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Research Article

EFFICIENT HYDROALCOHOLIC EXTRACTION FOR HIGHEST DIOSGENIN CONTENT FROM *TRILLIUM GOVANIANUM* (NAG CHHATRI) AND IT'S *IN VITRO* ANTICANCEROUS ACTIVITY

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ABSTRACT

Objective: The present study involves hydroalcoholic extraction of *Trillium govanianum* (Hindi name: *Nag chhatri*), which is a high-value medicinal plant found at the altitude of 2500-4000 m. Aiming in this direction, we performed hydrolysis of extract using response surface methodology (RSM) for optimizing diosgenin content. The extracts were evaluated for cytotoxicity.

Methods: In RSM, the cumulative effect of independent variables including time (minutes), temperature (°C), and solid-liquid ratio (g/ml) were investigated through central composite design. Cytotoxicity studies of crude rhizome and hydroalcoholic extract were carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on three cell lines, *viz*., MDCK, MCF-7, and MDA-MB-23.

Results: The diosgenin content obtained was 5.9%, which is reported for the first time in *T. govanianum.* Hydrolyzed extract showed less toxicity in MDCK (normal cell line) cells but significantly reduced the proliferation of MCF-7 and MDA-MB-231 cancer cells.

Conclusion: Hydrolysis method was optimized by RSM, which proved to be an efficient method for extraction of diosgenin from *T. govanianum*. Hydrolyzed extract showed antiproliferative activity on cancer cell lines with minimal effect on normal cells.

Keywords: *Trillium govanianum*, Diosgenin, *Nag chhatri*, Hydrolysis, MDCK, MCF-7, MDA-MB-231.

INTRODUCTION

The mountain region in India is spread over 13 states in the Indian Himalayan Region (IHR) covering a geographical area of 500,000 km^2. Over 51 million people (6% of total population of India) live in the IHR [1]. These millions of people rely on medicinal plants for their primary health care, income generation, and livelihood options. Medicinal plants are not only a major resource base for the traditional medicine and herbal industry but also provide livelihood and health security to a large segment of Indian population. Such increasing demand creates pressure on natural population and due to which many species declined in their number and abundance and entered into various degrees of threat [2].

Keeping in view the importance of high-value medicinal plant, the present study was carried out on *Trillium govanianum* Wall. Ex D. Don (Hindi name: *Nag chhatri*, Family: Trilliaceae) and found at Himalayan ranges from Pakistan to Bhutan at 2400-3500 AMSL is taking the front seat than all other medicinal plants of Himachal Pradesh [3]. The plant is a small herb preferring shady areas with stocky 15 g-25 cm purple-red stem carrying plain broadly ovate green leaves, powerfully deflexed, deep oxblood red and green flower at the apex. The underground part of the plant, i.e. rhizome is key material of trade containing trillarin which on hydrolysis yield diosgenin and used in preparation of steroidal and sex hormones [4]. The IHR is paradise for plants that can be used medicine and cultural practices, but the fear is that relentless smuggling will make them extinct before they can be cataloged and researched. The extraction and sale of such valuable species are banned by Indian law but for villagers who extract the plant is the sole source of income in summer months. However also, there are reports in daily newspaper for their illegal collection and trading.

