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GC/MS analysis, free radical scavenging, anticancer and β -glucuronidase inhibitory activities of *Trillium govanianum* rhizome

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Abstract

The current study aims to investigate the phytochemical constituents, antioxidant, β -glucuronidase inhibitory and anticancer activities of *Trillium govanianum* rhizome. Phytochemical screening revealed the presence of steroidal glycosides, saponins, sterols, flavonoids and carbohydrates. GC/MS analyses of *n*-hexane fraction identified 12 compounds, including 70% unsaturated and 30% saturated fatty acids. Higher free radical scavenging capacity was observed in *n*-hexane and chloroform fraction compared with the other fractions. Based on IC₅₀ (μ g/mL) values, antiproliferative activity on HeLa cells was observed for chloroform (0.8 ± 0.2), ethyl acetate (1.4 ± 0.1) and butanol (1.6 ± 0.3) fractions by comparison to anticancer drug doxorubicin (0.3 ± 0.0). Similarly, all fractions exhibited cytotoxicity on PC-3 cells. Moreover, the methanol extract (IC₅₀: 140.8 ± 3.8) and butanol fraction (196.2 ± 1.9) exhibited a moderate level of β -glucuronidase inhibitory activity. These findings may validate the folkloric uses of *T. govanianum* rhizome in cancer management, and can be a promising candidate as an anticancer agent.

Introduction

Medicinal plants are capitalized in cure of different ailments since time immemorial. This can be attributed to the presence of secondary metabolites, arising from primary metabolism by different biosynthetic pathways. Important classes of secondary metabolites include alkaloids, glycosides, tannins, steroids, terpenoids, saponins and phenolic compounds. These are biologically active molecules and potential activities of any medicinal plant are primarily due to these metabolites (Okigbo et al., 2009). After isolation of wide varieties of active compounds and exploration of their efficacies and safety profiles, it is proved that medicinal plants have a value in the modern synthetic era. Numerous drugs have entered the international phar-

macopoeias through the study of ethnopharmacology and traditional medicine (Patwardhan et al., 2005).

Keeping in view the importance and scope of medicinal plants, the present study was aimed to investigate the phytochemical constituents and biological activities of *Trillium govanianum* Wall. ex Royle, used in the traditional systems of medicine. *T. govanianum* belongs to the family Trilliaceae and is mainly distributed in South Asia, from Pakistan to Bhutan, at an altitude of 2700-3800 m (Muhammad, 2011). The genus *Trillium* includes long lived herbaceous flowering plants and different species are widely distributed throughout the world; the species reported from Pakistan is *T. govanianum* (Flora of Pakistan). The Asian species of *Trillium* produce secondary metabolites that are mainly exploit-



ted in cancer treatments (Huang et al., 2011). North American species of *Trillium* are known to have anti-fungal and antibacterial properties (Akihito et al., 2008). In folk medicine, *T. govonianum* is used to cure dysentery, in wound healing, inflammation, sepsis, menstrual and sexual disorders (Mahmood et al., 2013; Pant et al., 2010; Savita et al., 2013). It has been reported that the powdered plant is used as body and sexual tonic (Khan et al., 2013). Due to the folkloric knowledge of this plant, it is relevant to provide scientific evidence to its traditional uses, as well as to screen this valuable herb for any possible potential biological activity that will provide a base for isolation of targeted lead compounds. In this study, to the best of our knowledge we have explored for the first time, the antioxidant, anti-cancer and β -glucuronidase inhibitory activities of *T. govonianum* rhizome, its phytochemical constituents and proximate fatty acids composition.

Materials and Methods

Plant material

Plants of *T. govonianum* were collected from Dir Upper, Kohistan Valley (34° 54' and 35° 52' North latitudes and 72° 43' and 73° 57' East longitudes, at an altitude of 2700 -3800 m) in August, 2013. The plant was identified by Mr. Ghulam Jelani (Curator) of the Department of Botany, University of Peshawar and a voucher specimen [No. Bot. 20092 (PUP)] was deposited in the herbarium of the same department.

Extraction and fractionation

The shade dried rhizomes of *T. govonianum* (7 kg) were grounded and extracted with methanol (40 L) at room temperature for a period of 14 days (3 X 40 L). The combined methanolic extract was evaporated to dryness to yield a brownish gummy residue (512 g), which was further fractionated (solid-liquid partition) into *n*-hexane (81 g), chloroform (94 g), ethyl acetate (85 g) and *n*-butanol (115 g) fractions.

