SUPPORTING INFORMATION

Phytochemical profiling and evaluation of modified resazurin microtiter plate assay of the roots of *Trillium govanianum*

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ABSTRACT

Trillium govanianum Wall. ex D. Don (Melanthiaceae *alt.* Trilliaceae), is native to the Himalayas**.** The present study, for the first time, was undertaken to explore the antimicrobial potential, to determine the minimum inhibitory concentration (MIC) values of the methanol extract of the roots of *Trillium govanianum* and its solid phase extraction (SPE) fractions by using resazurin microtiter assay (REMA) against Gram positive and Gram negative bacterial registered strains and to carry out phytochemical analysis. The remarkable amount of gallic acid equivalent phenolic and quercetin equivalent flavonoid content was manifested by MeOH extract (20.27±3.03 mg GAE/ g DW and 9.25± 0.50 mg QE/ g DW respectively). The GC/MS analysis revealed the presence saturated and unsaturated components. Considerable level of antibacterial potential against Gram-positive bacteria (MIC: 2.5-0.009 mg/mL) than against Gram-negative bacteria (MIC: 2.5-0.165 mg/mL). The use of microtiter plates has the advantage of lower cost, fast and quantitative results.

Keywords: *Trillium govanianum; Melanthiaceae*; *GC/MS*; SPE; REMA; MIC.

Experimental

Reagents and chemicals

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Solvents were purchased from Fisher Scientific (Loughborough, UK); sterile resazurin tablets (Fischer Chemicals); UV spectrophotometer (Shimadzu); HPLC Agilent 1260 Infinity; Incubator (Binder) at 35 and 37 °C; vortex mixer (Labnet International); Eppendorf Centrifuge 5810 R (Fischer Scientific, UK); Shaker Incubator (Sartorius CERTOMAT); Four NCTC and One ACTC registered bacterial strains were obtained from the Microbiology labs of School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University.

Plant materials

Plant sample was collected from Muzaffarabad district of Pakistan-controlled Azad Kashmir and identified as *Trillium govanianum* Wall. by Dr Muhammad Zafar, Herbarium Botanist, Department of Plant Sciences, Quaid-I-Azam University, Islamabad, Pakistan. A herbarium specimen for this collection (voucher number: Acc no.128085) has been deposited and retained in the above herbarium.

Extraction and preparation of plant samples

Air-dried and finely ground roots (2.5 kg) of *T. govanianum* were macerated in MeOH (5 L) for 10 days at room temperature, filtered, and the solvent was concentered by using rotatory evaporator at maximum temperature of 45^{o}C to get a concentrated gummy crude extract.

Solid-phase extraction (SPE) and sample purification

The Strata C-18 cartridge (20 g) was first washed with MeOH (50 mL) and proceeded with water (100 mL) to saturate the cartridge. A portion of the dried MeOH extract (2 g) was mixed in 20 mL of HPLC grade water and loaded on the cartridge. The cartridge was eluted with a step gradient of MeOH-water mixture (20:80, 50:50, 80:20 and 100:0) of decreasing polarity to obtain four fractions coded as TGMF1, TGMF2, TGMF3 and TGMF4 respectively. All four fractions were evaporated to dryness using a combination of rotary evaporator and freeze-dryer, re-dissolved in MeOH (10 mg/mL).

Determination of total phenolic contents

Total phenolic contents of MeOH extract of the roots of *A. asparagus* and *T. govanianum* and its SPE were estimated by following the procedures of (Clarke et al., 2013) with slight modifications. Each test sample of 20 µl from 4 mg/ml DMSO stock solution was applied in respective well of 96 well plate and subsequently proceeded for further addition of 90µl of diluted folinciocalteu reagent followed by 5 min incubation. Further addition of 90µl of 6% sodium carbonate to each well of the plate and incubated for 1hr. The absorbance of each sample was taken at 630nm.

