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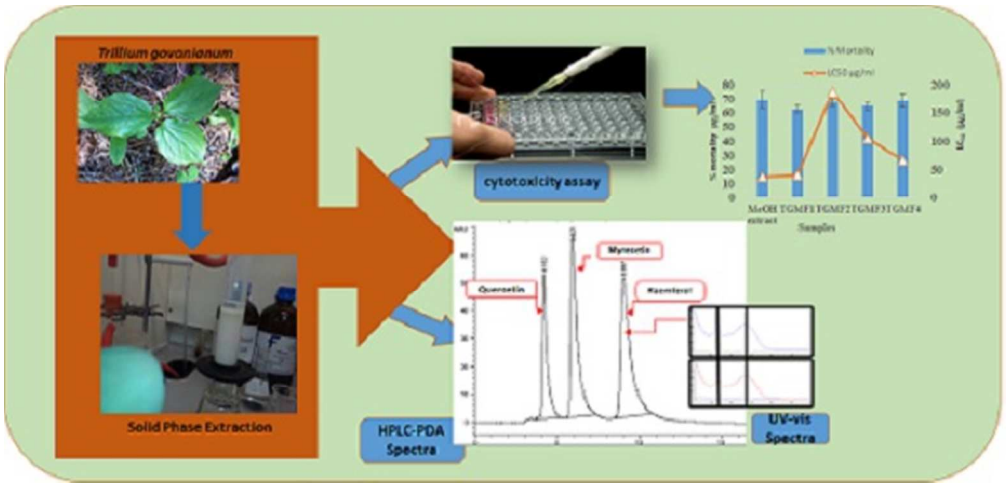

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Cytotoxicity, In Vitro Anti-Leishmanial and Fingerprint HPLC-Photodiode Array Analysis of the Roots of *Trillium govanianum*

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ABSTRACT

Trillium govanianum Wall. (Melanthiaceae *alt.* Trilliaceae), commonly known as "nagchhatry" or "teen patra", distributed from Pakistan to Bhutan about 2500-3800 m altitude is indigenous to Himalayas region. In folk medicine the plant has been reported for the treatment of wound healing, sepsis and in various sexual disorders. This paper reports, for the first time, to evaluate the cytotoxicity, in vitro anti-leishmanial and fingerprint HPLC-photodiode array analysis of the MeOH extract of the roots of *T. govanianum* and its solid phase extraction fractions. Reverse phase HPLC-PDA based quantification revealed the presence of significant amount of quercetin, myrecetin and kaemferol ranging from 0.221to 0.528 µg/mg DW. MeOH extract revealed distinguishable protein kinase inhibitory activity against *Streptomyces* 85E strain with 18 mm bald phenotype. The remarkable toxicity profile against brine shrimps and leishmanial was manifested by MeOH extract with LC_{50} 10 µg/ml and 38.5 µg/ml respectively.

Keywords: *Trillium govanianum; HPLC-PDA*; SPE; Protein kinase inhibition; Antileishmanial assay; LC_{50}.

1. Introduction

Trillium govanianum Wall. (Melanthiaceae *alt.* Trilliaceae), commonly known as "nagchhatry" in India and "teen patra" or "matar zela" in Pakistan, distributed from Pakistan to Bhutan about 2500-3800 m altitude is indigenous to Himalayas region ((Ismail et al. 2015). Since it is reported the presence of the steroid, trillarin, in this species, it has been enormously used in numerous traditional medicinal preparations that contain steroids and sex hormones, and now, it is one of the hot-selling herbal products in the Indo-Pak subcontinent. In folk medicine the plant has been reported for the treatment of wound healing, sepsis and in various sexual disorders (Pant and Samant 2010).

The reported studies on different species of the genus *Trillium* have revealed that this genus is ample in steroidal saponins, e.g., steroidal saponins were found in *T. erectum* L. (Hayes et al. 2009; Yokosuka and Mimaki 2008), *T. kamtschaticum* Pall. (Ono et al. 2003; Wei et al. 2012) and *T. tschonoskii* Maxim. (Man et al. 2010; Nakano et al. 1983; Wang et al.

2013). The dried rhizome of a few *Trillium* species that have been used traditionally for immuno-regulation, as an anti-inflammatory and anti-aging agent, were found to possess antitumor properties (Khan et al. 2016; Luo et al. 2006; Wang et al. 2013).

HPLC-PDA detection has grown into one of the frequently used technique for identification and quantification of flavonoids in plant extracts (Cai et al. 2006). Photodiode array (PDA) detector can collect multiple wavelength of chromatograms and corresponding spectra at the same time. The purity of the chromatographic peaks can also be determined and it can be used for spectra retrieval. Moreover, the qualitative information of chemical constituents can be obtained (Tan et al. 2014). Numerous phytochemical and biological assays are being used to estimate the medicinal characteristics of plants. These assays are more reliable and specific towards their reproducibility (Da Silva et al. 2000). Aromatic ring with one or more hydroxyl group in phenolic compounds are present in most of the plant species (Kim et al. 2016). Polyphenols are known as vital bioactive secondary metabolites of plants (Bahorun et al. 2004). Crude extract and their isolated compounds have been enormously screened to estimate their cytotoxicity (Meyer et al. 1982). Since biological screening is the leading thread of enthopharmacological approach and chemo-profiling the characteristic fingerprints for individual's plant parts could be very beneficial for the development of uniform standardization tools. Keeping in view the medicinal value of this plant the present study was designed to evaluate the cytotoxicity, in vitro anti-leishmanial and fingerprint HPLC- photodiode array analysis of the MeOH extract of the roots of *T. govanianum* and its SPE fractions.

2. Results and Discussion

2.1 Cytotoxicity assays: *Brine shrimp lethality assay*

In present study, cytotoxicity potential of the MeOH extract and its SPE fractions were tested against brine shrimp (*Artemia salina*) larvae to reveal its lethality profile. LC_{50} values below 100 µg/ml and ≤ 250 µg/ml were categorized as highly toxic and toxic respectively. The MeOH extract of roots of *T. govanianum* and its SPE exhibited different levels of Brine shrimp lethality. The LC_{50} value (µg/ml) of MeOH extract and its SPE fractions are shown in Table 1. The MeOH extract showed the most toxic exhibiting LC_{50} of 38.5 µg/ml followed by the SPE fractions TGMF1, TGMF2, TGMF3 and TGMF4 (LC_{50} = 40.5, 189.2, 105.6 and 66.5 µg/ml respectively). The SPE fraction TGMF1, which had the most polar components of the parent MeOH extract, showed quite similar toxic effects (LC_{50} 40.5 µg/ml) among other

SPE fractions. The positive control, doxorubicin demonstrated an LC_{50} value 1.98 µg/ml. These results are in agreement with the cytotoxicity of MeOH extract and its SPE fractions against four human carcinoma cell lines (IC_{50} = 5-16 µg/ml) (Khan et al. , 2016). The concentration of the extract determined the degree of lethality which was found directly proportional. The brine shrimp lethality assay is considered to be an appropriate tool for the primary estimation of cytotoxicity. The brine shrimp lethality assay determined the cytotoxicity of plant extract, if the LC_{50} value of less than 1000 µg/ml is observed. In present study, MeOH extract and its SPE fractions demonstrated LC_{50} values < 1000 µg/ml, indicated the presence of compounds having cytotoxic potential determined the observed cytotoxicity (Fatima et al. 2015).