Diosgenin is often used as a raw precursor for steroidal drug production such as testosterone, glucocorticoids, progesterone, also used in rheumatism, regulation of menstrual flow, and many more [5,6]. Plant is also reported to have govanoside, new steroidal saponins, and other components such as borassoside and pennogenin, which were explored further for antioxidant and anticancer activities [7,8]. These secondary metabolites produced in this species are analyzed specially for diosgenin, which is produced through steroid biosynthetic pathway. At present, natural diosgenin is from *Trigonella* species and the diosgenin content varies from 0.01% to 0.5% in aerial parts and seeds [9]. Tubers of Mexican yam (*Dioscorea* spp.) are also reported to have diosgenin contents as low as exemplify as with *Dioscorea polygonoides* (0.2%); [10] *Dioscorea althaeoides* (0.2-2.3%); *Dioscorea prazeri* (1.92%); *Dioscorea villosa* (1.3%); 2 years old *Dioscorea zingiberensis* (0.18-0.55%), several *Dioscorea* species (0.04-0.93%), and among others [11]. Production of diosgenin from Mexican yam is both time consuming and costly because yam tubers require 3 years to grow in mature size, and then, diosgenin can be extracted for use in commercial and pharmaceutical reagents [12-14]. Moreover, these raw materials are in short supply and hence developing the production of *Trigonella* and *Dioscorea* in every way; it is necessary to search for other plants as a source of steroids. Hence, *T. govanianum* can serve as an alternative for the production of diosgenin because of its shorter growing cycle, higher production [15]. Since the total content for diosgenin has not been reported in this very plant, so we need to explore the best protocol for the extraction of diosgenin from of dried rhizomes of *T. govanianum*. Therefore, it is necessary to establish an optimum extraction procedure for diosgenin which is less time-consuming and capable of detecting the true optimum conditions, especially due to the interactions among the factor [16]. The extraction protocol optimization has been widely performed in this study by using response surface methodology (RSM) [17,18]. The extraction efficiency is totally dependent on many factors, viz., solvent concentration, extraction time, temperature [19,20]. This statistical method was first introduced by Box and Wilson [21], and thereafter, considered as a most convenient method for optimizing processes with various conditions and simultaneously reduce the cost of production.

Thus, the aim of the present study was to optimize the best-suited method for the extraction of diosgenin from *T. govanianum* using RSM and evaluate its *in-vitro* cytotoxicity using MDCK, MCF-7, and MDA-MB231cell lines.

METHODS

Plant material and extraction procedure

Plants of *T. govanianum* were collected from Chamba region (altitude: 3500 m) of Himachal Pradesh, in the month of August 2014. The herb was identified by botanist Sheikh Gulzaar (JK Medicinal Plants Introduction Centre, Pampore; Jammu and Kashmir). The fresh rhizomes of *T. govanianum* were washed under running tap water, shade dried, and then grounded and subjected to hydroalcoholic (30:70; water:methanol) Soxhlet extraction for 72 hrs. Extracts were filtered while hot, concentrated under reduced pressure using rotary evaporator followed by lyophilization. Extractive yields were calculated, and extracts were stored at 4°C until used further.

Statistical optimization for maximum diosgenin content

To examine the highest diosgenin content, we took various extraction parameters, viz., incubation time (minutes), temperature (°C), and solid-liquid ratio (g/ml) and recorded their cumulative effect by employing RSM using a statistical software package Design Expert 10.0.0.3, Stat-Ease, Inc. A 2^3 full factorial central composite design (CCD) with 8 trials for factorial design, 6 trials for axial point, and 6 replicate trials at the central point, leading to a set of 20 experiments was designed. The response value from each experiment of CCD was the average of triplicates.

Hydrolysis and quantification for diosgenin by high-performance liquid chromatography (HPLC)

Extract hydrolysis was performed in slightly different methods as described by Drapeau *et al*. [22]. Extract volume (solid-liquid ratio), time (in minutes), and temperature (°C) were taken into consideration. Briefly, solid-liquid ratio ranging from 1:100 to 1:200 of extract was hydrolyzed in 150 ml of refluxing 20% H_2SO_4 in 70% isopropanol for 0-16 hrs at temperature ranging from 70-100°C. The extract was then filtered and extracted with hexane (50 ml × 3). The three hexane extracts were combined and rinsed thrice times with 5% alkali and then rinsed thrice with distilled water. The extract was then passed through a column of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at 40°C in a rotary evaporator. Rhizome samples were then fractionated with hexane in separating and funnel followed by freezing them in −80°C. Samples were lyophilized and then dissolved in MeOH for HPLC analysis. Diosgenin as standard was purchased from Chromadex, Bengaluru. A standard stock solution of diosgenin was prepared in HPLC grade methanol and then working solution was made from that.

Chromatographic conditions for HPLC

Chromatographic analysis was carried out with reverse-phase HPLC (Agilent 1200 series) equipped with HPLC pump, DAD photodiode array detector range from 190 to 800 nm. Diosgenin was estimated with a Zorbax-Eclipse XBD C-18 3.5 µm (4.6 mm × 150 mm). Solvent system used in a gradient mode to run the samples, i.e. 0.2% formic acid with HPLC grade water and methanol in a ratio of 30:70 (v/v). The column was eluted in the gradient mode with a flow rate of 0.8 ml/min. The diosgenin was detected at absorbance of 230 nm wavelength. The cycle time of analysis was 30 minutes at 25°C with injection volume of 10 µl. At the end of each run, the column was rinsed with pure solvents. The compounds were identified by retention time and comparison of ultraviolet spectra with the authentic standard from ChromaDex, Inc.