Determination of total flavonoid contents

Total flavonoid contents of MeOH extract and its SPE were estimated by using aluminum chloride colorimetric method reported by (Ul-Haq et al., 2012) with some modifications. From stock solution of 4.0 mg/ml DMSO, 20µl of sample were accurately applied to each well of 96 wells plate, subsequently followed by the addition of 10µl 10% aluminum chloride, 10µl 1M potassium acetate and 160µl distilled water. The incubation time of reaction mixture was 30 minutes and absorbance was measured at 415nm.

Gas chromatography/mass spectrometry analysis

The MeOH extract (5g) was fractionized through solvent-solvent extraction. Dried MeOH extract was dissolved in 250 mL of water and then resuspended in 250 mL of n-hexane in separating funnel. The n- hexane layer was filtered and evaporated under vacuum using rotary evaporated (<40°C) to get concentrated non-polar (n- hexane) fraction. The *n*-hexane fraction of MeOH extract of the roots of *Trillium govanianum* were analyzed using Thermo Scientific DSC-II gas chromatography-mass spectrometer (Thermo Scientific Co.) equipped with capillary column by slight modification reported by (Al Hashmi et al., 2013). Experimental conditions were as fellow: DB 5-MS capillary standard non-polar column (15m x 0.25mm x 0.25µm film thickness). The stationary phase is DB5 (95% methyl siloxane and 5% phenyl siloxane). Flow rate of mobile phase (carrier gas: He) was set at 1.0 mL/min. The gas chromatography part was fitted with a programmable temperature vaporizing injector and the analysis was carried out in EI mode at 70eV beam energy. The GC temperature was ramped from 50 to 300°C at a rate of 25°C per minute. Injection volume was 1.0 µl and mass scanning range was 50 –550 *m/z.* Total elution time was 14 mins. GC/MS is mostly used to identify the fatty profile of non-polar fraction of plant extracts. The spectra of GC/MS analysis of tested compounds was compared with the inbuilt library of

National institute standard and technology (NIST), which have approximately more than 62,000 patterns. On the basis of retention time and molecular weight, the sample components were identify.

Resazurin microtiter assay (REMA)

The *in vitro* susceptibility testing was performed using a 96-well microtiter plate with resazurin. A stock solution having concentration of 128 µg/ml were prepared by dissolving in sterile distilled water. Plant extracts stock solution having concentration of 10 mg/ml were prepared with 10% DMSO (Sarker et al., 2007). Sterile Distilled water was used to dissolve resazurin dye to obtain 0.02 % and the solution was then sterilized by filtration. The MIC assay was carried out in according to CLSI guideline for microdilution test. Briefly, the stock antibiotics and plant extracts were serially twofold diluted with cation-adjusted Mueller hinton broth (CAMHB). The additional 60 µL of the CAMHB and 20 µL of 0.02% resazurin were added to all wells. An overnight culture of test bacteria, after centrifugation at 4,000 rpm for 10 min, was harvested with with NaCl, which were centrifugation at 4,000 rpm for 5 min each. The pellet collected was then adjusted approximately 0.5 standard McFarland equivalent (1 x 108 CFU/mL), diluted to give 5 x 10^6 CFU/mL, and then 20 µL will be transferred to the well so that the final concentration of inoculum was approximately 5 x 10^5 CFU/mL. The total volume in each well was 200 µL and the final concentration of antibiotics and the extracts were 0.06-64 µg/mL and 0.005-5 mg/mL, respectively. Wells without antibacterial agents and bacterial strain were used as controls. The 96-well microplate was then incubated at 37 °C for 24 h. The lowest concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was noted as the MIC. Each test was carried out in triplicate. The average values were calculated for the MIC of test material.

Statistical analysis

The results obtained for cytotoxic and antileishmanial analysis were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey and Duncan's test using the statistical package IBM Statistics 23 and $P < 0.05$, $P < 0.01$, or $P < 0.001$ was considered as significant when appropriate. Data were expressed as mean ± SD.