Phytochemical screening

Different qualitative chemical tests of the *n*-hexane, chloroform, ethyl acetate, *n*-butanol and methanol fractions were performed for the determination of plant metabolites like alkaloids, tannins, phenolic compounds, flavonoids, saponins, sterols and carbohydrates, according to the recommended standard protocols (Shome and Joshi, 1984).

Gas chromatography/mass spectrometry (GC/MS)

GC/MS analysis was carried out on a 6890N Agilent gas chromatograph coupled with a JMS 600 H JEOL mass spectrometer. The compound mixture was separated on a fused silica capillary SPBI column, 30 m x 0.32 mm, 0.25 μ m film thicknesses, in a temperature program from 50 to 256°C with a rate of 4°C/min with

2 min hold. The injector was at 260°C and the flow rate of the carrier gas (helium) was 1 mL/min. The EI mode of JMS 600 H JEOL mass spectrometer has ionization volt of 70 eV, electron emission of 100 μ A, ion source temperature of 250°C and analyzer temperature of 250°C. Sample was injected manually in split mode. Total elution time was 90 min. MS scanning was performed from *m/z* 85 to *m/z* 390 (Faizi et al., 2014).

Derivatization

The *n*-hexane fraction was esterified to the more volatile methyl esters by methanol boron trifluoride method. Hexane solution (5 mL) obtained from the extraction was treated with boron trifluoride methanol (10%) solution and refluxed for 2 min on a water bath, and then 5 mL *n*-hexane was added, after an another 1 min of reflux, the solution was treated with 15 mL saturated NaCl solution under vigorous stirring. The organic layer was then separated and dried over anhydrous CaCl₂ (Botinestean et al., 2012).

GC/MS identification of components

Identification of proximate fatty acid components of the non-polar fraction (*n*-hexane) was based on the computer evaluation of mass spectra of sample through NIST-based AMDIS (automated mass spectral deconvolution and identification software), direct comparison of peaks and retention times with those for the standard compounds as well as by following the characteristic fragmentation patterns of the mass spectra of particular classes of compounds.

DPPH free radical scavenging assay

The *in vitro* antioxidant activity was evaluated by DPPH free radical scavenging assay according to method described elsewhere (Lue et al., 2010) with slight modifications. Two milliliters of 0.1 mM DPPH free radical solution in methanol were added to 1 mL of different concentrations (1, 10, 30, 50, 100, 200 μ g/mL) of the fractions or standards in methanol. The solutions were shaken thoroughly on a vortex (Gyromixer, Pakland Scientific Production, Pakistan) and incubated in the dark at ambient temperature for 30 min. Absorbance was then measured at 517 nm using UV/Visible spectrophotometer (Lambda 25, PerkinElmer, USA) against control which consisted of 0.1 mM DPPH free radical solution without extracts or standards. Blank consisted of methanol alone. Ascorbic acid and BHT were used as standards. The percent DPPH free radical scavenging was calculated using the following formula: DPPH (%) = [(A_I-A_{II}/A_I) x 100], where A_I is the absorbance of the control reaction and A_{II} is the absorbance in the presence of the sample.

Anticancer assay

The cytotoxic activity of samples was determined by the MTT assay, according to Baydoun and Mosmann with slight modification (Baydoun et al., 2013;

Mosmann, 1983), on two cancer cell lines, i.e. HeLa (cervical cancer cells) and PC-3 (prostate cancer cells). For the assay, cells were grown in DMEM (Dulbecco's modified Eagle medium) and MEM (modified Eagle's medium) containing 10% FBS and 2% antibiotic (penicillin and streptomycin) and maintained at 37°C, in 5% CO₂, for 24 hours, in a flask. Cells were plated (1 × 10⁵ cell/mL) in 96-well flat bottom plates and incubated for 24 hours for cell attachment. Various concentrations of test sample/fractions ranging from 1.25-20 μM were added into the well and incubated for 48 h. A 50 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; 0.5 mg/mL] aliquot was added to each well 4 hours before the end of incubation. Medium and reagents were aspirated and 100 μL DMSO was added and mixed thoroughly for 15 min to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Spectra Max 340; Molecular Devices, CA, USA). Finally, IC₅₀ values were calculated. Doxorubicin was used as the positive control (Baydoun et al., 2013).