2.2. *Protein kinase inhibition assay*

In current exploration, the zones recorded in protein kinase inhibition activity for the MeOH extract and its SPE fractions are summarized in Table 1. The MeOH extract of roots of *T. govanianum* and its SPE exhibited different levels of protein kinase inhibition zones. Among all the MeOH extracts and its SPE fractions, a significant inhibition zone of 18 mm bald, 8 mm clear phenotype was observed around the MeOH extract, while the SPE fractions TGMF1 (18 mm bald, 11 mm clear) showed the most noteworthy hyphae formation inhibition straggled by TGMF2 (13 mm bald, 7 mm clear), TGMF3 (11 mm bald, 7mm clear) and TGMF4 (11 mm bald, 7 mm clear). The positive control (surfactin) showed prominent 22 mm bald growth inhibition zone, while negative control (DMSO) showed no zone of inhibition establishing its non-toxic effect. The results of the current study revealed that the most promising kinase inhibitory was shown by MeOH extract and SPE fraction TGMF1, which had the most polar components of the parent MeOH extract. Rest of SPE fractions (TGMF2, TGMF3 and TGMF3) shown mild kinase inhibitory activity. The biological processes like apoptosis, cell differentiation and cell proliferation, which are regulated by the protein phosphorylation through protein kinases needs the surge for the development of protein kinases inhibitors especially from natural origin. In this regard, the protein kinases inhibitor are established as a potential target for the cancer treatment (Yao et al. 2011).

2.3. *In vitro* antileishmanial analysis

Antileishmanial capability of MeOH and its SPE fractions was evaluated for the first time in the present study. The antileishmanial potential of MeOH extract of and its SPE fractions against *Leishmania tropica* KWH23 strain shown in Figure 1. The MeOH extract exhibited

the most significant results with 70% mortality at 38.5 µg/ml (control: 0.36 µg/ml). The SPE fraction TGMF1, which had the most polar components of the parent MeOH exhibited as good result as its parent MeOH extract (LC_{50} 40.5 µg/ml). Rest of SPE fractions TGMF2, TGMF3 and TGMF4 (LC_{50}= 189.5, 105.6 and 66.5 µg/ml respectively) also showed considerable good results. The SPE fraction TGMF4, which contained the least polar components of the parent MeOH extract, exhibited the notable antileishmanial potential (LC_{50}: 66.5 µg/ml). In the previous findings flavonoids exhibited good antileishmanial activity by forming a complexes with the parasite cell wall (Wabwoba et al., 2010). Flavonoids like quercetin equivalent flavonoid compounds that have been reported to be effective as antitrypanosomal and antileishmanial agents and/or glycosides, limonoids or steroid compounds (Khan et al. 2014; Tasdemir et al. 2006).

2.4. Performance Liquid Chromatography –Photodiode array detection

HPLC-PDA analysis on the SPE fractions of the MeOH extract of the roots of *T. govanianum* was performed to obtain insights into the possible chemical composition of the fractions, particularly, to have an indication whether they contain phenolic and flavonoids as possible contributors to the significant cytotoxic and anti- leishmanial activity of the extract and its fractions. The chromatographic conditions were optimized by method development. A linear gradient elution with water and MeOH containing 1% TFA as the mobile phase offered the best resolution. Reverse phase HPLC-PDA based profiling was used for quantitative analysis of selected plant phenolics and the chromatographic finger printing was done by comparing the retention time and UV spectra of the reference compounds with those of the test sample, the results of which are summarized in Table 2, 3. A significant amount of quercetin (0.221 µg/mg DW), myrecetin (0.09 µg/mg DW) and kaemferol(0.528 µg/mg DW) were quantified from SPE fraction TGMF1 and TGMF2 (Table 3). Typical chromatograms of fractions (Fig. S10, S12) were recorded by using PDA detector at 220, 254, 360 nm to provide a real time chromatograms and on-line Ultraviolet (UV) spectra from 200-500 nm were recorded (Table S1) for identification of different groups and classes of compounds. The possible presence of compounds such as flavonoids, quercetin, myrecetin and kaemferol in SPE fractions draw a parallel correlation of plants potential with their know bioactivities. Typical chromatograms of standard and as well as compounds detected from various SPE fractions are presented in (Fig. S1, S5 & S7).

3. Experimental

3.1 *Reagents and chemicals*

All chemicals and reagents used were of analytical grade. Solvents used for the extraction were acquired from Merck (Darmstadt, Germany), quercetin, myrestine, kaemferol were purchased from Sigma–Aldrich (Steinheim, Germany), unless stated otherwise.

3.2 *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.

3.3 *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.

3.4 *Solid-phase extraction (SPE) and sample purification*

The Strata C-18 cartridge (20 g) was first washed with MeOH (50mL) 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), centrifuged at 12,000 rpm for 3 min, filtered through 0.20 µm sterile syringe filter for injection (20 µL) into the HPLC-PDA system.

3.5 *High Performance Liquid Chromatography –Photodiode array detection (HPLC-PDA)*

An analytical Agilent 1260 Infinity was used. Reversed-phase chromatography was performed on a Phenomenex Gemini-NX 5 U C_{18} column (250 x 4.6 mm). The column temperature was set at 25^{o}C. A variable wavelength UV-Vis detector was set at 220 nm, 254nm and 360nm. An elution gradient was used with solvent A (1% trifluoroacetic acid in water) and solvent B (1% trifluoroacetic acid in MeOH). The initial mobile phase composition was 70% of A and 30% B at 0 min, then linear gradient to 100% of B over 30

min and held at that composition for 5 min before to returning to start conditions and column equilibration at flow rate of 0.800 mL/min. The chromatograms were monitored as 220 nm, 254 nm and 360 nm.

3.6 *Cytotoxicity assays: Brine shrimp lethality assay*

The cytotoxic effect of MeOH extract and its SPE were measured in a 96 well plate by brine shrimp lethality bioassay (Ul-Haq et al. 2012). Eggs of test organism *Artemia salina* (Ocean 90, USA) were retained for 24–48 hours hatching period in simulated sterile sea water (38 g/l supplemented with 6 mg/l dried yeast) with constant oxygen supply. Each fraction was tested at three graded concentrations (1000µg/ml, 500µg/ml and 250µg/ml). Doxorubicin (4mg/ml) and DMSO were used as standard (positive control) and negative control respectively. In each well of 96 wells plate, the mature phototropic nauplii were then harvested and artificial sea water (200µl) was added to dissolve the fraction and volume was adjusted to 300µl. The incubation time was 24 hours and the degree of lethality exhibited was determined by counting the number of shrimps per well for each test samples. Table curve 2D v5.01 software was used to calculate the median lethal concentration (LC_{50}) of each test sample with ≥ 50 % mortality.