Results

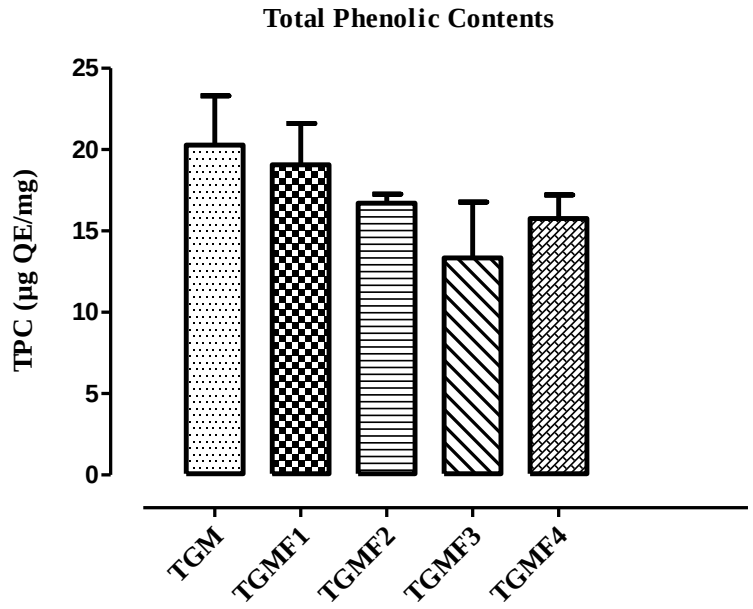

Figure S1: Total phenolic contents of *Trillium govanianu*m

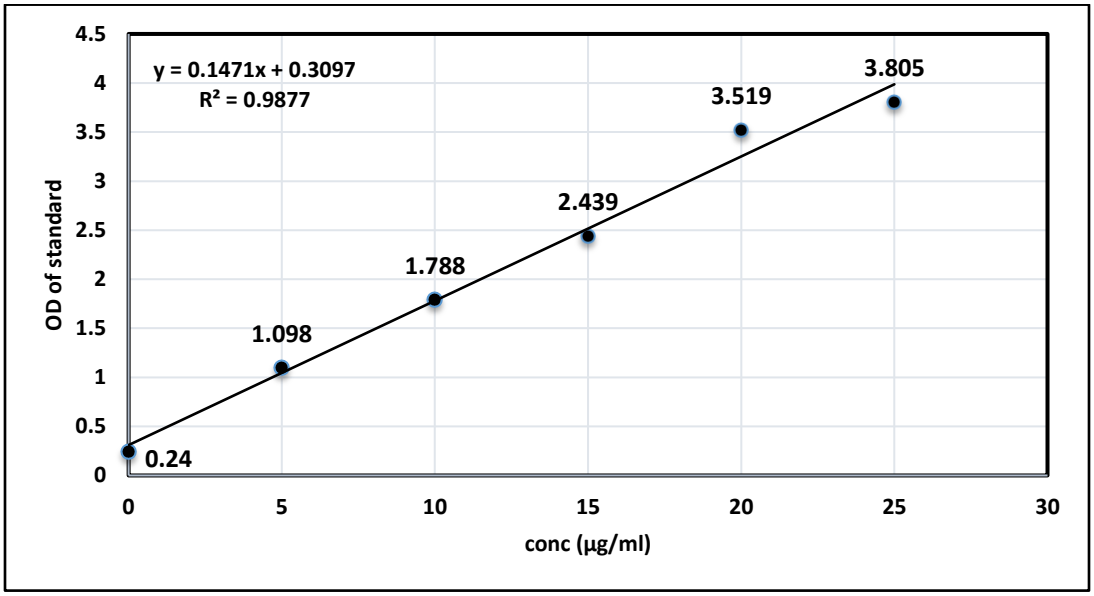

Figure S2: Regression line of Galli acid