β-Glucuronidase assay

The β-glucuronidase assay was performed according to the method described previously, using p-nitrophenyl β-D-glucuronide as substrate (Collins et al., 1997). The enzyme mixture (total volume 0.25 mL) contained 50 mL of p-nitrophenyl glucuronide, 190 mL of acetate buffer, 5 mL enzyme and 5 mL of inhibitor. The assay mixture was incubated at 37°C for 40 min, the reaction was stopped by the addition of 50 mL of 0.2M Na₂CO₃, and the absorbance was measured at 405 nm. D-saccharic acid-1,4-lactone was used as a standard inhibitor. The percent inhibitory activity (%) was calculated using the formula; [(E-S)/EX100], where "E" is the

activity of enzyme without test material and "S" is the activity of enzyme with test material.

Chemicals and reagents

Boron trifluoride solution in methanol (10%) was purchased from Fluka Chemie (Buchs, Switzerland). Sodium hydroxide solution (methanolic; 0.5N) and sodium chloride (analytical grade) were obtained from Merck (Darmstadt, Germany), while methanol (HPLC grade) and *n*-hexane (HPLC grade) were from Fischer Scientific (Leicestershire, UK). Helium gas (purity 99.9999%) was procured from Pak Gas (United Arab Emirates). Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) were from Sigma-Aldrich (Germany).

Results

Phytochemical screening

The preliminary (qualitative) phytochemical tests on *T. govonianum* rhizome revealed the presence of secondary metabolites in methanol extract and its fractions, such as glycosides, steroidal saponins, tannins, sterols and flavonoids (Table I). Noteworthy, samples were very rich in steroids, steroidal glycosides and saponins.

Gas chromatography/mass spectrometry (GC/MS)

The proximate fatty acid composition of *n*-hexane fraction was carried out by GC/MS analysis. Twelve compounds were identified by comparison of *n*-hexane GC/MS spectra with the mass library search (NIST based AMDIS), as shown in Table II. Unsaturated fatty acids (70%) were more abundant than saturated fatty acids (30%). Among the unsaturated fatty acids, high

Table I

Preliminary phytochemical profile of *T. govonianum*

Phytochemical	Qualitative test	Methanol extract	<i>n</i> -Hexane fraction	Chloroform fraction	Ethyl acetate fraction	<i>n</i> -Butanol fraction
Alkaloids	Mayer's test	-	-	-	-	-
	Wagner's test	-	-	-	-	-
Glycosides	Keller Killiani test	+	-	+	+	+
Tannins	Ferric chloride test	+	-	+	-	+
	Lead acetate test	+	-	-	-	+
Flavonoids	Ferric chloride test	+	-	+	-	+
	Sodium hydroxide test	+	-	+	+	+
Carbohydrates	Molisch's test	+	-	+	+	+
Sterols	Liebermann-Burchard test	+	+	+	+	+
	Salkowski's test	+	+	+	+	+
Saponins	Frothing test	+	-	+	+	+

Table II

Chemical composition of *n*-hexane fraction of *T. govaniatum*

Name	Formula	Molecular Weight	R.T. (min)	Abundance (%)
2,4-Decadienal	C ₁₀ H ₁₆ O	152	18.11	2.07
Pentanoic acid-5-hydroxy-2,4-di- <i>t</i> -butylphenyl ester	C ₁₉ H ₃₀ O ₃	306	23.2	7.83
Ethyl 13-methyl-tetradecanoate	C ₁₇ H ₃₄ O ₂	270	33.59	6.53
Hexadecanoic acid methyl ester	C ₁₇ H ₃₄ O ₂	270	34.35	7.19
9-Hexadecenoic acid methyl ester	C ₁₇ H ₃₂ O ₂	268	35.15	9.65
2-Methyl hexadecanoic acid methyl ester	C ₁₈ H ₃₆ O ₂	284	35.58	15.00
9,12-Octadecadienoic acid methyl ester	C ₁₉ H ₃₄ O ₂	294	36.25	12.50
9,12-Octadecadienoic acid ethyl ester	C ₂₀ H ₃₆ O ₂	308	37.58	3.98
9,12-Hexadecadienoic acid methyl ester	C ₁₇ H ₃₀ O ₂	266	38.03	13.86
9-Octadecanoic acid methyl ester	C ₁₉ H ₃₆ O ₂	296	36.22	2.50
<i>cis</i> -13-Eicosenoic acid	C ₂₀ H ₃₈ O ₂	310	39.42	7.27
9,12-Octadecadienoic acid-2-hydroxy-1-(hydroxy methyl) ethyl ester	C ₂₁ H ₃₈ O ₄	354	43.25	4.53