3.7 *Protein kinase inhibition assay*

The protein kinase inhibition assay of MeOH extract and its SPE were performed thrice by observing hyphae formation in purified isolates of Streptomyces 85E strain (Yao et al. 2011). Refreshed culture of Streptomyces were used on sterile plates to develop a bacterial lawn. From stock solution 20 mg/ml of DMSO, 5 µl of each sample was poured onto the 6mm sterile filter paper discs. The sterile plates were seeded with Streptomyces 85E and soaked paper discs having concentration of 100 µg/disc were placed on the surface directly. The incubation time for plates was 72 hours at room temperature and results were depicted as bald zone of inhibition around test samples and soaked discs. Positive control (surfactin) and negative control (DMSO) soaked discs were placed.

3.8 *In vitro* antileishmanial analysis

The *in vitro* antileishmanial analysis of MeOH extract and its SPE were estimated by the method described by (Pulivarthi et al. 2015). A medium containing 10% fetal bovine serum was used to kept *Leishmania tropica* KWH23 strain for 24 hours incubation for 7days. Stock solution was prepared in DMSO (10 mg/ml DMSO) and 96 wells plate was serially diluted. Each 96 wells of plate contained $5x10^6$ promastigotes and samples were tested at three

different concentrations (1000, 100, 10 µg/ml). For positive control and negative control Amphotericin B and 1% DMSO in PBS were used respectively. The incubation temperature for 96 wells plates were at 24^{o}C for 72 hours. For the count of surviving promastigotes about 15 µl of test sample were transferred to neubauer counting chamber (Marien, Germany) under light microscope. Afterwards table curve 2D v5.01 software was used for the calculation of LC_{50}.

3.9 *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.

4. Conclusion

The findings of current study support the notion that the use of different fractions on the basis of different solvent systems truly retrieves a complete phytochemical and biological profiling of plants. The current study concludes that the Reversed-phase HPLC-PDA has proved to be the method of choice for the separation and quantification of a verity of flavonoids in different fraction. Similarly MeOH extract of this plant is exceptionally effective against Leishmaniasis and lethal to brine shrimps as well as oncogenic kinases inhibitor, which signify its cytotoxic potential. The present study may proceeded for further investigation to target the isolation of secondary metabolites responsible for the observed activity.The given effective MeOH extract and its SPE fractions could serve as novel scaffolds in drug discovery. To our best knowledge, this is the first report showing significant phytochemical and biological potential of *Trillium govanianum* indigenous to Pakistan.

Disclosure statement

The authors have declared that there is no conflict of interest.

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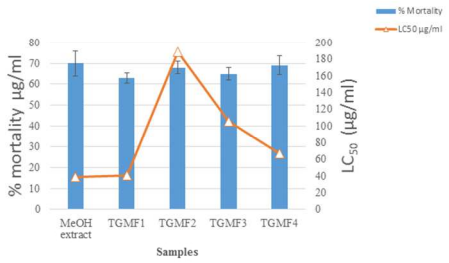

Figure 1. Antileishmanial potential of MeOH Extract of the roots of T. govanianum and its SPE fractions. Values are presented as mean ± standard deviation of triplicate analysis.

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Table 1. Brine shrimp lethality assay and Streptomyces hyphae formation inhibition potential of MeOH Extract of the roots of *T. govanianum* and its SPE fractions. Values are presented as mean ± standard deviation of triplicate analysis.

	Brine shrimp lethality assay % mortality µg/ml				Streptomyces hyphae formation inhibition Diameter of growth inhibition zone	
	1000	500	250	LC_{50} µg/ml	Clear Zone (mm ± SD)	Bald Zone (mm ± SD)
MeOH Extract	70 ± 0^{a}	68 ± 2.5^{b}	59 ± 1.1^{c}	10.1	$8 \pm 0.5_{a}$	$18 \pm 0.5_{b}$
TGMF1	70 ± 0^{a}	63 ± 2.8^{b}	58 ± 2.0^{c}	77.5	$11 \pm 0.5_{a}$	$18 \pm 0.5_{b}$
TGMF2	70 ± 0^{a}	63 ± 1.5^{b}	58 ± 1.5^{c}	38.2	$7 \pm 1.0_{a}$	$13 \pm 1.0_{b}$
TGMF3	70 ± 0^{a}	63 ± 1.3^{b}	60 ± 1.6^{c}	93.1	$7 \pm 0.5_{a}$	$11 \pm 1.1_{b}$
TGMF4	70 ± 0^{a}	63 ± 0.5^{b}	58 ± 1.2^{c}	22.7	$7 \pm 0.5_{a}$	$11 \pm 0.5_{b}$
Negative Control	0	0	0	-	-	-

Initially, the samples were evaluated at single highest concentration and the samples which showed more than 50% inhibition/significant activity were tested at lower concentrations to find their LC_{50}.

Negative control: DMSO.

LC_{50} of Doxorubicin (positive control employed in the brine shrimp lethality assay) was 1.98 µg/ml. Growth inhibition zone exhibited by surfactin (positive control in streptomyces hyphae formation inhibition assay) was 22 ± 1.01 mm (bald zone).

Values (mean ± SD) are average of three samples of each MeOH extract and its SPE, analyzed individually in triplicate (n = 1 × 3). $^{a\text{-}c}$ Means difference is significant at $p < 0.05$.

Table 2. Retention time (*tR*), calibration curve parameters, limit of detection (LOD), limit of quantification (LOQ) for the standards.

Standard	*tR* (min)	Calibration curve equation	Correlation coefficient (r^2)	LOD (µg/ml)	LOQ (µg/ml)
Quercetin	4.162	y = 20.26x + 10.207	0.9989	3.64	11.04
Myrecetin	5.921	y= 17.796x + 3.0343	0.9988	3.85	11.66
Kaemferol	8.997	y = 18.395x - 58.817	0.9963	6.7	20.31

Table 3. Chemical profiling of SPE fractions of MeOH Extract of the roots of *T. govanianum* using HPLC-PDA

	Flavonol flavonoid (µg/mg DW)								
	Quercetin			Myrecetin			Kaemferol		
SPE Fractions	*tR* (min)	λ (nm)	Quantified Amount (µg/mg)	*tR* (min)	λ (nm)	Quantified Amount (µg/mg)	*tR* (min)	λ (nm)	Quantified Amount (µg/mg)
TGMF1	4.102	360	0.221±0.011	-	-	-	-	-	-
TGMF2	-	-	-	6.504	360	0.09 ±0.010	8.887	360	0.528 ±0.011
TGMF3	-	-	-	-	-	-	-	-	-
TGMF4	-	-	-	-	-	-	-	-	-

Supporting Information

Cytotoxicity, In Vitro Anti-Leishmanial and Fingerprint HPLC-Photodiode Array Analysis of the Roots of *Trillium govanianum*