Cell lines and culture

MDCK, MCF-7, and MDA-MB-231 were obtained from the NCCS, Pune. Cells were grown in T25 culture flasks containing Dulbecco's modified Eagle medium for MDCK and MCF-7 likewise L-15 (Leibovitz) for MDA-MB-231 supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). MDCK and MCF-7 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. On reaching confluency, the cells were trypsinized and passaged.

Cytotoxicity studies

The cytotoxicity studies were performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to a well-established method with slight modification at laboratory conditions [23,24] on three cell lines (MDCK, MCF-7, and MDA-MB-231). The cells line were seeded at a density of 1×10^4 cells/well in 96-well plate and incubated at 37°C till confluence reached 70-80%. The cells were then treated with different concentrations of crude extract and 8 hrs hydrolyzed extract (1 ng/ml, 500 ng/ml, 1 µg/ml, 500 µg/ml, and 1 mg/ml) were incubated for 24 hrs. After stipulated period of treatment, MTT (40µl) was added to each well and further incubated at 37°C for 4 hrs. Metabolically active cells were able to reduce MTT with the help of enzyme succinate dehydrogenase and form purple-colored insoluble formazan. Dimethyl sulfoxide (100 µl) was added to each well to solubilize the formazan crystal and kept at room temperature for 15-20 minutes. Absorbance (A) was taken at 570 nm as test wavelength and 630 nm as reference wavelength to evaluate cell cytotoxicity using microplate reader (Bio-Rad). Triplicate wells were assayed for each condition and standard deviations were determined.

A decrease in absorbance in this assay measures the extent of decrease in the number of viable cells on exposure to the test substances. The cell cytotoxicity was calculated using the following formula:

$$\% \text{ cell cytotoxicity} = \left[\frac{\text{Abs.test}}{\text{Abs.control}} \times 100 \right] \quad (1)$$

Acridine orange (AO) and ethidium bromide (ETBR) live-dead staining

Further, the best-selected concentration was analyzed for its cell morphological changes in MDCK, MCF-7, and MDA-MB-231 cell line by dual AO and ETBR staining. Briefly, cells were seeded at a density of 2×10^5 cells/well in 6-well plate comprising 1 ml of growth medium. Till the confluence reached 70-80% cells were incubated at 37°C in CO_2 incubator. Cells were then treated with the best-selected concentration and incubated for 24 hrs. After desired period, the cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 4% of paraformaldehyde for 30 minutes. Afterward, the supernatant was discarded, and cells were treated with 1% triton ×-100 for 15 minutes followed by washing of cells with PBS. Cells were stained with dye mixture; comprising 5 µl of AO (1 mg/ml) and 5 µl of ETBR (1 mg/ml) for 5 minutes and then washed thrice with PBS. The stained cell with morphological changes was observed by fluorescence microscope at ×200 (Nikon Eclipse-80i, Japan).

RESULTS AND DISCUSSION

Extraction from rhizomes

Hydroalcoholic (30:70) Soxhlet extraction is one of the best techniques to prepare plant extract, which has been widely used globally [25]. Extraction was successfully carried in Soxhlet apparatus, and total extractive yield of raw hydroalcoholic extract was found to be 42.2%.

Hydrolysis and quantification for diosgenin by HPLC

Diosgenin is an important bioactive chiral compound originated from *Dioscorea* spp., and *Trigonella* spp. has many applications in steroidal pharmaceutical industry. However, due to ever-rising pollution and low extraction yield during hydrolysis of plants materials by unfavorable conventional methods, the demand of diosgenin is increasing day by day. Extraction of diosgenin is reported from *Dioscorea* spp., in which they found that concentration of diosgenin in two Indian species, *viz*., *Dioscorea deltoidea* and *D. prazeri* ranges from 0.32% to 1% [26]. Reports are lying with the new approach for the cleaner and more efficient extraction of diosgenin from the tubers of *Dioscorea zingiberensis* by extraction of glycosides from plant materials, and their

hydrolysis combined with the extraction of diosgenin in one step, which has greatly simplified the process. Then, in the second step, biphase immiscible solvent system composed of non-polar polyethylene terephthalate can maintain the stability of diosgenin and thus make its extraction feasible [27].

