ANALYSIS OF DIOSGENIN CONTENT AND PHARMACOLOGICAL EXPLORATION OF ENDANGERED MEDICINAL HERB- *TRILLIUM GOVANIANUM* (NAG CHHATRI)

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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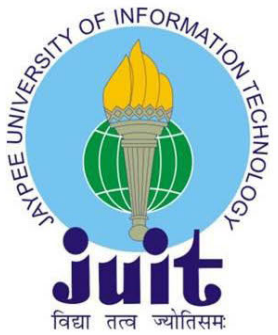

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

MARCH 2017

DECLARATION

I certify that:

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor, Dr. Hemant Sood and Prof.(Dr.) Rajinder Singh Chauhan
b. The work has not been submitted to any other organization for any degree or diploma.
c. Wherever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

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CERTIFICATE

This is to certify that the thesis entitled, **"Analysis of diosgenin content and pharmacological exploration of endangered medicinal herb- *Trillium govanianum* (Nagchhatri)"** which is being submitted by **Shivam Sharma (Enrollment No. 126558)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by him under our supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Shivam Sharma

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LIST OF ABBREVIATIONS

IUCNNR	International Union for Conservation of Nature and Natural Resources
GC-MS	Gas chromatography mass spectrometry
RSM	Response surface methodology
WHO	World Health Organization
1H-NMR	Proton Nuclear Magnetic Resonance
13C- NMR	Carbon 13Nuclear Magnetic Resonance
COSY	Corelation spectroscopy
NOESY	Nuclear Overhauser effect spectroscopy
HSQC	Heteronuclear single quantum coherence spectroscopy
HMBC	Heteronuclear multiple bond correlation
HR-FAB	High resolution fast atom bombardment
IR	Infrared spectroscopy
UV	Ultraviolet spectroscopy
FAB	Fast atom bombardment
26S	26S rRNA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase
SQS	Squalene synthase
FPPS	Farnesyl pyrophosphate
BETA	26-O- beta- glucosidase
CAS	Cycloartenol synthase
PMK	Phosphomevalonate kinase
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
DXPR	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
ISPD	2-C-methylerythritol 4-phosphate cytidyl transferase
ISPE	4-(Cytidine-5-diphospho)-2-C-methylerythritol kinase
CNS	Central nervous system

IGF	Insulin like growth factor
MDR	Multidrug resistance
HCC	Hepatocellular carcinoma
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
MS	Murashige and Skoog medium
BAP	6- Benzylamino purine
NAA	1- Napthaleneacetic acid
IPP	Isopentenyl diphosphate
GPP	Geranyl diphosphate
FPP	Farnesyl diphosphate
UDP	Uridine diphosphate
MDCK	Madin-Darby Canine Kidney Epithelial Cells
MCF-7	Michigan Cancer Foundation-7
NCCS	National Center for Cell Science
CCD	Central composite design
MSA	Multiple sequence alignment
PCR	Polymerase chain reaction
dNTPs	Deoxynucleotide Solution Mix
EDTA	Ethylenediaminetetraacetic acid
BLAST	Basic local alignment search tool
NR	Non- redundant Database
NCBI	National Center for Biotechnology Information
RT-qPCR	Real time quantitative PCR
ANOVA	Analysis of variance
DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
MTT	3-)4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMSO	Dimethyl sulfoxide
AO	Acridine orange

EtBr	Ethidium Bromide
PBS	Phosphate buffer saline
CPCSEA	Control and Supervision of Experiments on Animals
CMC	Carboxymethylcellulose

ABSTRACT

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Trillium govanianum Wall. ex. D. Don, is a high value medicinal herb of North-Western Himalayan regions of India having plethora of medicinal properties like Cancer, Sexual disorders, GI disorders, skin infections, Anthelmintic, etc. The rhizome part of the plant containing trillarin is of commercial value to pharmaceutical industry. Diosgenin which is a phytoestrogen can be chemically converted into antifertility drugs having their steroidal base such as progesterone, corticosteroids and anabolic steroids. This species has high folkloric value and rhizome of this plant species are used for treating wounds, dysentery, skin boils, infections, menstrual and sexual disorders. Over exploitation of these species necessitates the development of conservation strategies and enhanced production of diosgenin at commercial scale.

Since limited studies have been carried on *T. govanianum* rhizome and soxhlet extraction and optimization, we studied in detail and devised a novel methodology for the same. The detailed study was carried on getting the highest extractive yield of 43.56% (30:70; Water: Methanol) using different solvent systems depending upon eluotropic series w.r.to maximum diosgenin content (2.4 %). Finally by using response surface methodology (RSM) best condition viz. incubation time for hydrolysis (12 hr), temperature (85^0C) and solid- liquid ratio (234.09 gm/ml) were optimized giving maximum diosgenin content (5.99 %).

Molecular basis of diosgenin biosynthesis was explored in *T. govanianum* for which, partial gene sequences of 5 genes encoding key enzymes viz. HMG Co-A reductase (*HMGR*), squalene synthase (*SQS*), farnesyl pyrophosphate synthase (*FPPS*), 26-O-beta-glucosidase (*BETA*) and cycloartenol synthase (*CAS*), involved in diosgenin biosynthesis pathway were cloned through comparative genomics. The RT-qPCR analysis revealed that all five genes viz. *HMGR*, *FPPS*, *SQS*, *BETA* and *CAS* involved in diosgenin biosynthesis had relatively higher expression in the rhizomes of Chamba (2.4%) (upto 3.2 folds) ($p<0.05$) as compared to Lahaul and Spiti (0.7%).