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Cytotoxicity, In Vitro Anti-Leishmanial and Fingerprint HPLC-Photodiode Array Analysis of the Roots of *Trillium govanianum*

ABSTRACT

Trillium govanianum Wall. (Melanthiaceae *alt.* Trilliaceae), commonly known as "nagchhatry" or "teen patra", distributed from Pakistan to Bhutan about 2500-3800 m altitude is indigenous to Himalayas region. In folk medicine the plant has been reported for the treatment of wound healing, sepsis and in various sexual disorders. This paper reports, for the first time, to evaluate the cytotoxicity, in vitro anti-leishmanial and fingerprint HPLC-photodiode array analysis of the MeOH extract of the roots of *T. govanianum* and its solid phase extraction fractions. Reverse phase HPLC-PDA based quantification revealed the presence of significant amount of quercetin, myrecetin and kaemferol ranging from 0.221to 0.528 µg/mg DW. MeOH extract revealed distinguishable protein kinase inhibitory activity against *Streptomyces* 85E strain with 18 mm bald phenotype. The remarkable toxicity profile against brine shrimps and leishmanial was manifested by MeOH extract with LC_{50} 10 µg/ml and 38.5 µg/ml respectively.

Keywords: *Trillium govanianum; HPLC-PDA*; SPE; Protein kinase inhibition; Antileishmanial assay; LC_{50}

Results

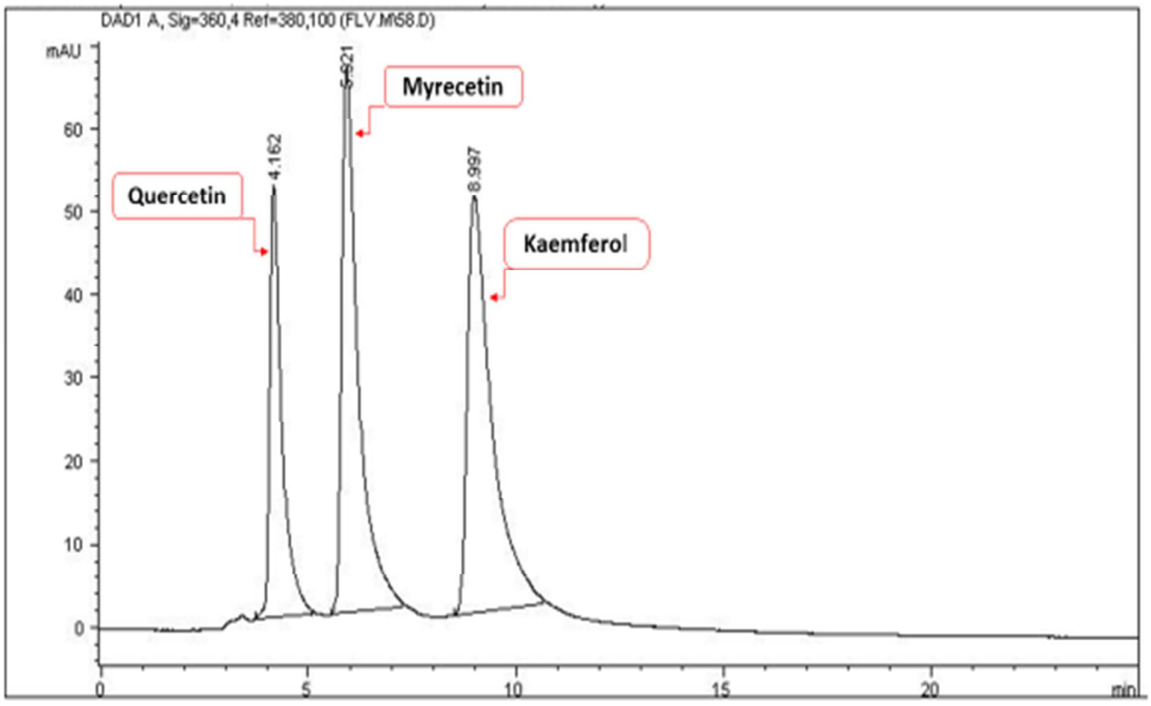

Figure S1: HPLC-PDA Chromatogram of standard phenols monitored at 360nm.

