(1->2)-gentiobiosyl-(1->6)-glucoside	Flavonoid	C40 H52 O26	948.27	8.03	947.26
8	Brassicoside	Flavonoid	C34 H42 O22	802.21	8.15	801.20
9	Kaempferol 3-(2G-glucosylrutinoside)	Flavonoid	C33 H40 O20	756.21	8.37	755.20
10	Isorhamnetin 3-glucosyl-(1->2)-[rhamnosyl-(1->6)-galactoside]	Flavonoid	C34 H42 O21	786.22	8.41	785.21
11	Robinin	Flavonoid	C33 H40 O19	740.21	8.59	739.20
12	Robinetin 3-rutinoside	Flavonoid	C27 H30 O16	610.15	8.62	609.14
13	Isorhamnetin 3-glucosyl-(1->6)-galactoside	Flavonoid	C28 H32 O17	640.16	8.67	639.15
14	Luteolin 7-rhamnosyl(1->6)galactoside	Flavonoid	C27 H30 O15	594.15	8.89	593.15
15	6-C-Rhamnopyranosylrhamnetin 3-O-glucopyranoside	Flavonoid	C28 H32 O16	624.16	8.93	623.16
16	Vitisifuran A	Flavonoid	C56 H40 O12	904.25	8.99	903.24
17	Tricetin 7-methyl ether 3'-glucoside-5'-rhamnoside	Flavonoid	C28 H32 O16	624.17	9.21	623.16
18	6-Hydroxyluteolin 5-rhamnoside	Flavonoid	C21 H20 O11	448.10	9.27	447.09
19	6-Methoxykaempferol 3-rhamnoside-7-(4'''-acetylrhamnoside)	Flavonoid	C30 H34 O16	650.18	9.30	649.17
20	Laricitrin 3-rhamnoside	Flavonoid	C22 H22 O12	478.11	9.40	477.10
21	Tomentin 4'-glucoside	Flavonoid	C23 H24	508.12	9.46	507.11

			O13			
22	Kaempferol 3,4'-diglucoside-7-(2''-ferulylglucoside)	Flavonoid	C43 H48 O24	948.25	9.55	947.24
23	Isovitexin 2''-O-(6'''-(E)-p-coumaroyl)glucoside 4'-O-glucoside	Flavonoid	C42 H46 O22	902.24	9.58	901.24
24	Kaempferol 3-neohesperidoside-7-(2''-ferulylglucoside)	Flavonoid	C43 H48 O23	932.25	9.90	931.25
25	Isovitexin 7-(6'''-sinapoylglucoside) 4'-glucoside	Flavonoid	C44 H50 O24	962.26	9.98	961.26
26	Kaempferol 3-rhamnoside-7-[6'''-ferulyglucosyl-(1->3)-rhamnoside]	Flavonoid	C43 H48 O22	916.26	10.11	915.25
27	oleanolic acid 3-O-beta-D-glucosiduronic acid	Triterpenoid	C36 H56 O9	632.39	14.62	631.38

T_R: retention time

Table S3. Antioxidant activities of methanol and DCM extracts of *B. glabra* flower.

Samples	Radical scavenging activity				Reducing power		Total antioxidant capacity (TAC)	Ferrous chelating
	%RSC (1mg/mL)	DPPH IC_{50} (µg/mL)	ABTS (mgTE/g extract)	AAEAC (mg AAE/g)	CUPRAC (mgTE/g extract)	FRAP (mg GAE/g)	Phosphomolybdenum (mg GAE/g)	Metal Chelating (mgEDTA/g)
BGF-M	*86.2±0.08 **a**	85.40±1.93 a	111.32±5.82 a	45.35±1.01 **a**	147.94±7.02 a	73.95±0.39 **a**	16.97±1.05 **a**	9.16±1.41 b
BGF-D	61.01±0.7 **b**	542.19±2.64 b	40.89±1.52 b	7.13±0.03 **b**	66.57±3.20 b	13.67±0.23 **b**	40.15±0.30 **b**	17.51±0.64 a
Ascorbic acid	89.96±1.60 **a**	16.82±069 e	nt	nt	nt	nt	nt	nt

* Values are expressed as means ± S.D. of three replicates, means with different superscript letters in the same column are significantly ($p < 0.05$) different, nt: not tested. RSC: radical scavenging capacity; AAEAC: Ascorbic acid equivalent anti-oxidant capacity; CUPRAC: Cupric reducing antioxidant capacity; FRAP: ferric reducing anti-oxidant power; TE: trolox equivalent; GAE: gallic acid equivalent

Table S4. Percentage enzyme inhibition (0.5 mg/mL) and IC_{50} (µg/mL) of methanol and DCM extracts of *B. glabra* flower.