Biological activities of crude extracts for toxicity were investigated on cancer cell lines and anti-fertility studies on female rat model. Crude rhizome extract of *T.govanianum* showed less

Biological activities of crude extracts for toxicity were investigated on cancer cell lines and anti-fertility studies on female rat model. Crude rhizome extract of *T.govanianum* showed less toxicity on MDCK cells (Normal cell line) with IC_{50} value of 2.1 µg/ml, compared to cell viability of MCF-7 and MDA-MB-231 cells (breast cancer cell lines), which showed IC_{50} values of 2.03 and 1.8 µg/ml, respectively. Likewise, hydrolyzed extract of *T. govanianum* was observed to be less toxic towards normal MDCK cells, with IC_{50} value of 1.7 µg/ml. A significant reduction in the proliferation of MCF-7 and MDA-MB-231 cells was observed with IC_{50} values of 1.11 and 0.495 µg/ml, respectively. These results suggest that extract is less toxic against normal cells but showed a significant anti-proliferative activity on cancer cells (MCF-7 and MDA-MB-231). TG extract showed very strong dose dependent anti-implantation effect. Number of implantation sites was significantly reduced in animals treated with 125 mg/kg extract and none of the females was observed to have any implants in the uterus when treated with 250 mg/kg extract. Extract treatment resulted in 94.87% and 100% inhibition of implantation at 125 and 250 mg/kg dose respectively. Hence, the current study has revealed the potentiality of herbal extract of *T. govanianum* for anti-fertility thus proving to be novel effective herbal contraceptive supplemented with anti-cancerous effect.

CHAPTER 1

INTRODUCTION

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Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other are used for medical purposes (Levine, 2000). It is estimated that world market for plant derived drugs accounts for nearly Rs. 3,00,000 crores in which Indian contribution is about Rs. 3,000 crores approximately. It has been estimated that in developed countries such as United States plant drugs constitute as much as 30% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80% (Farnsworth and Soejarto, 1991). Thus, the economic importance of medicinal plants is much more in developing countries compared to rest of the world. Amongst many useful herbs, *Trillium govanianum* Wall. ex D. Don, popularly known as Nagchhatri (Trilliaceae), is a high value medicinal herb found in North-West Himalayan region of India. It is a threatened plant species found at an altitude of 2400-3500 m above msl (Vidyarthi et al. 2013) (Fig. 1). The plant is a small herb which propagates through roots and seeds. The underground part of the plant, i.e. rhizome is key material of trade containing trillarin, which on hydrolysis yields diosgenin – a corticosteroid hormone (Fig. 2). The cortico-steroid hormone isolated from the plant is used in various preparations like sex hormones; cortisone and allied preparations used in rheumatism, birth control, regulation of menstrual flow and in stomach related problems (Chauhan 1999).

Fig. 1.1. Mature field grown plants of *T. govanianum*

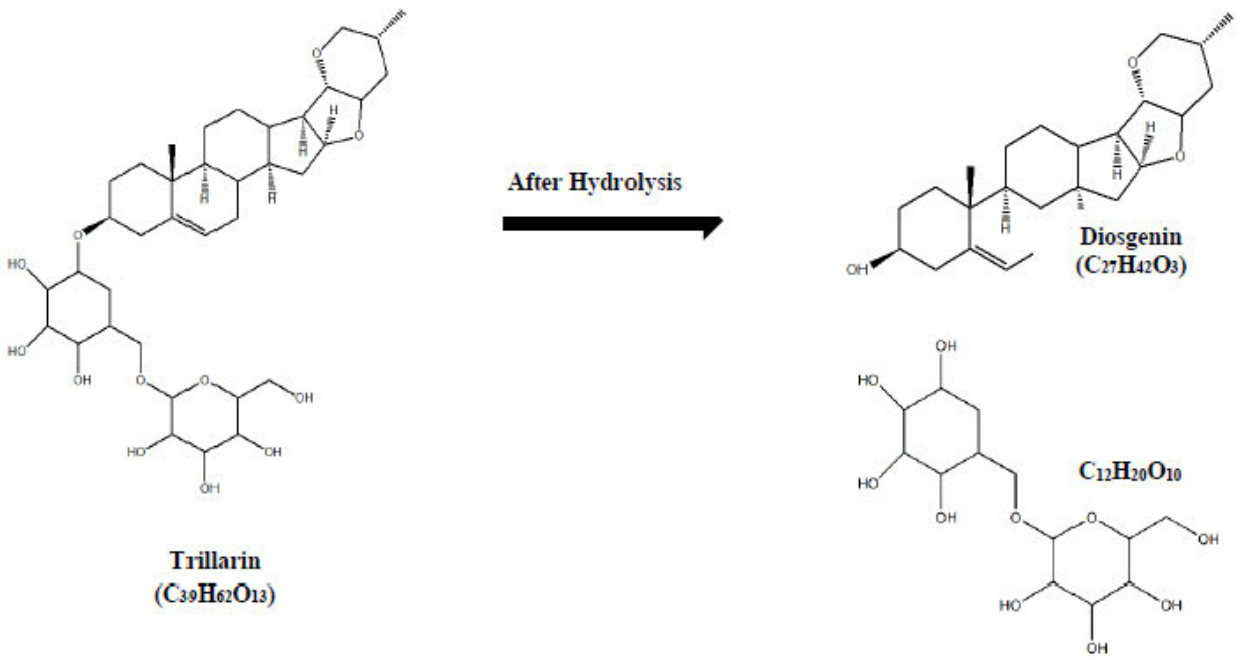

Fig. 1.2. A schematic hypothesized view for the production of Diosgenin from Trillarin after hydrolysis