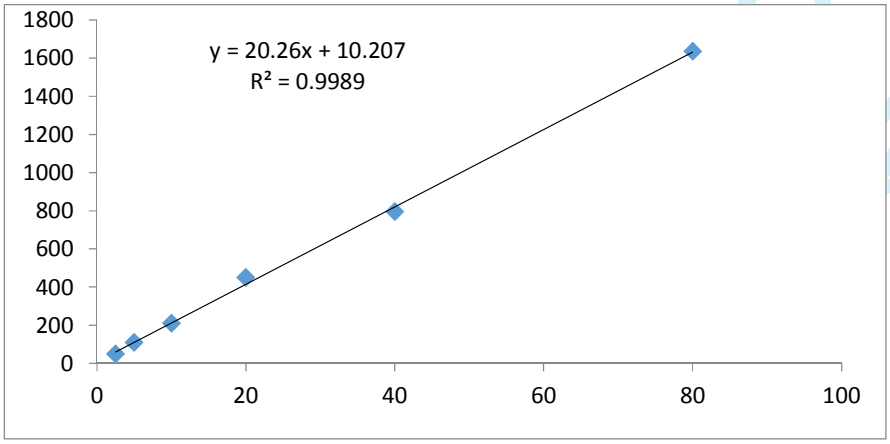

Figure S2: Calibration curve of standard Quercetin

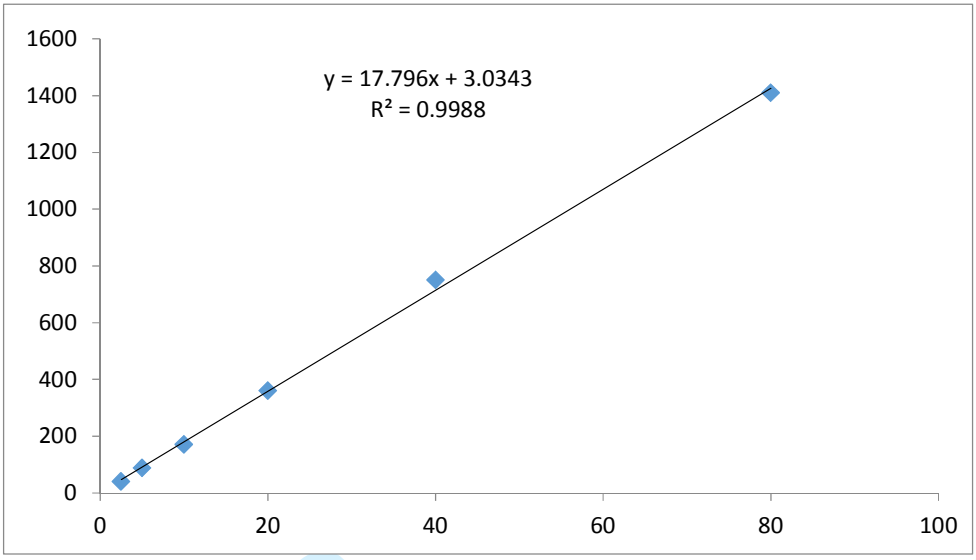

Figure S3: Calibration curve of standard Myrecetin

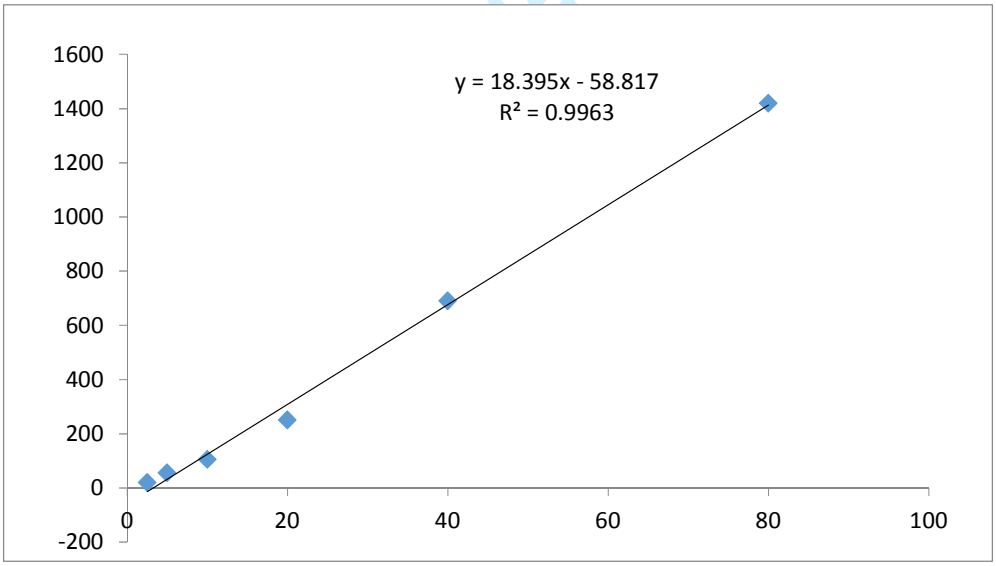

Figure S4: Calibration curve of standard Kaemferol

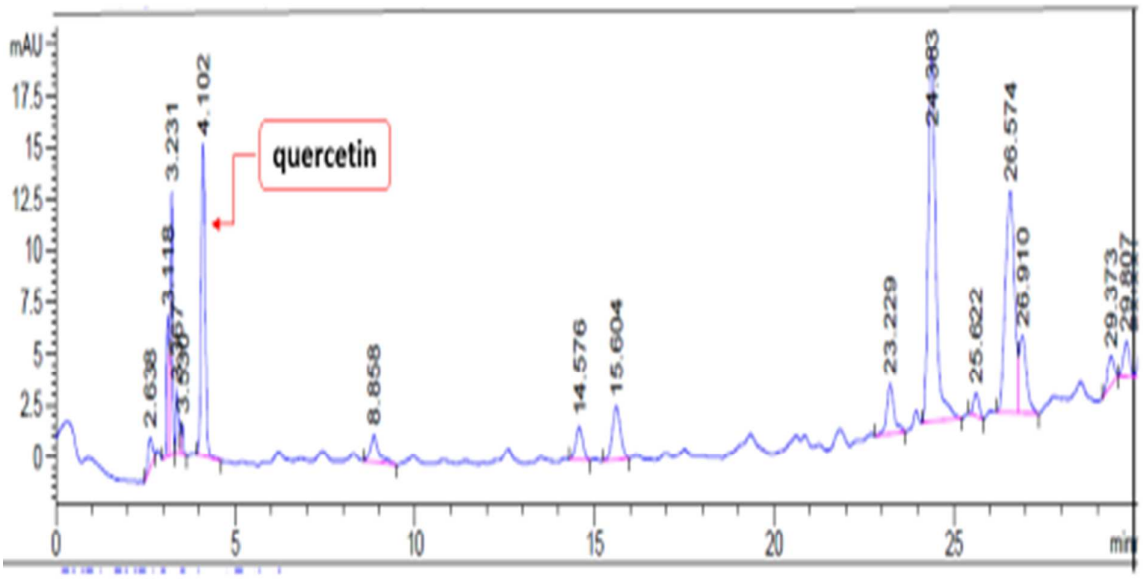

Figure S5: HPLC-PDA Chromatogram of detected compound from TGMF1 of *Trillium govanianum* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

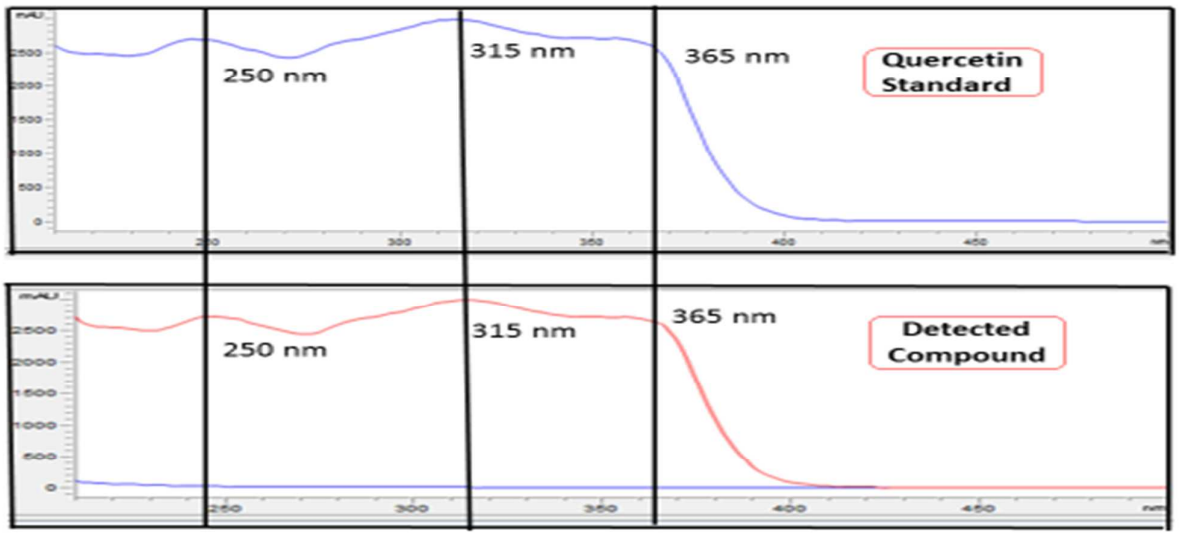

Figure S6 : Comparison of UV-vis spectra of a reference standard and detected compound.