*Name in bold is a saturated fatty acid

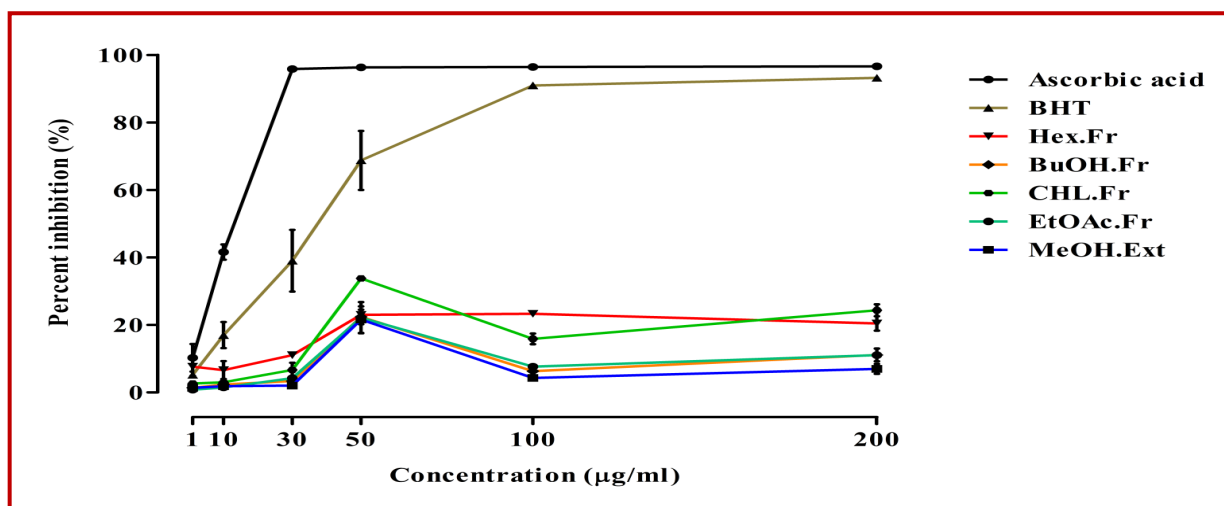


Figure 1: Percent of DPPH free radical scavenging activity of extract/fractions or standards (ascorbic acid and BHT). Results are mean \pm SEM of three different experiments

(C₁₉H₃₀O₃), 9-hexadecenoic acid methyl ester (C₁₇H₃₂O₂) and *cis*-13-eicosenoic acid (C₂₀H₃₈O₂) were detected, where as 2-methyl hexadecanoic acid methyl ester (C₁₈H₃₆O₂) and ethyl 13-methyl tetradecanoate (C₁₇H₃₄O₂) represented the saturated fatty acids present at higher concentrations.

DPPH free radical scavenging assay

Antioxidants from natural sources are effective in reducing the toxic effects in human due to xenobiotic exposure (Wong et al., 2006). DPPH free radical scavenging assay is considered as a standard method for the assessment of the antioxidant activity of pure compounds, natural extracts and fractions (Loo et al., 2007; Lue et al., 2010; Rufino et al., 2009). Our results indicated that a higher scavenging capacity was

measured in Hex-Fr and CHL-Fr (Figure 1 and 2) compared with the other fractions. This can be attributed to the presence of fatty acids, i.e. 9,12-octadecadienoic acid and hexadecanoic acid, in Hex-Fr, and glycosides, saponins and flavonoids in CHL-Fr, as previously reported on diverse plant species (Table II) (Bodoprost et al., 2007; Ha YL et al., 1990; Naga et al., 2012; Ying et al., 2014).