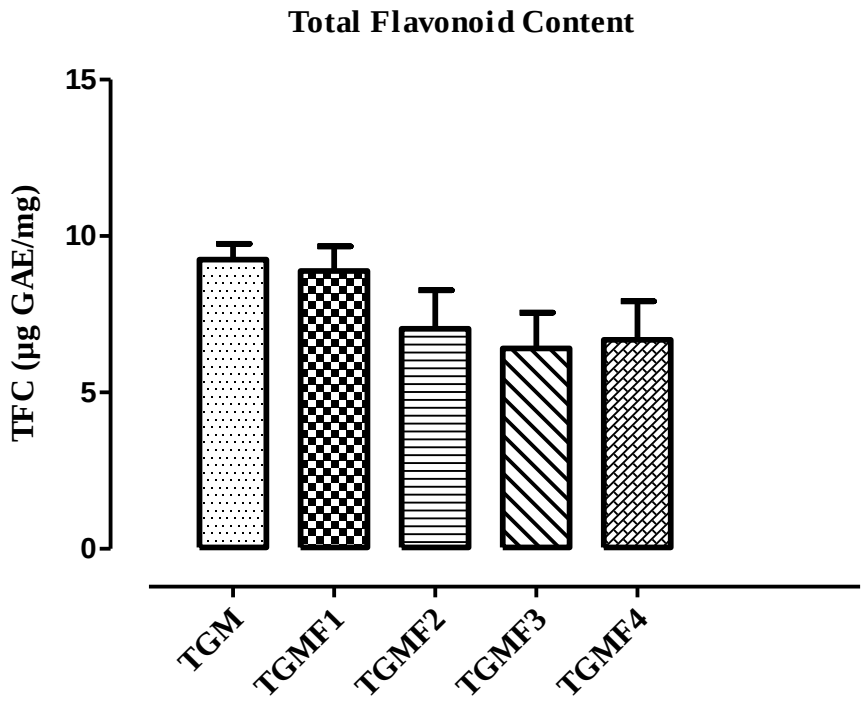

Figure S3: Total flavonoid contents of *Trillium govanianum*

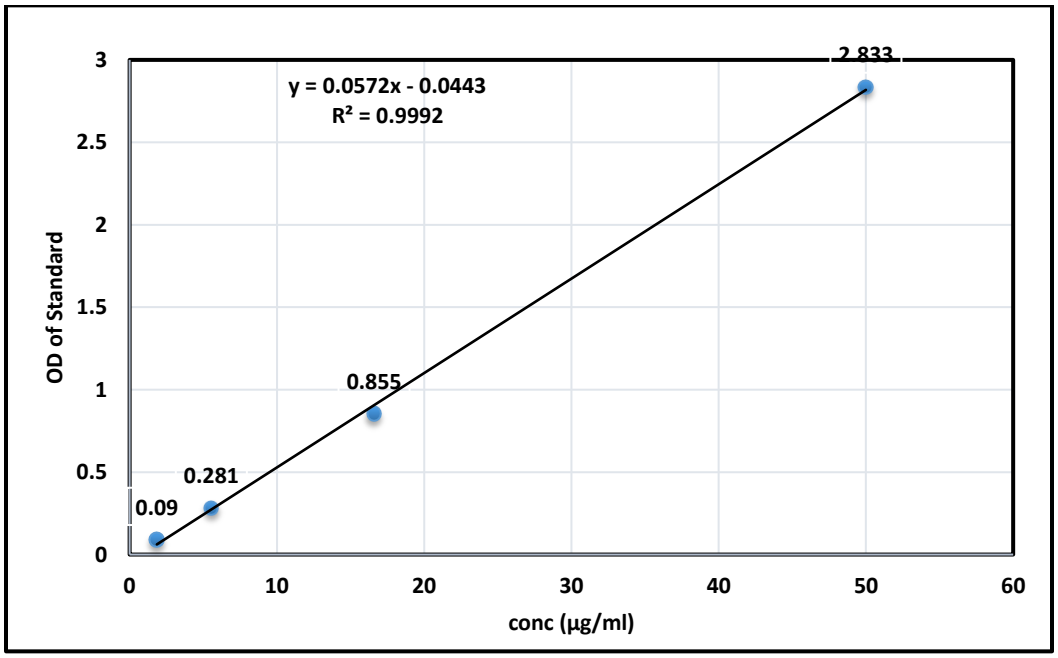