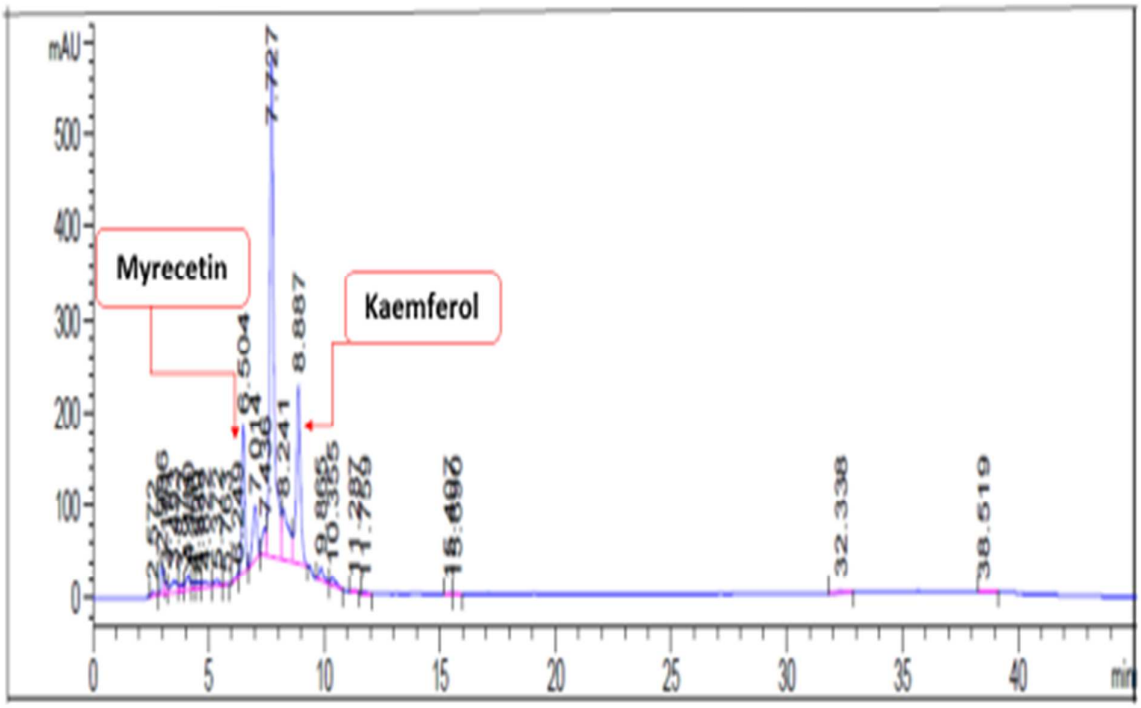

Figure S7: HPLC-PDA Chromatogram of detected compounds from TGMF2 of *Trillium govanianum* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

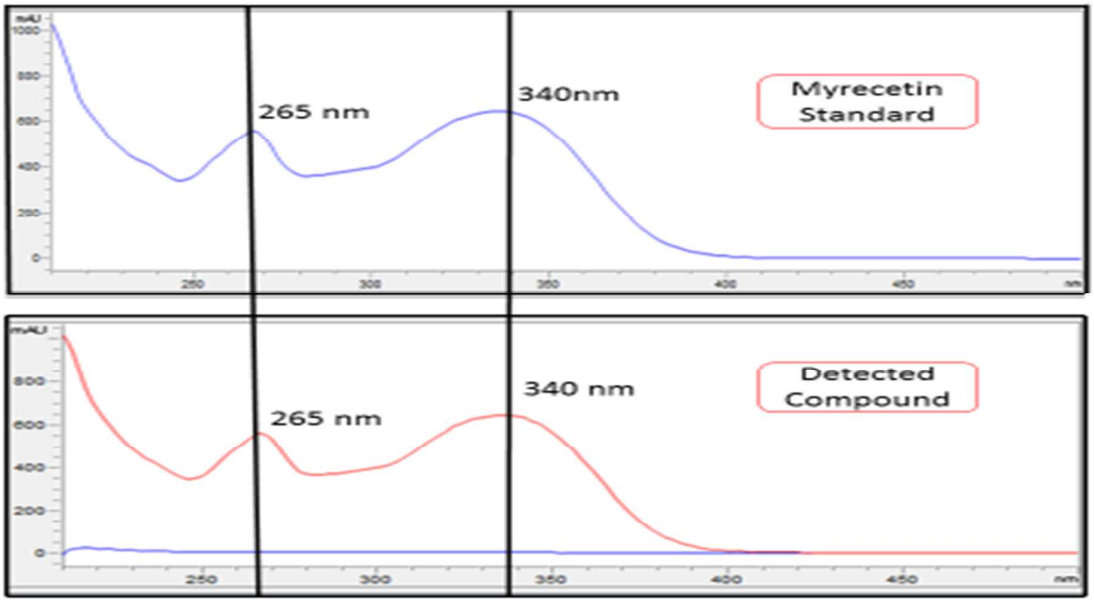

Figure S8 : Comparison of UV-vis spectra of a reference standard and detected compound.

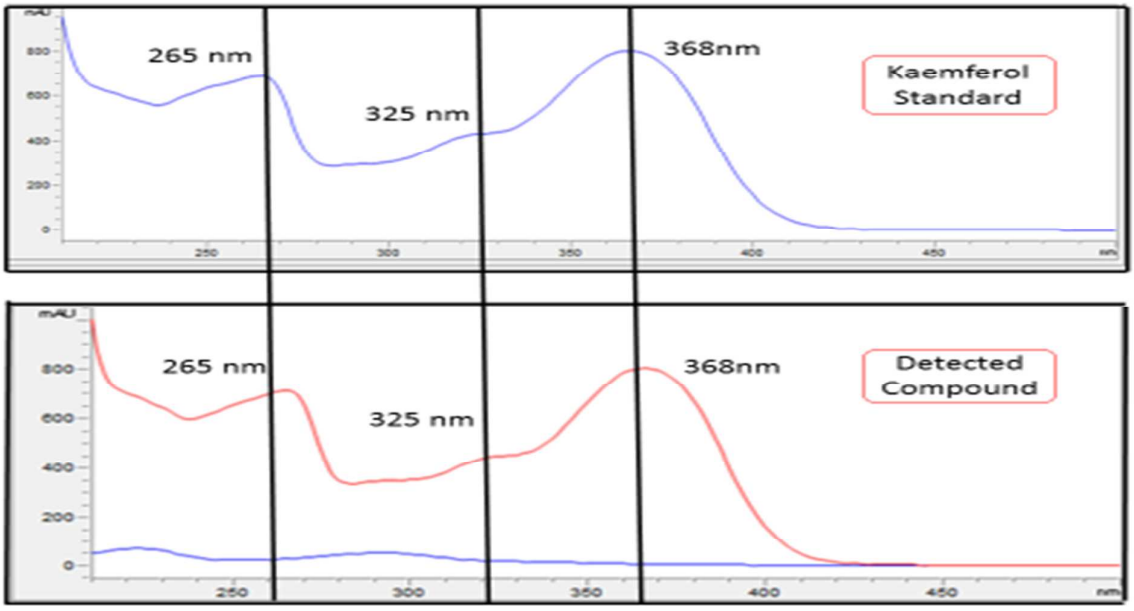

Figure S9: Comparison of UV-vis spectra of a reference standard and detected compound.