Samples	AChE		BChE		α-Glucosidase		Urease	
	% inhibition	IC_{50} (µg/mL)	% inhibition	IC_{50} (µg/mL)	% inhibition	IC_{50} (µg/mL)	% inhibition	IC_{50} (µg/mL)
BGF-M	*21.52±0.29	>500**	37.62±0.45	>500**	15.62±1.19	>500**	92.42±0.57	87.45±0.42
BGF-D	32.68±0.24	>500**	67.27±0.86	227.56±0.71	91.34±2.67	15.80±2.35	43.25±0.46	>500**
Eserine	91.27±1.17	0.04±0.0	82.82±1.09	0.85±0.01	nt	nt	nt	nt
Acarbose	nt	nt	nt	nt	92.83±0.18 mM	37.45±0.16µM	nt	nt
Kojic acid	nt	nt	nt	nt	nt	nt	98.21±0.18	21.25±0.15

*Values are expressed as means ± S.D. of three replicates, nt; not tested

**The IC_{50} value was higher than 500 µg/mL.

Eserine is control for AChE, BChe, Acarbose for α-glucosidase and Kojic acid for urease. AChE; Acetylcholinesterase, BChE; Butrylcholinesterase

Table S5. Cytotoxicity (IC50 µg/mL) of methanol and DCM extracts of *B. glabra* flower.

Cell lines	IC_{50} value (µg/mL)	
	BGF-M	**BGF-D**
MCF-7	*105.7	>500**
MDA-MB-231	300.6	>500**
CaSki	88.49	180.1
DU-145	129.9	180.9
SW-480	>500**	304.7

Values are expressed as means of three replicates,

IC_{50} value represents concentration that reduces cell viability to 50%. **The IC_{50} value was higher than 500 µg/mL

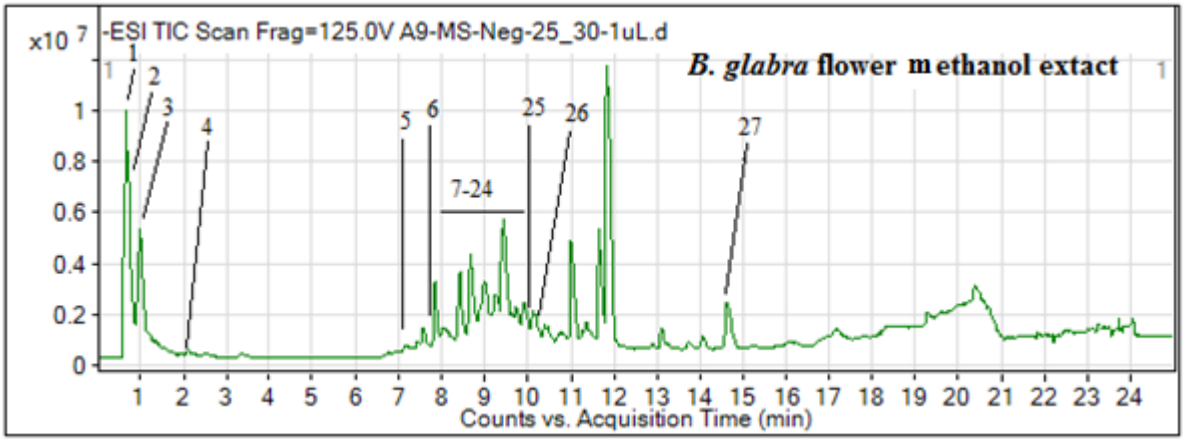

Figure S1. LC-MS chromatogram for methanol extract of *B. glabra* flower.

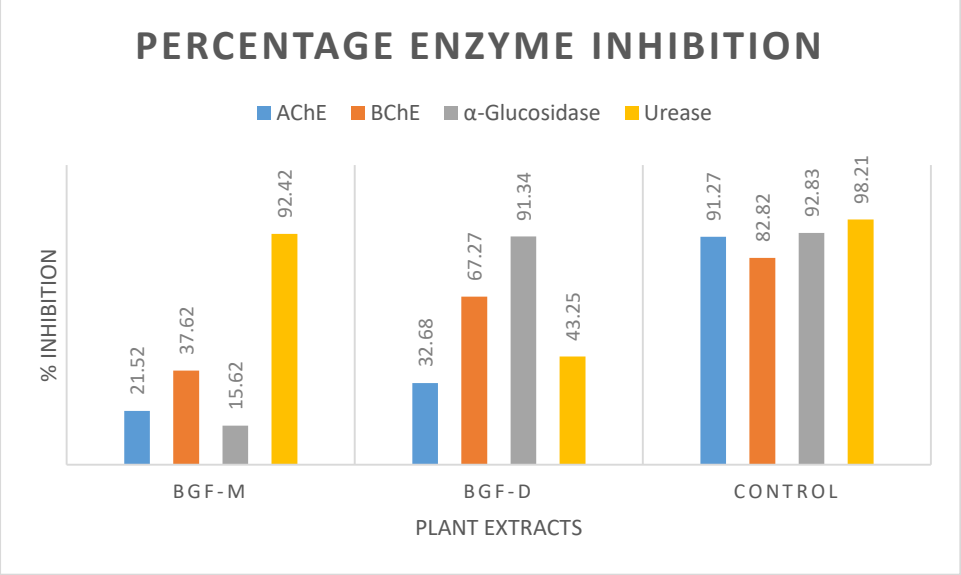

Figure S2. In vitro inhibition results of *B. glabra* flower extracts against AChE, BChE, α-glucosidase and urease expressed as percent inhibition as compared with control.