At present times, some other yams including tuber of *Dioscorea* sp. and *Trigonella* sp. are an important source of diosgenin [5,28]. Yams with high diosgenin content are grown for steroid preparation. Out of 26 *Dioscorea* available in India, *D. prazeri* and *D. deltoidea* are used to manufacture diosgenin [29]. Edible yams are having very low concentration of diosgenin, and the study has reported that the highest content was observed the left twining species of *Dioscorea bulbifera* (1.35 %) followed by *Dioscorea hispida* (0.825%) and *Dioscorea pentaphylla* (0.818%) [30]. Among right twining species, *Dioscorea oppositifolia* contains the highest amount in diosgenin, i.e., 0.658%, and among others, *Dioscorea alata* has the lowest amount of diosgenin, i.e., 0.095% ascended by *Dioscorea belophylla* (0.12%) and *Dioscorea wallichii* (0.129%). All compound leaves *Dioscorea* contain higher amount of diosgenin. It is observed that *Dioscorea* species with higher diosgenin content are less tasty, and their content varies from species to species [31].

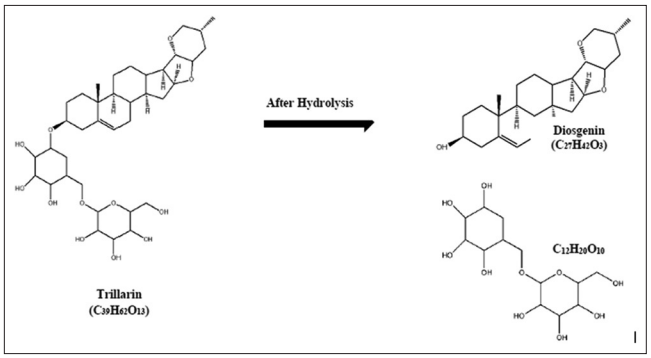

Fig. 1: A schematic hypothesized view for production of diosgenin from trillarin after hydrolysis

Trigonella has also received considerable attention as a source of diosgenin [32]. Aerial parts of *Trigonella spicata*, *Trigonella anguina,* and *Trigonella caerulea* showed a higher level of diosgenin as compared to *Trigonella foenum-graecum* [9] Hypothesized schematic representation is showed in Fig. 1. The highest diosgenin levels were observed in *T. spicata*. However, none of the species are commercially viable for diosgenin production and thereby search for new plant variety is needed to be introduced in drug market. Our report for *T. govanianum* having diosgenin is much higher than other species containing diosgenin. The hydrolysis was performed under optimized condition by RSM, and the optimum diosgenin content was recorded as 5.99%. The HPLC chromatogram is provided in Fig. 3.

Analysis of RSM

The three factors and lower, middle, and upper design points for RSM along with the experimental results of diosgenin content (%) are shown in Table 1. A polynomial quadratic equation for the total diosgenin content (%) was predicted from multiple regression coefficient determined by employing least squares technique. The polynomial equation was Diosgenin content (%) = 4.97 + 2.27A − 0.22B + 0.70C − 0.14AB + 0.14AC + 0.061BC − 1.89A^2 − 10.94B^2 − 0.25C^2