Anticancer assay

The cytotoxic activity of methanol extract and fractions against two cancer cell lines, HeLa (cervical cancer cells) and PC-3 (prostate cancer cells), was evaluated by MTT assay. All fractions exhibited cytotoxicity against both cancer cell lines (Table III). In particular, anticancer activity of chloroform fraction on HeLa cells was

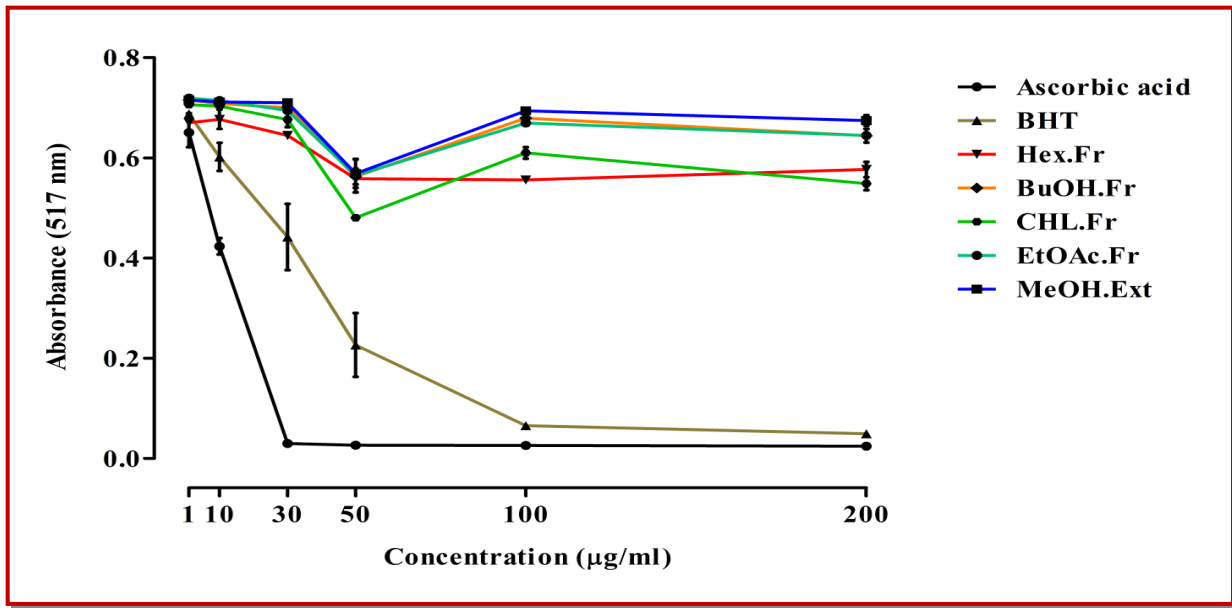


Figure 2: DPPH free radical scavenging activity of extracts/fractions or standards (ascorbic acid and BHT). Results are mean \pm SEM of three different experiments

slightly lower than that of doxorubicin, with IC_{50} of 0.8 ± 0.2 and 0.3 ± 0.0 , respectively. Similarly, this fraction was also most effective against PC-3 cells ($IC_{50} = 2.7 \pm 0.3$), though to a lesser extent than doxorubicin ($IC_{50} = 1.4 \pm 0.2$). Moreover, the butanol fraction, although possessed moderate cytotoxic effect against the HeLa cells ($IC_{50} = 1.6 \pm 0.3$) but was less effective in inhibiting the PC-3 cells ($IC_{50} = 4.0 \pm 0.3$).

β -Glucuronidase inhibitory assay

Based on the IC_{50} values ($\mu\text{g/mL}$) the methanol extract (140.8 ± 3.8) and butanol fraction (196.2 ± 1.9) exhibited a moderate level of enzyme inhibitory activity in comparison to the standard D-saccharic acid-1,4-lactone (Table IV).

Discussion

In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A lot of research has been conducted on exploring the phytochemical and pharmacological characteristics of medicinal plants to provide a scientific rationale to their folkloric uses (Adonizio et al., 2008; Saeed et al., 2010). Keeping in view, the importance of medicinal plants and folkloric uses of *T. govianum* rhizome, the present research was undertaken for the scientific exploration and validation of crude drug i.e. rhizome.