Relentless collection and smuggling of *Trillium govanianum* because of its high price (Rs 2,500-5,000) have made it a highly prized medicinal herb in Himalayas. At present, natural diosgenin is extracted from Trigonella species and the diosgenin content varies from 0.01% to 0.5% in aerial parts and seeds (Dangi et al. 2014). Diosgenin content varies in *Dioscorea* spp with *Dioscorea polygonoides* (0.2%); *Dioscorea althaeoides* (0.2-2.3%); *Dioscorea prazeri* (1.92%); *Dioscorea villosa* (1.3%); *Dioscorea zingiberensis* (0.18-0.55%) (Niño et al. 2007). Production of diosgenin from *Dioscorea* spp is both time consuming and costly because tubers require ~3 years to grow to mature size (Šavikin-Fodulović et al. 1998, Acharya et al. 2008, Zerbino & Birney 2008). Moreover, these raw materials are in short supply and production strategies of Trigonella and Dioscorea are not enough for meeting the present demand, so there is a need for alternative sources for steroids. *T. govanianum* can serve as an alternative for higher production of diosgenin in shorter growing cycle (Vidyarthi et al. 2013). Due to immense medicinal importance, small population, limited niche, low degree of genetic diversity, low set rate of seed, reckless collection and escalating industrial demands, *T. govanianum* has been listed as an endangered medicinal plant species by the International Union for Conservation of Nature and Natural Resources (Hu- Tian-yin and Qian Li-hua 2009).

Different tissue culture techniques including micropropagation, synthetic seed production, conservation of germplasm through encapsulated microshoots, plant regeneration via direct and indirect organogenesis can be employed for conservation of *T. govanianum* but no significant research work has been done towards development of effective approach for enhancing secondary metabolite production in this plant species under *in vitro* conditions.

Hence immediate attention needs to be paid for conservation, micropropagation, cultivation and *in vitro* production of secondary metabolites in *T. govanianum*.

T. govanianum is less explored in the identification of govanoside, new steroidal saponins, and other components such as borassoside and pennogenin, which have been explored for antioxidant and anticancer activities (Ur-Rahman et al. 2015). These secondary metabolites produced in this species are analyzed specially for diosgenin, which is produced through steroid biosynthetic pathway. Recently, GC/MS analysis of rhizomes of *T. govanianum* has been carried out to investigate the phytochemical constituents & explored its antioxidants, anticancer activities (Ur-Rahman et al., 2015). Other pharmacological properties reported for *T. govanianum* are Anthelmintic, Anti- analgesic, Anti-inflammatory, Anti- diarrheal and Anti-septic.

The availability of predicted pathway for diosgenin biosynthesis by Vaidya et al. (2013) and Diarra et al. (2013) is expected to help in discerning molecular basis of diosgenin biosynthesis in *Trillium govanianum* (Fig. 3).

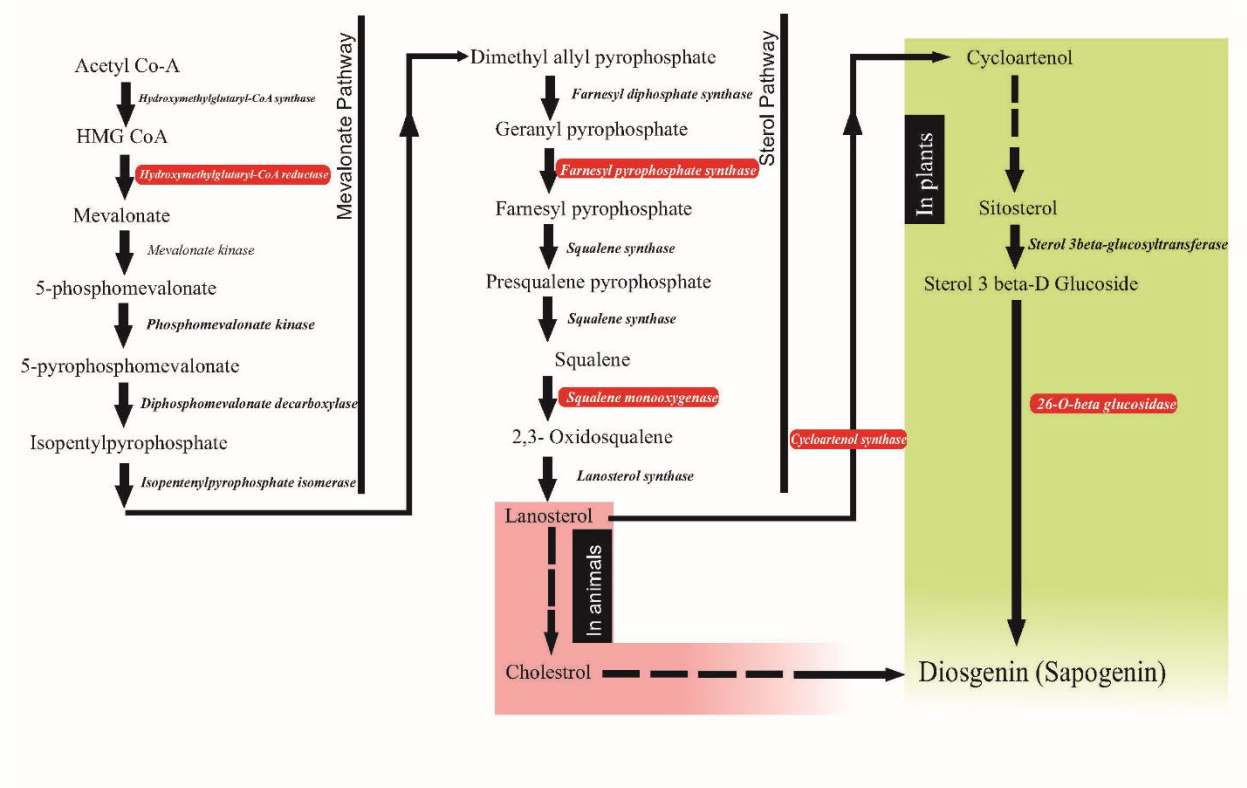

Fig. 3. Proposed pathway for diosgenin biosynthesis in *T. govanianum* (Vaidya et al. 2013; Diarra et al. 2013)