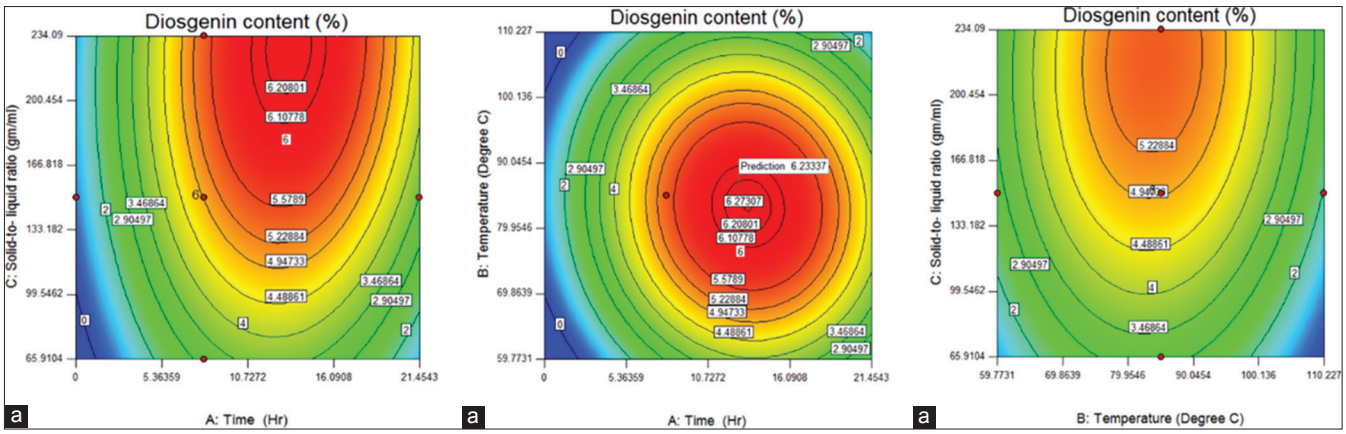

Fig. 2: Effect of (a) time and temperature, (b) time and solid-liquid ratio, and (c) temperature and solid-liquid ration on the yield of diosgenin (%)

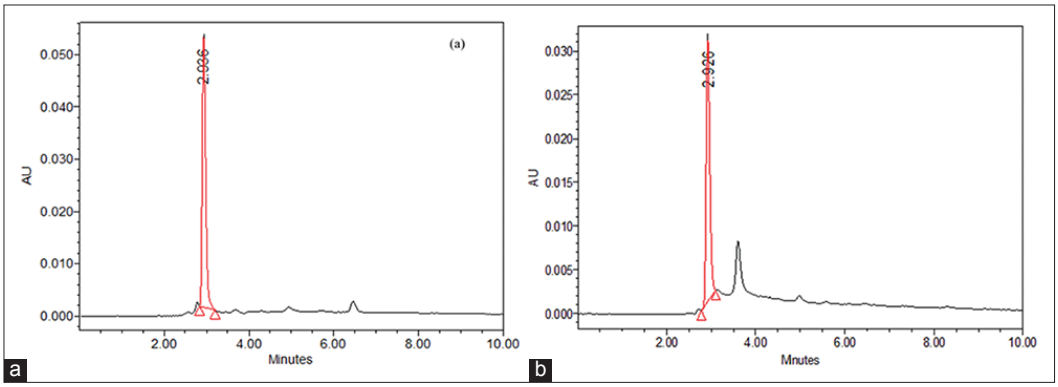

Fig. 3: Chromatogram of (a) standard diosgenin was detected at 2.9 minutes retention time and (b) chromatogram of 8 hrs hydrolyzed rhizome samples of *Trillium govanianum*

Table 1: Factors and levels in the response surface CCD arrangement along with the experimental and predicted results for diosgenin content (%)

No.	X_1	X_2	X_3	Experimental	Predicted
1	0	100	200	0	0.16
2	21.45	85	150	2.2	3.45
3	0	70	100	0	0
4	8	85	150	5.1	4.96
5	8	59.77	150	2.1	2.67
6	8	85	150	5.1	4.96
7	8	85	65.91	1	3.09
8	8	85	150	5.1	4.96
9	16	70	200	5.3	5.29
10	0	85	150	0	0.80
11	8	110.22	150	1	1.96
12	8	85	234.09	5.99	5.43
13	16	70	100	4.99	3.74
14	16	100	200	5	4.71
15	8	85	150	5.1	4.96
16	8	85	150	5.1	4.96
17	0	70	200	0	0.19
18	0	100	100	0	0
19	16	100	100	4.2	2.91
20	8	85	150	5.1	4.96

X_1=Incubation time (hr); X_2=Temperature (°C); X_3=Solid-liquid ratio (g/ml)

Where, A is incubation time (hr.), B is temperature (°C), and C is solid-liquid ratio (g/ml). The model equation was statistically significant as determined by analysis of variance. The level of variability in the model was explained by coefficient of determination (R^2), which was found to be 0.87 in our model. It has been reported that the R^2 should be >0.75 indicated that our model equation was significant [18].