The phytochemical analysis of rhizome revealed the presence of secondary metabolites like steroids, glycolides and saponins, and these metabolites have been previously reported in the genus *Trillium* (Akihito et al.,

Table III

IC_{50} values of different extracts of *T. govianum* and doxorubicin against cancer cells

Extracts	IC_{50} ($\mu\text{g/mL}$)	
	HeLa cells	PC-3 cells
Methanol	3.1 ± 0.7	6.5 ± 0.5
Chloroform	0.8 ± 0.2	2.7 ± 0.3
Ethyl acetate	1.4 ± 0.1	5.1 ± 0.3
Butanol	1.6 ± 0.3	4.0 ± 0.3
Doxorubicin	0.3 ± 0.0	1.4 ± 0.2

Results are mean \pm SEM of three independent experiments; PC-3 = human prostate cancer cells; HeLa = human cervical cancer cells

Table IV

IC_{50} values of different extracts of *T. govianum* against β -glucuronidase

Extracts	IC_{50} ($\mu\text{g/mL}$)
Methanol	140.8 ± 3.8
Chloroform	>200
Ethyl acetate	>200
Butanol	196.2 ± 1.9
D-saccharic acid-1, 4-lactone	46.7 ± 2.2

Results are mean \pm SEM of three independent experiments

2008; Jiang et al., 2014), which includes species traditionally used in the treatment of different diseases by virtue of these phytochemicals (Abbasi et al., 2010; Andreana et al., 2002; Rahman et al., 2011; Ullah et al., 2013).

GC/MS analysis of *n*-hexane fraction showed the presence of saturated and unsaturated fatty acids, and thus *n*-hexane fraction represents the biologically active compounds with relevant antibacterial, antifungal and anticancer activities (Ching et al., 2008; Qiong et al., 2011). Therefore, the presence of these fatty acids in *T. govonianum* rhizome supports its potential uses as an antimicrobial and anticancer agent.

As the antioxidant activity of the extract as well as its fractions was lower than BHT and ascorbic acid. This low scavenging capacity of the extract or its fractions might be attributed to the presence of large sized fatty constituents as revealed from their phytochemical and GC-MS analyses. The DPPH assay is limited by steric accessibility, thus small molecules having low molecular weight have better access to the DPPH molecules than larger ones having high molecular weight and therefore possessed strong scavenging capacity (Bergeron et al., 2012). Moreover, there is a non-linear relationship between antioxidant activity and hydrophobicity with an increase of alkyl chain length results in low scavenging capacity (Laguerrre et al., 2013).

β -Glucuronidase enzyme has been found in animals, plants and bacteria. This enzyme catalyzes the hydrolysis of β -glucuronides conjugates of exogenous and endogenous compounds produced in the body (Fishman, 1974). From the sub-cellular fractionation studies of mammalian tissues, it was seen that β -glucuronidase is a typical lysosomal enzyme (Fishman, 1974). Increased level of β -glucuronides in blood has been seen in liver injury. Over expression of this enzyme may also be related to liver cancer. Similarly β -glucuronidase of intestinal bacteria in human and rats is correlated to colon cancer. In addition to this, β -glucuronidase of bacteria, which are found in the biliary tract is also associated with gall stone formation (Dong et al., 1999; Muhammad et al., 2002).

So far, a number of anticancer metabolites have been reported in the genus *Trillium* (Jiang et al., 2014; Ono et al., 2007; Ono et al., 1986) including steroidal glycosides and saponins isolated from *T. erectum* and *T. tschonoskii* (Akihito et al., 2008; Zhao et al., 2011). As the phytochemical analysis revealed the presence of steroidal glycosides, flavonoids and saponins, therefore the potent anticancer and moderate β -glucuronidase inhibitory activity in extract and fractions of *T. govonianum* rhizome may be attributed due to the presence of these secondary metabolites. Therefore, the rhizomes of this plant specie may be effective in the treatment of prostate and cervical cancers and also in the management of liver and colon cancers associated with an increase activity of β -glucuronidase.

Conclusion

To the best of our knowledge, this is the first report on

the phytochemical analysis, antioxidant, β -glucuronidase inhibitory and anti cancer activities of this plant species. These findings indicated that *T. govonianum* rhizome possesses a promising potential as anticancer medicinal plant, even if further studies, now in progress, are necessary to isolate active constituents responsible for the observed efficacy.

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Conflict of Interest

The authors declare no conflict of interest.

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