It is well established that Glycolytic, mevalonate, and steroid biosynthesis pathways are involved in diosgenin biosynthesis but complete pathway in *T. govanianum* is still not elucidated. It has been reported by Dewick (2002) that cholesterol is the also one of the precursors of diosgenin biosynthesis pathway where it might be formed from 2,3- Oxidosqualene in two ways i.e either from lanosterol via formation of cholesterol or from cycloartenol via formation of sitosterol.

Thus, the current work is focused on the extraction procedures for the diosgenin content where so far limited attempts were reported. Optimization of effective extraction protocol and quantification of diosgenin from different parts of the plant achieve maximum yield of diosgenin was quantified using response surface methodology (RSM). Expression of the key genes of steroidal pathway was carried out. Further, biological activities of crude extracts for toxicity were investigated on cancer cell lines and anti-fertility studies on female rat model were performed.

Therefore, the current study was undertaken with the following objectives:

Objective 1: Optimization of diosgenin extraction from *T. govanianum*

Objective 2: Discerning the expression status of steroidal pathway genes for diosgenin biosynthesis in *T. govanianum*

Objective 3: To investigate anti-cancerous and anti-fertility potential of *T. govanianum* extract

CHAPTER 2

REVIEW OF LITERATURE

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Man has been using herbs and produce from plants for combating various diseases since long time. Indian subcontinent is enriched by variety of Flora- both aromatic and medicinal plants which is due to the wide range of climatic conditions in India ranging from deserts to swamplands. The indigenous system of medicine viz. Ayruveda, Unani, Sidha still depends on the collection of herbal raw materials from the wild (Mukherjee, 2002). In spite of several advancements in the field of synthetic drug chemistry and antibiotics, plants continue to be one of the major raw materials for drugs treating various ailments of humans (Sati, 2013).

The traditional system of medicine is so entrenched in our culture that even today around 75% of Indian population depends upon indigenous system for relief. The World Health Organization is now actively encouraging many developing countries for the use of herbal medicine and even they have identified 3000 plants from forests of India and other tropical countries which can be used as a medicine. The pharmacologically active compounds are worth near Rs 2,000 crores for the US market alone and nearly 8 times that for the world market. (Hoareau and DaSilva, 1999; Rustogi R.P 1980). Now these demands are so high that world marker for plant derived drugs by year 2050 is likely to reach ~ US $ 5 trillion. Therefore, the alarming situation has come towards the high demand of high value medicinal plant (Sarasan, 2006).

In India around 20 well established manufacturers of herbal drugs are existing with 140 medium or small scale manufacturers and about 1200 licensed small manufacturers on record, with annual production of herbal drugs is around Rs. 100 crores. Herbal medicines represent an estimated $60-billion a year global market, are 1650 herbal formulation in the Indian market and 540 major plants involved in their formulation (Ramawat and Goyal, 2008).

The world is gradually turning to herbal formulations which are known to be effective against a large repertoire of diseases and ailments. More importantly, they are not known to cause any notable side effects; and are readily available at affordable prices.

The pharmacologically active compounds are derived from different parts of plants like, leaves, stem, bark, root, flower, seed or whole plant which is further used for preparation of herbal drug

formulation (Kapoor, 2000). Also, non- availability of quality rich unadulterated plant raw material for formulation of drugs is a matter of concern as industrial demands are ever increasing day by day. Therefore, latest biotechnological and pharmaceutical drug tools offer an excellent platform for carrying out effective extraction of chemical constituents and molecular understanding so as to utilize the immense plant wealth. Molecular pharmacological procedures like *in vitro*/*in vivo* experiments in mice and rats are used nowadays to screen herbal drug compounds/ extract efficacy (White, 2000).

Out of many important medicinal plants, *Trillium govanianum* finds a key position for conservation and enhancement of secondary metabolite production. The current status of literature on various aspects of *Trillium govanianum* research has been reviewed as under:

2.1 *Trillium govanianum*

T. govanianum is a perennial herb and propagates through seeds and rhizomes. It is a small herb, 15-25 cm in height, with a central inconspicuous purple brown flower borne at the apex of stem surrounded by three broadly ovate, acute leaves with a conspicuous petiole. This species is unusual in that the sepals and petals resemble each other, giving a six-petalled appearance. These contrast well with the prominent yellow anthers and red style that sits atop the flower. The flower is followed by a grape-red fruit, sitting at the center of the three leaves. Flowering is from May to June and but due to huge market demand plant is manually extracted in the month after snow starts melting at higher altitudes. *Trillium govanianum* is a native species of Himalyan region and distributed in the Himalaya, Bhutan, Nepal, China between the elevation range of 3,000 to 4,000 m. Vernacular name include "Nag chhatri" or "Matar jarai" in Nepal and Hindi. It prefers humus rich soil and shady places.

Fig. 2.1 Mature plants of *T. govanianum*

The transverse section of rhizome shows the presence of cortex cells, trichomes, carinal canal, sclereids, vascular bundles, fibers and other starch grains (Rahman Ur, 2015).