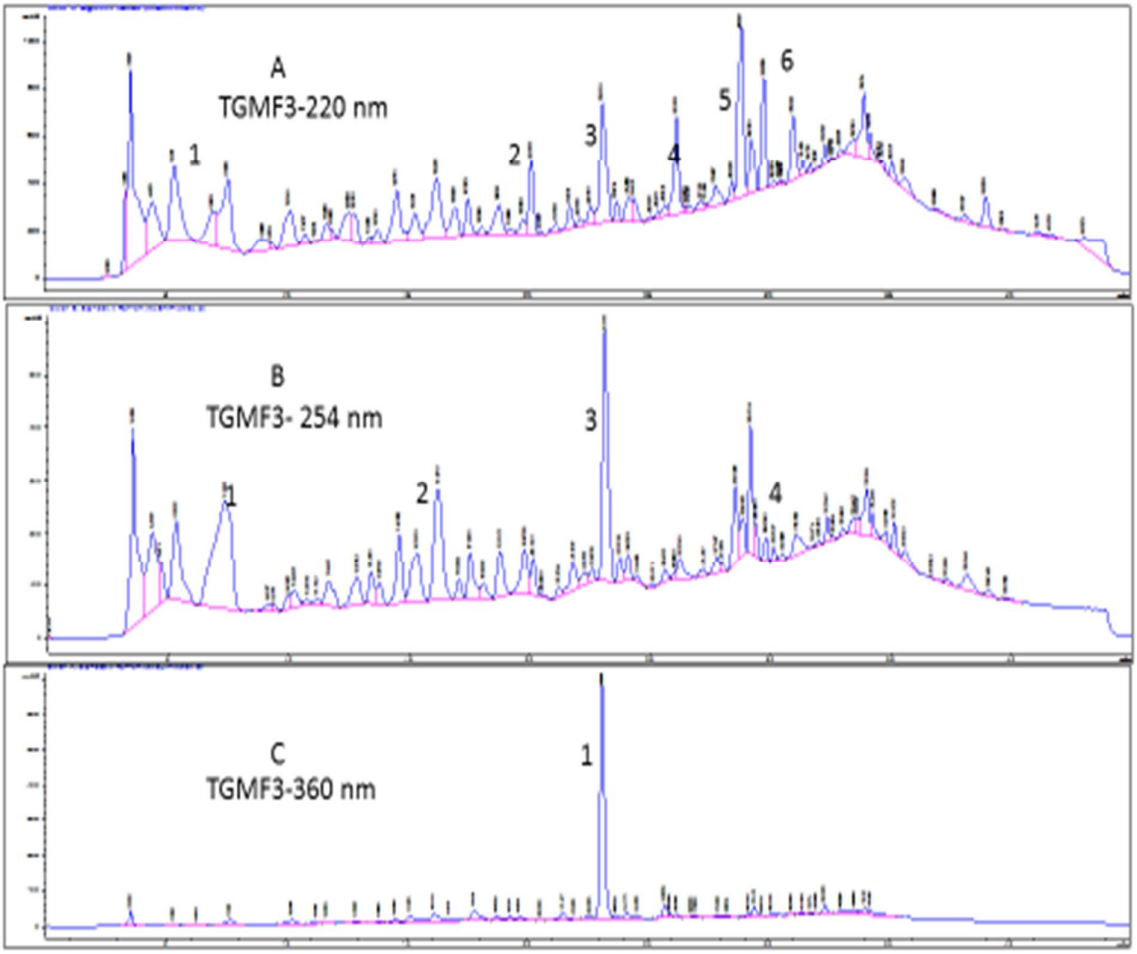

Figure S10: HPLC-PDA Chromatogram of TGMF3 of *Trillium govanianum* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

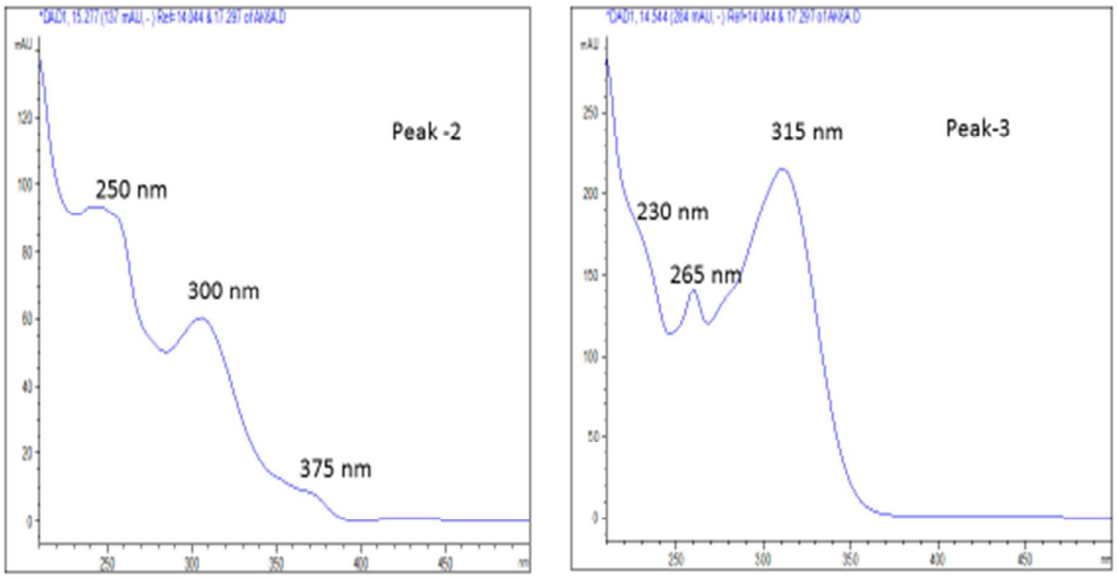

Figure S11: Corresponding UV-vis absorbance (TGMF3) at multiple wavelengths of the peaks separated by HPLC-PDA.