BGF-M: *B. glabra* flower methanol extract; BGF-D: *B. glabra* flower DCM extract

Eserine was control for AChE and BChE, Acarbose for α-glucosidase and Kojic acid for urease.

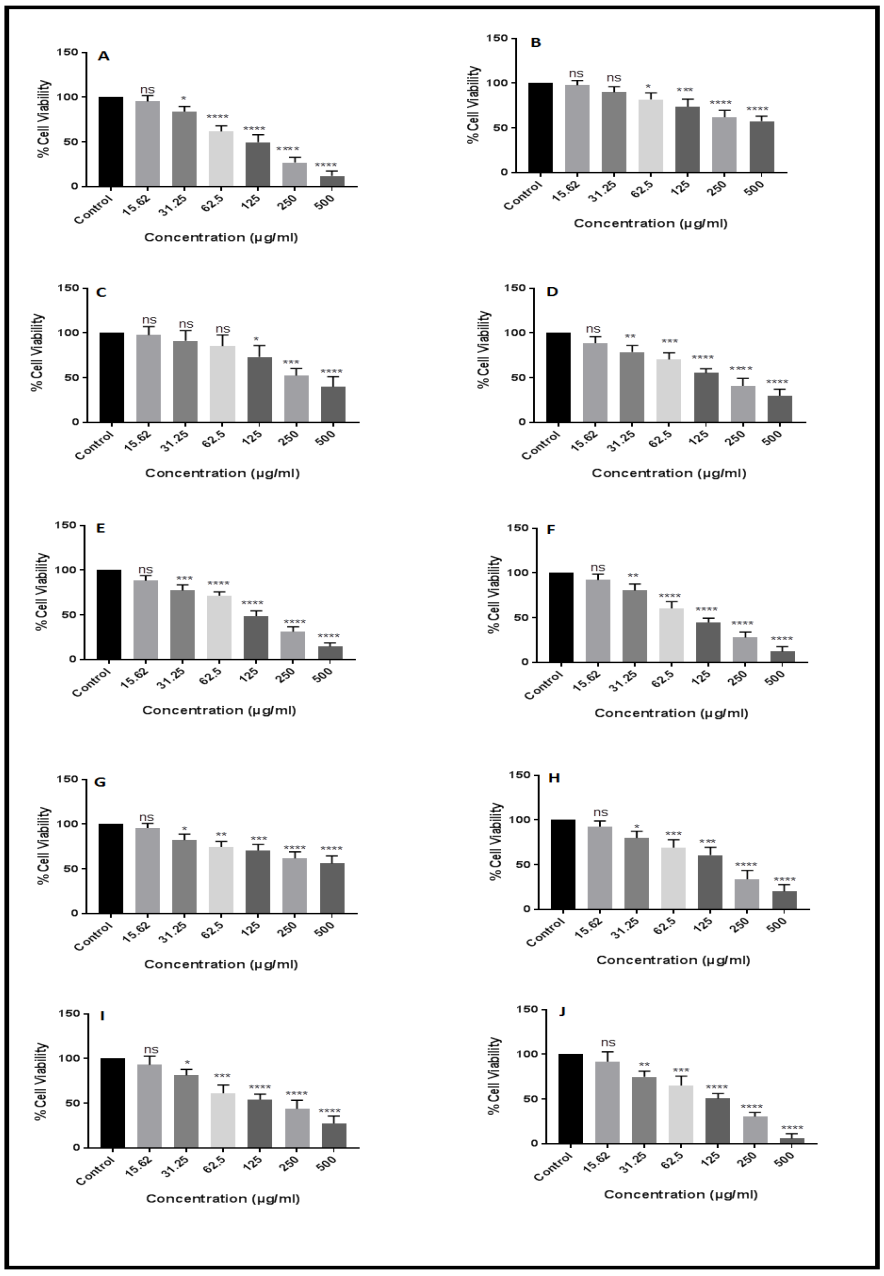

Figure. S3. Cytotoxicity of methanol and DCM extracts of *B. glabra* flower.

The graphs shows cytotoxic effects as **(A)** MCF-7 (BGF-M), **(B)** MCF-7 (BGF-D), **(C)** MDA-MB-231 (BGF-M), **(D)** MDA-MB-231 (BGF-D), **(E)** CaSki (BGF-M), **(F)** CaSki (BGF-D), **(G)** DU-145 (BGF-M), **(H)** DU-145 (BGF-D), **(I)** SW-480 (BGF-M) and **(J)** SW-480 (BGF-D) **** indicates significant difference when compared with untreated (control) cells (p value < 0.05)