T. govanianum grows naturally in the dry North- Western Himalayan region at an altitudinal range from 2,400 to 3,500 m above msl.

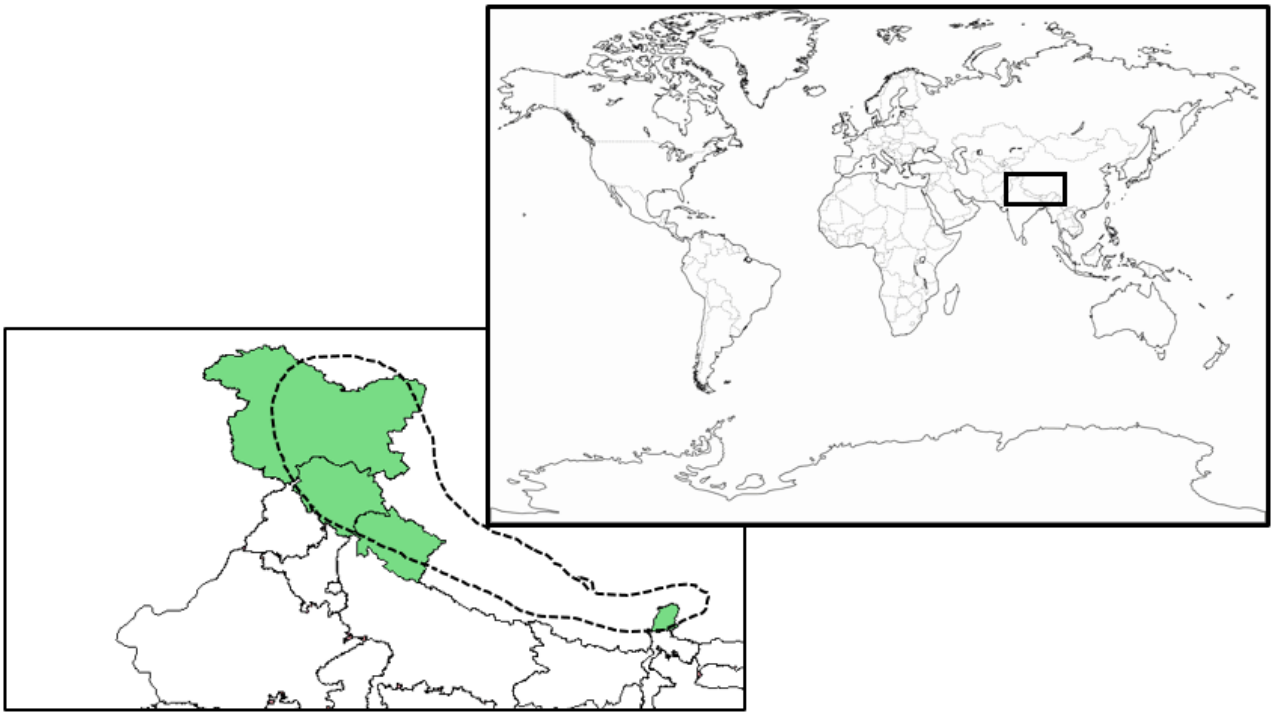


Fig. 2.2 Distribution of *Trillium govanianum* in the Himalayan region: ▬ ▬ *T. govanianum*.

Table 2.1 Distribution of *T. govanianum* in the Himalayan region

	State/Country	Location	Reference
T. govanianum	Jammu & Kashmir	Doda and Kishtwar, Bandipora district	Lone et al. (2014)
		Kanzalwan, Phalgham, Poonch, Gurez (Kashmir)	News (JKMPIC)
	Himachal Pradesh	Lahaul, Kinnaur, Kulu, Rohru, Kangra, Pangi, Bharmour	Uniyal and Datta (2012)
		Upper Beas Valley, Kothi, Banjar Valley, Parbati Valley	Vidyarthi et al. (2013)
	Uttarakhand	Harsil, Raithal, Sukhi, Sayara, Tehri-Garhwal in Bhagirathi Valley	Kala (2016)
	Pakistan	Khyber Pakhtunkhwa, Dir Upper, Kohistan Valley	Rahman et al. (2015)
	China	Qinba mountain of Shaanxi Province	Chai et al. (2014)

2.2 Phytochemistry of *T. govanianum*

This genus contains steroidal saponins, reported from *T. tschonoskii* Maxim. (Nohara et al., 1975a; Wang et al., 2007; Wei et al. 2012, Chai et al. 2014), *T. kamtschaticum* Pall. (Konyukhov et al., 1973; Kim et al., 1991; Ono et al., 2003, 2007a, 2007b) and *T. erectum* L. (Yokosuka and Mimaki, 2008; Hayes et al., 2009).

Using different chromatographic techniques eight compounds from CHL-fr and two compounds from BoOH-fr were isolated. The chemical structures of isolated compounds were elucidated using spectroscopic and spectrometric techniques i.e 1H-NMR, 13C-NMR, COSY, NOESY, HSQC, HMBC, FAB, HR-FAB, IR and UV. Only 4 compounds are reported till date from *T. govanianum* namely Govanoside A, Borassoside E, Pennogenin, Diosgenin. *Trillium* genus is having around 31 species widely distributed from the Western Himalayas to Japan, China, Kamchatka (Russia) and North America, and out of them only selected species are found to possess medicinal value with wide application in pharmaceuticals (Li, 1952; Gracie & Lamont, 2012). The major class of chemical compounds isolated from these are steroids, glycosides, terpenoids, sterol, saponins,

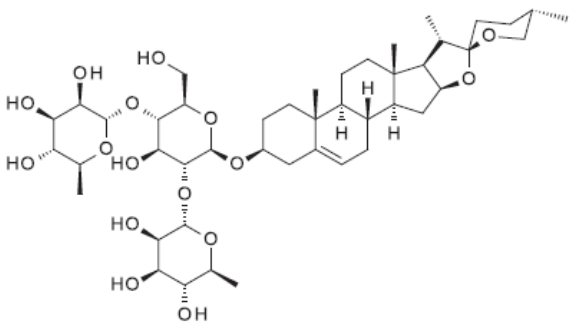
sapogenins and flavonoids.