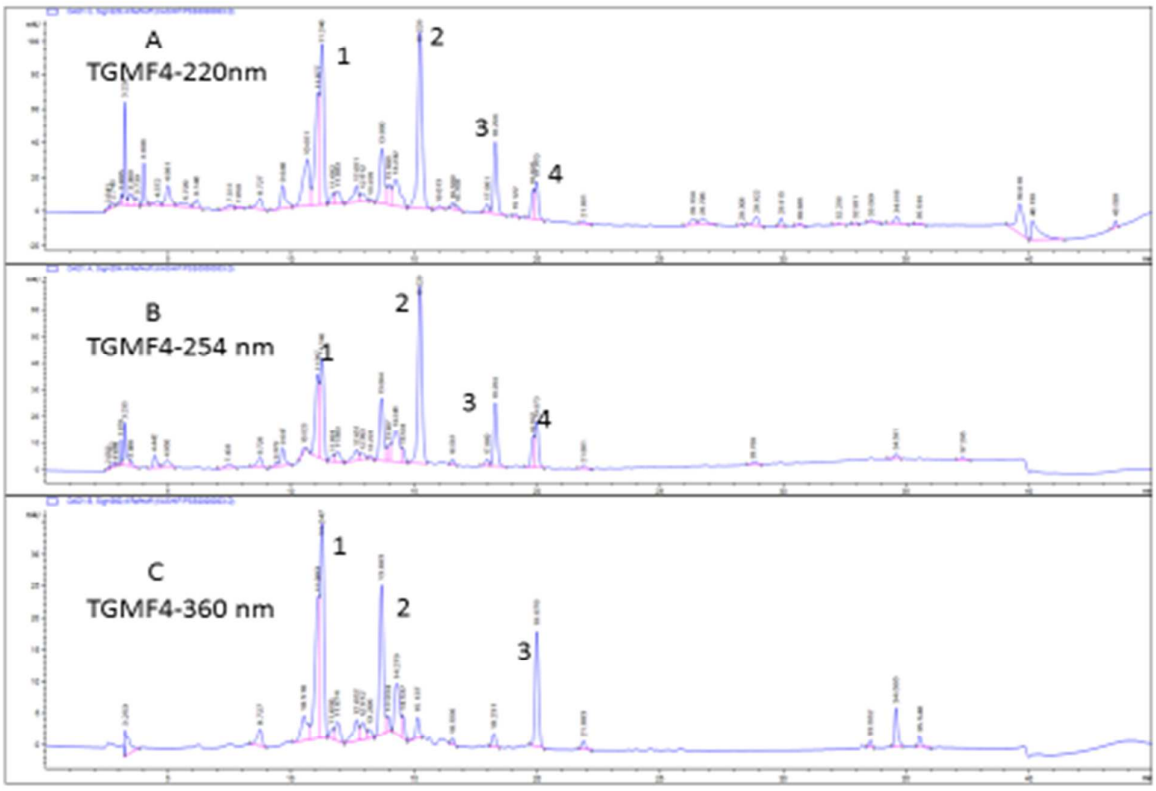

Figure S12: HPLC-PDA Chromatogram of TGMF4 of *Trillium govanianum* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

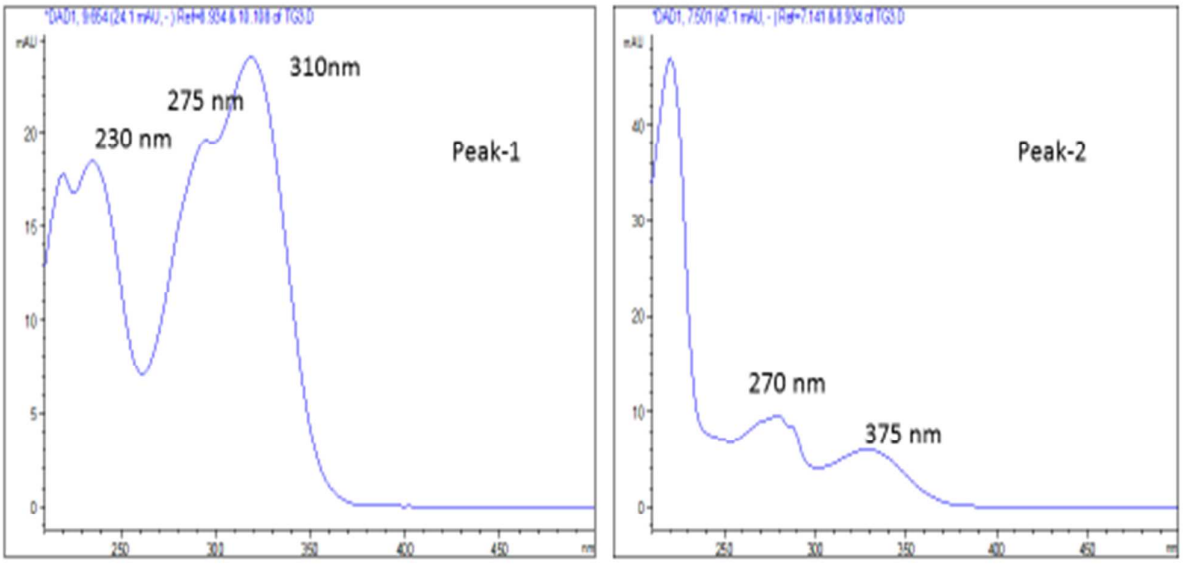

Figure S13: Corresponding UV-vis absorbance (TGMF4) at multiple wavelengths of the peaks separated by HPLC-PDA.

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Table S1. Retention times (t_R) and corresponding UV-vis absorbance at multiple wavelengths of the peaks separated by HPLC of SPE fractions of the MeOH extract of the roots of *Trillium govanianum*

TGMF1		**220nm**						**254nm**						**360nm**					
Peaks	t_R in (min)						Peaks	t_R in (min)					Peaks	t_R in (min)					
1[a]	3.23	230	270	295	320		1	7.47	220	280			1[a]	12.85	240	260	290	310	
2[a]	4.1	230	250	285	315	365	2[a]	8.42	225	270	320		2[a]	16.66	285	325	360		
3	14.57	220	275				3[a]	12.91	260	295	310								
4	15.60	230	290				4	26.44	240	290									
5[a]	23.22	230	265	290	285	445	5[a]	28.34	265	290	390								
6[a]	24.38	230	265	295	390	440	6	32.76	230	260									
7	26.57	220	270				7[a]	36.21	295	375	390								
TGMF2																			
1[a]	6.5	230	265	320	340		2[a]	8.42	225	270	320		1[a]	12.85	240	260	290	310	
2[a]	7.72	240	260	290	310	340	5[a]	28.3	265	290	390		2[a]	16.66	285	295	325	360	
3[a]	8.88	230	265	290	325	368	8[a]	36.2	295	375	390		3[a]	28.34	265	295	385	410	
TGMF3																			
1a	6.25	235	285	320			1	6.56	225	265	290		1[a]	24.57	230	280	330	365	
2	20.36	230	260				2a	17.18	250	285	355								
4a	24.45	230	280	330	365		3[a]	24.89	235	280	330	365							
5[a]	26.57	245	290	320	360		4[a]	29.47	240	335	360	380							
TGMF4																			
1[a]	11.84	225	265	285	330	360	1[a]	11.41	225	270	290	335	1[a]	11.52	220	270	310	390	
2[a]	15.69	220	260	335	365		2[a]	15.36	222	260	310	360	2[a]	13.54	225	255	310	380	
3	18.57	225	255	290			3	18.57	225	250	285		3	20.21	235	260	290		
4	20.25	235	265				4	20.67	230	260	280								

[a]Possible Phenolic Compounds