The cumulative effect of the selected variables, i.e. incubation time, temperature, and solid-liquid ratio are represented as contour plots (two-dimensional) as shown in Fig. 2. From Fig. 2a, the cumulative effect of incubation time and temperature revealed that total diosgenin content was minimum at low and high levels of incubation time and temperature. The contour plot showed that the maximum yield of diosgenin was obtained when hydrolysis was carried out at 85°C for 12 hrs. In contrast to this, (Fig. 2b) showed maximum diosgenin content at high levels of solid-liquid ratio while minimum at low and high levels of incubation time. From (Fig. 2c), it was concluded that when temperature was at a certain value, the diosgenin content increased with the increase of solid-liquid ratio. Further, the experimental values determined by the experiments were in close agreement with the statistically predicted values which validated the experimental model. Thus, using RSM optimized conditions (incubation time: 12 hrs, temperature: 85°C, and solid-liquid ratio: 1:234 g/ml), the maximum diosgenin content was 5.99% which was higher than the corresponding value of 2.1% obtained under un-optimized condition (incubation time: 8 hrs, temperature: 60°C, and solid-liquid ratio: 1:150 g/ml). Thus, RSM was successfully used to determine the optimum extraction conditions for diosgenin, and the results are in congruence with the previous reports showed a significant increase in metabolite content using RSM [17,20].

Effect of *T. govanianum* extract on cell viability

Crude rhizome extract of *T. govanianum* showed less toxicity on MDCK cells (Fig. 4a) with inhibitory concentration 50% (IC_{50}) value of 2.1 µg/ml, and cell viability of MCF-7 and MDA-MB-231 cells were having IC_{50} value 2.03 and 1.8 µg/ml, respectively. Likewise, hydrolyzed extract of *T. govanianum* was less toxic in MDCK cells (Fig. 4a) with an IC_{50} value of 1.7 µg/ml but significantly reduces proliferation of MCF-7 (Fig. 4b) and MDA-MB-231 cells (Fig. 4c) with IC-50 value 1.11 and 0.495 µg/ml, respectively. This suggests that the extract is less toxic against normal cells but showed a significant antiproliferative activity on cancer cells (MCF-7 and MDA-MB-231). On the contrary, hydrolyzed extract is also having a less toxicity toward normal cells were as considerably reduces cancer cell viability. Cytotoxicity of diosgenin has

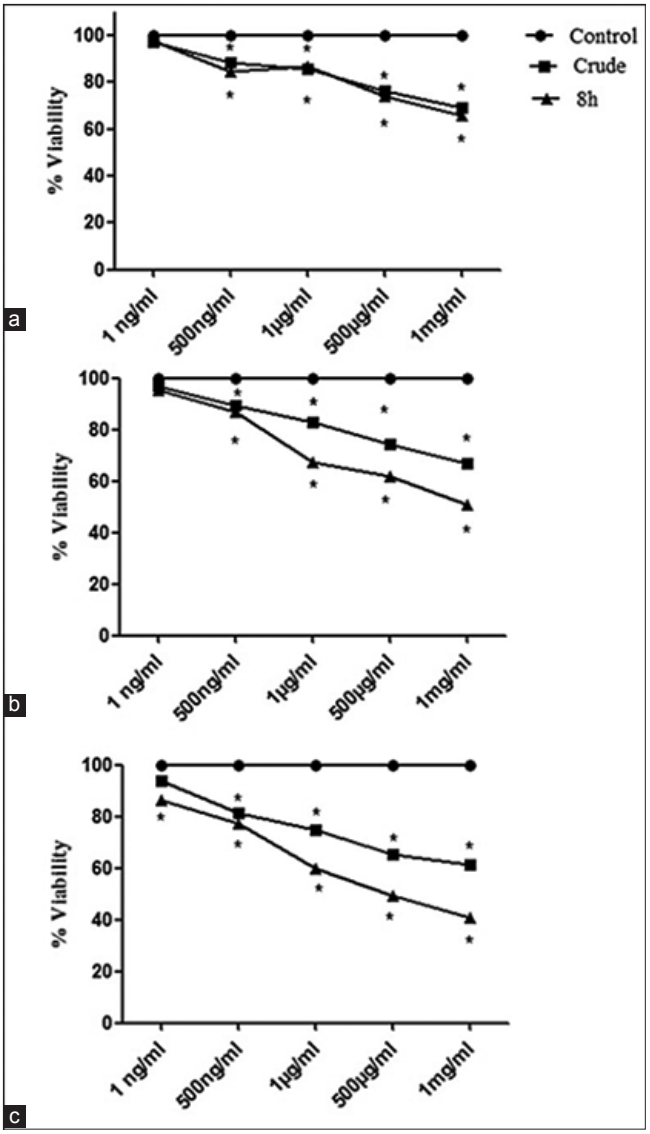

Fig. 4: Inhibition of proliferation in MDCK (a), MCF-7 (b), and MDA-MB-231 (c) cells after treatment. Results are presented as percentage of control (Untreated cells), and values are expressed as means ± standard deviation of six experiments (*p value relative to control group, p<0.05)