Figure S4: Regression line for flavonoid contents

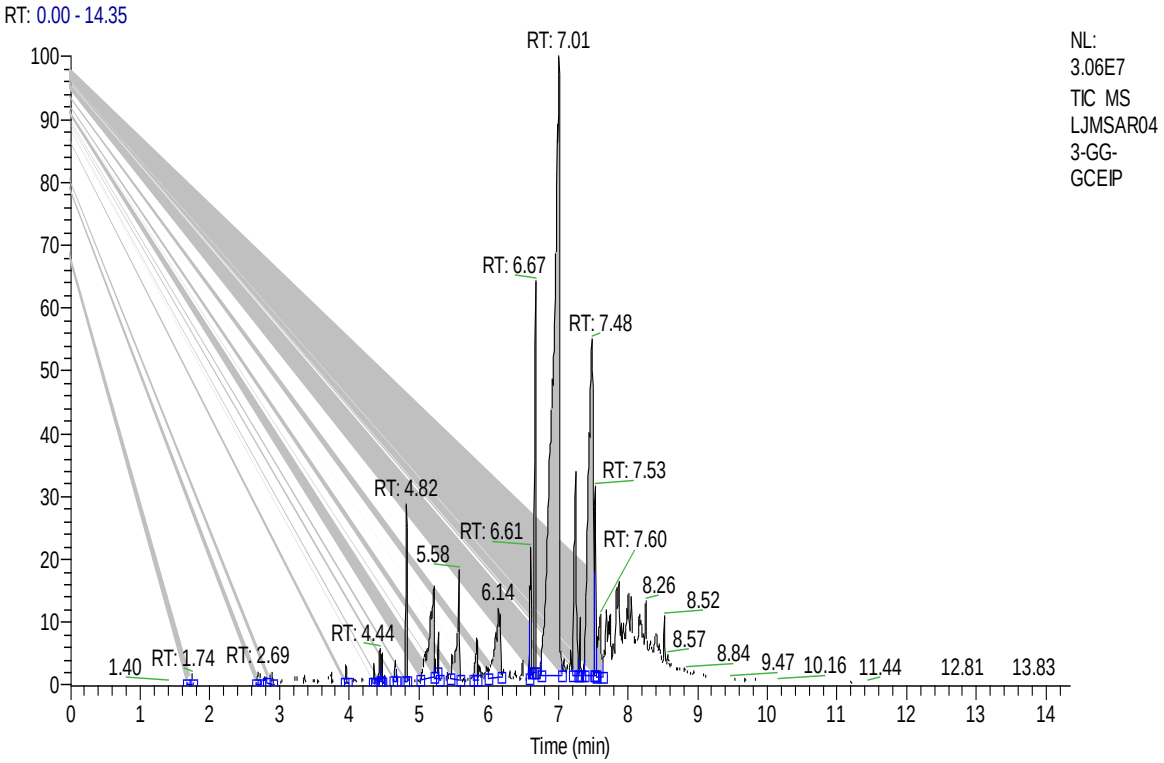

Figure S5: Typical Gas Chromatogram of *Trillium govanianum* showing separation of chemical components