Table 2.2 Various chemical constituents isolated from *Trillium* species

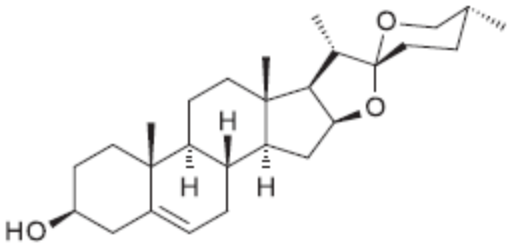
Class	Compound	Species	Reference(s)
Sterol Saponin	Govanoside A	*T. govanianum*	Ur-Rahman et al. (2015)
	Borossoside E	*T. govanianum*	Ur-Rahman et al. (2015)
	Pennogenin	*T. govanianum*	Ur-Rahman et al. (2015)
	Diosgenin	*T. govanianum*	Ur-Rahman et al. (2015)
Steroidal glycosides	(25S)-17a,27-dihydroxyspirost-5-en-3b-yl O-a-Lrhamnopyranosyl-(1?2)-b-D-glucopyranoside	*T. erectum*	Ono et al. (2007)
	(25R)-17a-hydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-b-D-glucopyranoside	*T. erectum*	Nohara et al.(1975)
	(25R)-17a-hydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?4)-b-D-glucopyranoside	*T. erectum*	Mahato et al., 1981
	25R)-17ahydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-O-[a-Lrhamnopyranosyl-(1?4)]-b-D-glucopyranoside	*T. erectum*	Nohara et al. (1975) Nakano et al. (1989)
	(25R)-17a-hydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?4)-O-a-L-rhamnopyranosyl-(1?4)-b-D-glucopyranoside	*T. erectum*	Mimaki et al. (2000)
	(25R)-17a-hydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-O-[O-a-L-rhamnopyranosyl-(1?4)-a-L-rhamnopyranosyl-(1?4)]-a-D-glucopyranoside	*T. erectum*	Nohara et al. (1975)
	(25R)-spirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-b-D-glucopyranoside	*T. erectum*	Nohara et al., (1975) Espejo et al.(1982)

(25R)-spirost-5-en-3b-yl O -a-L-rhamnopyranosyl-(1?2)-O-[a-L-rhamnopyranosyl-(1?4)]-b-D-glucopyranoside (dioscin)	*T. erectum*	Nohara et al. (1975) Espejo et al.(1982) Nakano et al. (1989)
(25R)-spirost-5-en-3b-yl O-a-Lrhamnopyranosyl-(1?2)-O-[O-a-L-rhamnopyranosyl-(1?4)-a-Lrhamnopyranosyl-(1?4)]-b-D-glucopyranoside	*T. erectum*	Nohara et al.(1975)
(25R)-26-[b-D-glucopyranosyl]oxy]-22a-methoxyfurost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-O-[a-L-rhamnopyranosyl-(1?4)]-b-D-glucopyranoside (methylprotodioscin)	*T. erectum*	Hirai et al. (1984) Teshima et al.(1986)
(25R)-26-[b-D-glucopyranosyl] oxy]-17a-hydroxy-22b-methoxyfurost-5-en-3b-yl O-a-Lrhamnopyranosyl-(1?2)-b-D-glucopyranoside	*T. erectum*	Nakano et al. (1984)
(25R)-26-[b-D-glucopyranosyl]oxy]-17a-hydroxy-22amethoxyfurost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-O-[a-Lrhamnopyranosyl-(1?4)]-b-D-glucopyranoside	*T. erectum*	Nakano et al. (1982)
(25R)-26-[b-D-glucopyranosyl]oxy]-17a-hydroxy- 22a-methoxyfurost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-O-[O-a-L-rhamnopyranosyl-(1?4)-a-L-rhamnopyranosyl-(1?4)]-b- D-	*T. erectum*	Nakano et al. (1982)
(25R)-26-[b-Dglucopyranosyl] oxy]-3b-[(O-a-L-rhamnopyranosyl-(1?2)-b-D-glu copyranosyl)oxy]-cholesta-5,17-diene-16,22-dione	*T. erectum*	Nohara et al. (1975)
(25S)-Spirost-5-ene-3b,17a,27-triol	*T. erectum*	Yokosuka and Mimaki (2008)
(25S)-27-[(b-D-Glucopyranosyl)oxy]-17a-hydroxyspirost-5-en-3b-yl O-a-L-rhamnopyranosyl-(1?2)-b-D-glucopyranoside	*T. erectum*	Yokosuka and Mimaki (2008)