been previously established on many cell lines such as V79 fibroblast and K562 cells [33,34]. Our results were consistent to previously reported findings, which did not show any significant toxicity on MDCK but comparatively effective on MCF-7 and MDA-MB-231. This may be due to the presence of diosgenin and other steroidal, saponins compounds in *T. govanianum* as they have been reported previously for their anticancer activity [7].

Cell and nuclear morphology

Morphological analysis for MDCK, MCF-7, and MDA-MB-231 cells was performed, and photographs were taken under a fluorescence microscope at 200 X (Nikon Eclipse-80i, Japan). The cells were treated with respective IC_{50} value of crude extract and 8 hrs hydrolyzed extract concentration for 24 hrs and further compared with untreated cells using AO/ETBR dual staining. AO/ETBR is generally used to examine nuclear morphological changes in apoptotic cells. AO dye is well reported, as it binds to both live and dead cells, were as ETBR stain those cells which lose their membrane integrity. Cell stained with green fluorescence represents the viable cells, whereas the yellow staining signifies early apoptotic cells, and orange cell denotes late apoptotic

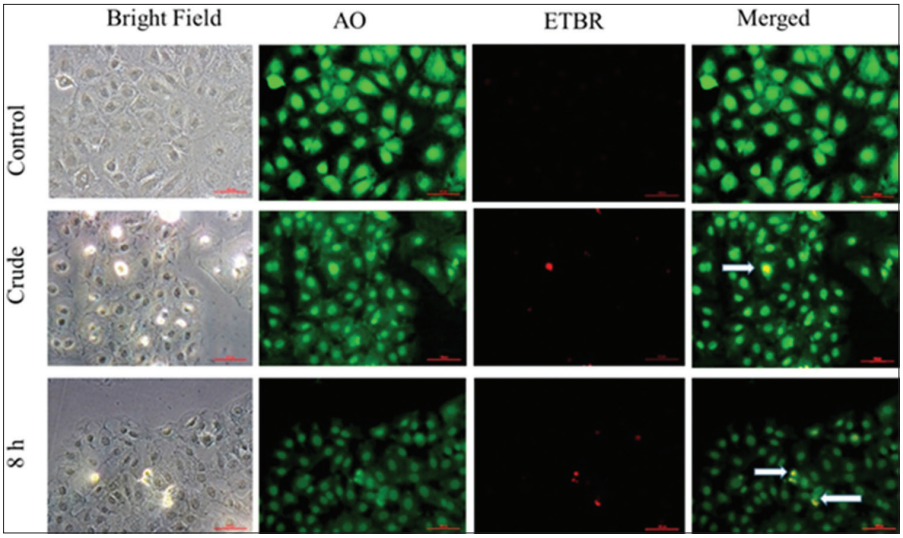

Fig. 5: Effect of *Trillium govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDCK cells through acridine orange/ethidium bromide staining, viewed under a fluorescence microscope (×200)