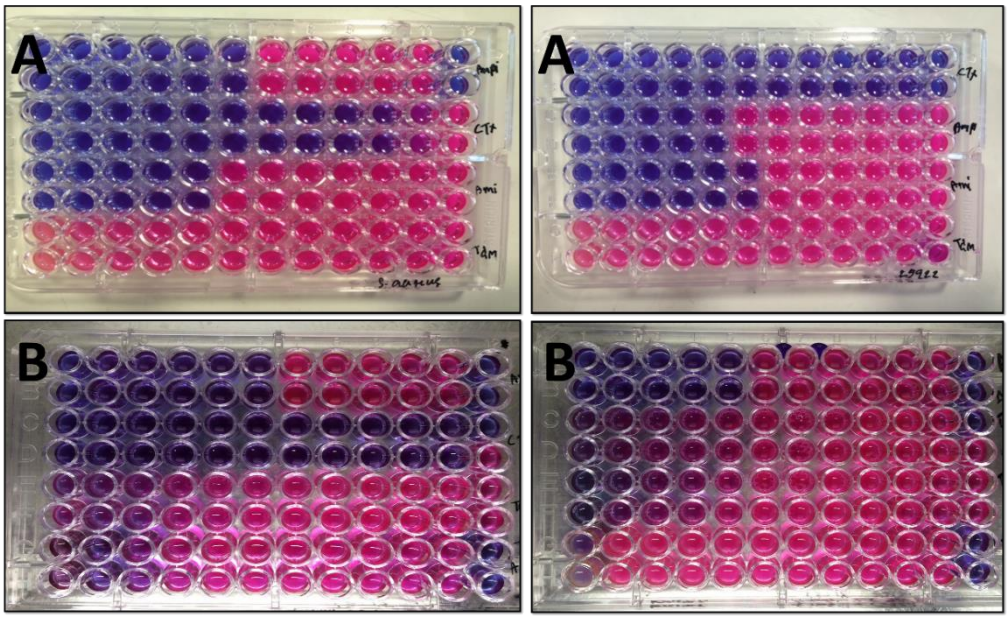

Figure S6. Typical 96-well plates shows the result after 24 hours

Table 1S. The MIC (mg/mL) values of the MeOH extract of the roots of *T. govanianum* and its SPE fractions by using the resazurin assay

Test sample	Bacterial strains (MIC mg/mL)				
	SA	BS	ML	EC	KO
TGM (mg/mL)	2.5	0.078	0.156	2.5	0.165
TGMF1(mg/mL)	2.5	2.5	0.156	1.25	2.5
TGMF2 (mg/mL)	2.5	0.615	1.25	2.5	2.5
TGMF3 (mg/mL)	0.31	0.0195	0.039	1.25	0.615
TGMF4 (mg/mL)	0.615	0.039	0.156	≥10	≥10
CTX[b](µg/mL)	2	0.25	0.25	≤0.06	≤0.06
AMP[c] (µg/mL)	0.125	≤0.06	0.125	4	2
AMX[d] (µg/mL)	4	0.5	4	2	2

SA, *Staphylococcus aureus* NCTC 7508; BS, *Bacillus subtilis* NCTC 1604; ML, *M. luteus* NCTC 7508; EC, *Escherichia coli* ATCC 25922; KO, *K. oxytoca* NCTC 8017; AAM[a] *(Asparagus adscendens* methanol extract), CTX[b] (Cefotaxime), AMP[c] (Ampicillin), AMX[d] (Amoxicillin)

Table S2: Compound identified by GCMS *Trillium govanianum*

Compound No	t_R (min)	Chemical Formula & Mol. Mass	Compound
1	5.21	$C_{12}H_{22}O_3$ 214.31	Isopropyl 9-oxononanoate
2	5.58	$C_{10}H_{18}O_4$ 202.25	Nonanedioic acid, monomethyl ester
3	6.14	$C_{10}H_{20}O_3$ 188.27	10-Hydroxydecanoic acid
4	6.61	$C_{17}H_{32}O_2$ 268.44	9-Hexadecenoic acid, methyl ester
5	6.67	$C_{17}H_{34}O_2$ 270.46	Hexadecanoic acid, methyl ester
6	7.01	$C_{16}H_{32}O_2$ 256.43	Hexadecanoic acid
7	7.25	$C_{19}H_{36}O_2$ 296.50	6-Octadecenoic acid, methyl ester
8	7.48	$C_{18}H_{32}O_2$ 280.45	9,12-Octadecadienoic acid
9	7.53	$C_{18}H_{36}O_2$ 284.48	Octadecanoic acid

References

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