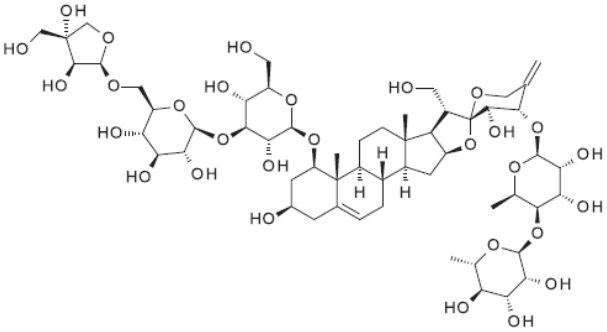
	(25S)-27-[(b- D-glucopyranosyl)oxy]-17a,27-dihydroxyspirost-5-en-3b-yl O-(4- O-acetyl-a-L-rhamnopyranosyl)-(1?2)-b-D-glucopyranoside	*T. erectum*	Yokosuka and Mimaki (2008)
	(25S)-27-[(b-D-glucopyranosyl)oxy]-17a,27- dihydroxyspirost-5-en-3b-yl O-a-D-glucopyranosyl-(1?6)-O-[a-rhamnopyranosyl-(1?2)]-b-D-lucopyranoside	*T. erectum*	Yokosuka and Mimaki (2008)
	(25S)-17a,27-dihydroxyspirost-5-en-3b-yl b-D-glucopyranoside	*T. erectum*	Yokosuka and Mimaki (2008)
	(25S)-17a,27-dihydroxyspirost- 5-en-3b-yl O-(4-O-acetyl-a-L-rhamnopyranosyl)-(1?2)-b- D-	*T. erectum*	Yokosuka and Mimaki (2008)
	7- b -hydroxy trillenogenin 1-O-b -D-apiofuranosyl-(1→3)-a -rhamnopyranosyl-(1→2)-[b -D-xylopyranosyl-(1→3)]-a -Larabinopyranoside.	*T. tschonoskii*	Wang et al. (2007)
	Trillenoside A.	*T. tschonoskii*	Wang et al. (2007)
	(23S,24S,25S)-spirost-5-en-1b,3b,21,23,24-pentaol-1-O-b-D-xylopyranosyl-(1!3)-[O-a-Lrhamnopyranosyl-(1!2)]-O-a-L-arabinopyranoside	*T. tschonoskii*	Chai et al. (2014)
Sesquiterpe-noid glycoside	(2,3-S-trans,10R,6E)-7,11-dimethyl-3-methylene-1,6-dodecadien-10, 11-diol 10-O-b-D-glucopyranosyl-(1!4)-O-b-D-glucopyranosyl-(1!4)-O-b-D-glucopyranoside	*T. tschonoskii*	Chai et al. (2014)