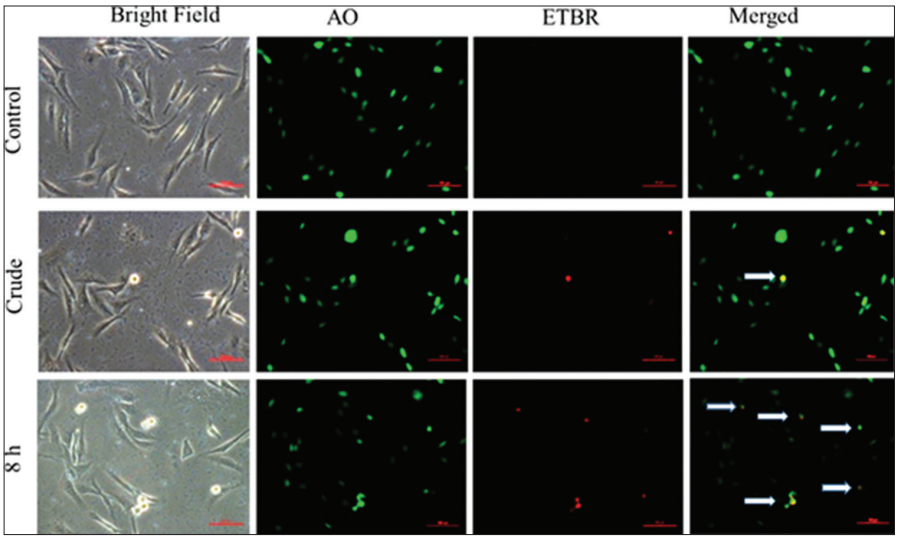

Fig. 6: Effect of *Trillium govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDA-MB-231 cells through acridine orange/ethidium bromide staining, viewed under a fluorescence microscope (×200)

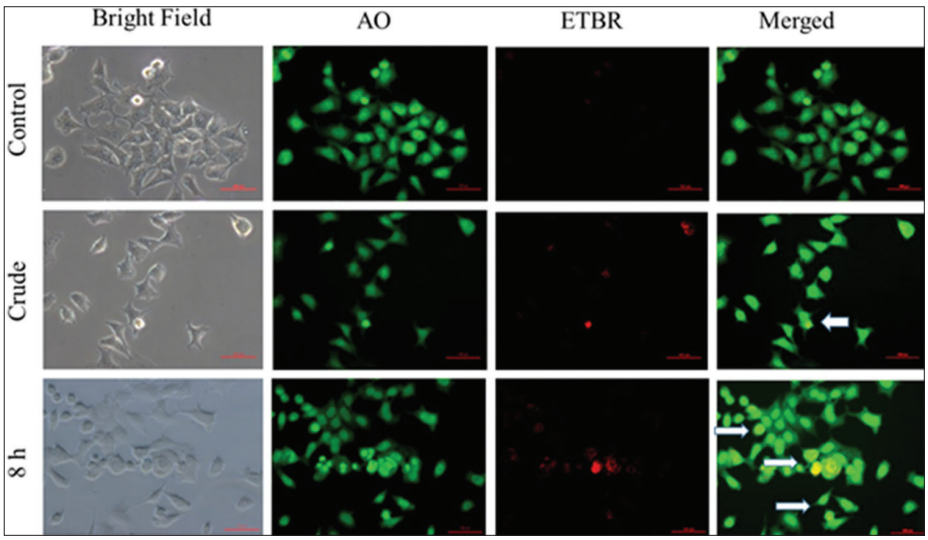

Fig. 7: Effect of *Trillium govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MCF-7 cells through acridine orange and ethidium bromide staining, viewed under a fluorescence microscope (×200)

cells. Likewise, in untreated control cells, uniform green fluorescence was observed which signifies the cell with normal nuclear morphology, whereas in treated cells yellow and orange stains were observed (Figs. 5-7).

Till now, a number of secondary metabolites from *Trigonella* and *Trillium* genus have been reported to possess anticancer activity [35]. These anticancer metabolites include steroidal glycosides, saponins, and flavonoids [36-39]. Moreover, we also observed the decline in cell density from normal cells to cancer cells which further suggests that hydrolyzed extract induced apoptosis in breast cancer cells. AO/ETBR fluorescent staining is widely used technique to detect cellular morphology, apoptosis and to differentiate between normal and dead cells [40]. These results demonstrate that this species can be an alternative source for novel anticancer lead molecules.

CONCLUSIONS

T. govanianum is an indigenous medicinal herb of the Indian subcontinent and is illegally uprooted for its steroidal content. It can be concluded from the observed results that rhizomes of *T. govanianum* contain a high amount of diosgenin (5.99%) which was obtained from hydrolysis of hydroalcoholic extract. The best method for getting the highest diosgenin content was found to be 12 hrs hydrolysis at 85°C with sold-liquid ratio of 1.234 g/ml. Crude and hydrolyzed extract did not show any toxicity in normal cells. However, the hydrolyzed extract showed significant anticancer activity against MCF-7 and MDA-MB-231 cells, which was not pronounced for crude extract.

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