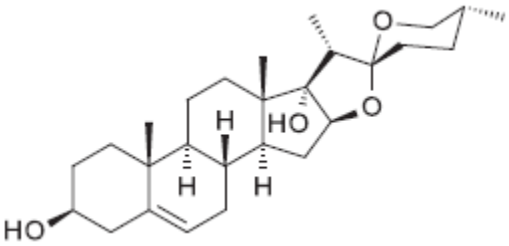
Borassoside E



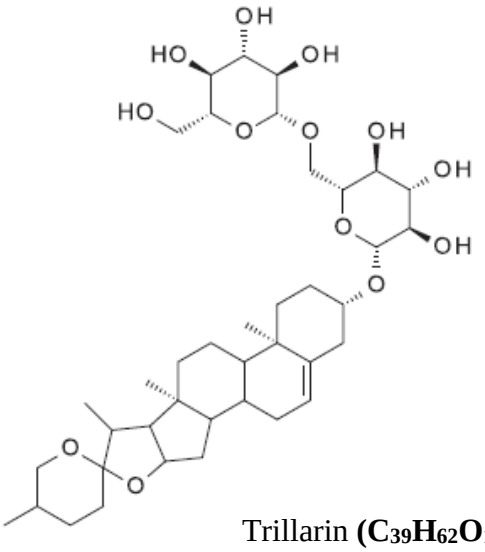
Diosgenin **($C_{27}H_{42}O_3$)**



Govanoside A



Pennogenin



Trillarin **($C_{39}H_{62}O_{13}$)**

Fig 2.3. Chemical structures of some active compounds from *T. govanianum*

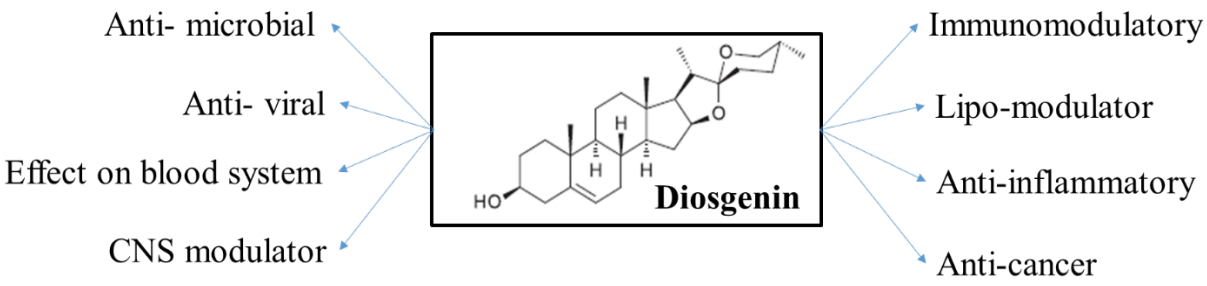


Fig 2.4. Pharmacological activities of diosgenin

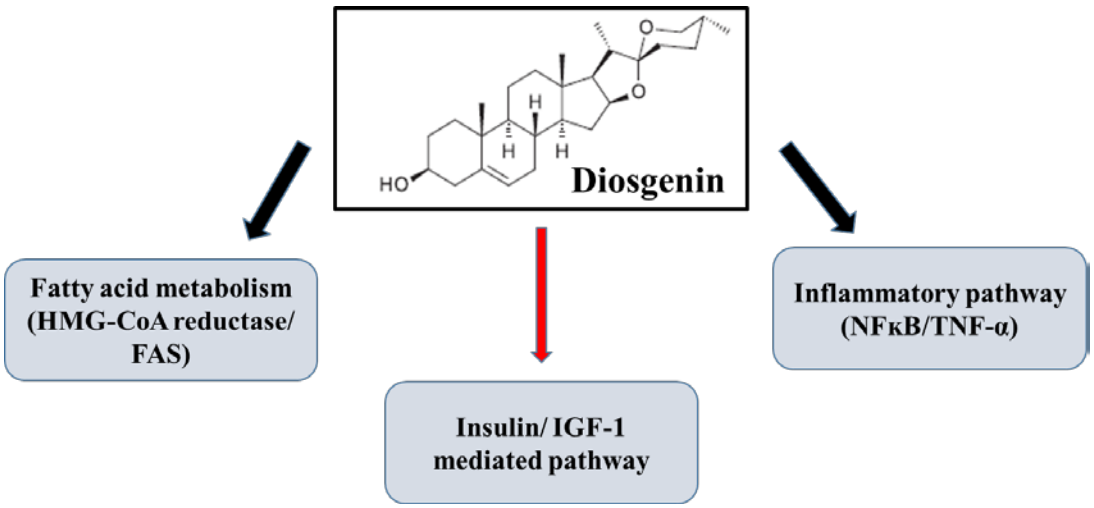


Fig 2.5. Representation depicting the molecular mode of action of diosgenin in control of metabolic pathways

2.3 Diosgenin production in other species

Various established methodologies and new technologies are intervened to overcome the processing challenges postured by nature and uniqueness of natural complex compounds.

Two-third of the total world consumption of steroid is coming from diosgenin (Thomas et al. 2000). Annual demand in the world is around 3000 tons that of India is 150 tons. Total production being only 30 tons in Indian subcontinent draws our attention towards searching of alternative sources (Dasgupta and Pandey, 1970). China and Mexico are the two top countries with richest yam resource in the world and yield accounts for 67% of world production. Yam is also used a traditional medicine in various area of China, Nepal, Pakistan and India (Li et al. 2010). Diosgenin

is found in several plant species namely *Trigonella, Trillium, Allium fuscoviolacenum, Allium narcissifolium, Costus speciosus, Smilax menispermoidea, S. aspera, exfelsa, many species of Dioscorea* (yam) like *D. deltoidea, floribunda, gracillima, prazeri, zingiberensis, Funkia ovata, Kallstroemia pubescens, Paris polyphyalla, Polygonatum latifolium, Polygonatum multiflorum, Smilax exfelsa, Solanum introsum, Solanum indicum, Tamas communis* and *Tribulus terrestris, Trigonella foenum* and *Trigonella tschonoskii.* Production of diosgenin is mainly from *Dioscorea* spp which is both time consuming and costly because tubers require ~3 years to grow to mature size (Šavikin-Fodulović et al. 1998, Acharya et al. 2008, Zerbino & Birney 2008). Moreover, these raw materials are in short supply and production strategies of Trigonella and Dioscorea are not enough for meeting the present demand, so there is a need for alternative sources for steroids. Diosgenin content varies in *Dioscorea* spp with *Dioscorea polygonoides* (0.2%); *Dioscorea althaeoides* (0.2-2.3%); *Dioscorea prazeri* (1.92%); *Dioscorea villosa* (1.3%); *Dioscorea zingiberensis* (0.18-0.55%) (Niño et al. 2007). Diosgenin content varies from 0.01% to 0.5% in aerial parts and seeds of Trigonella species (Dangi et al. 2014).

Table 2.3 Major medicinal plants containing diosgenin

S. No.	Name of Plant	Part Used
1	*Dioscorea spp.*	Rhizome (Diosgenin Content ~2%)
6	*Trigonella spp.*	Leaves and Seed (Diosgenin Content ~0.5%)
9	*Solanum spp.*	Fruit (Diosgenin Content ~0.2%)
11	*Smilax spp.*	Leaves (Diosgenin Content ~0.4%)
12	*Paris polyphyalla*	Leaves (Diosgenin Content ~0.8%)
13	*Polygonatum spp.*	Leaves (Diosgenin Content ~2.1%)
15	*Costus speciosus*	Rhizome (Diosgenin Content ~0.152%)
16	*Kallstroemia pubescens*	Whole Plant (Diosgenin Content ~2.2%)

Overall, various studies have been reported on different aspects of *T.govanianum* but limited efforts have been made for cost effective and efficient extraction protocol for metabolite extraction from dried rhizomes. Also, molecular studies are required for better understanding of diosgenin biosynthesis and ascertaining the role of genes involved in diosgenin production in *T.govanianum*. According to folkric knowledge at village level plant raw powder is also used as an alternative for contraceptive capsule. So, under this umbrella we carried out some pharmacological explorations in mice models.

2.4 Medicinal properties of pharmacologically active compounds from Trillium species

Hufford et al. (1988) reported anti-fungal activity from rhizomes of *T. grandiflorum*. This *in vitro* activity was against *Candida albicans*. Bioassay fractionation was also done which resulted in isolation of two active components. The study was then followed by NMR for structure elucidation which revealed the presence of sapogenins and hydrolysis later produced an aglycone identical glycoside diosgenin.

Pence and Soukup (1993) reported the most important conservation technology i.e Tissue culture for mass multiplication of *T. erectum* and *T. grandiflorum*. Study was based on factors affecting the initiation of mini-rhizomes from these species *in vitro*. The best response for both the species was found to be half-strength MS basal medium supplemented with auxins and cytokinin. *T. erectum* was more efficiently responsive than *T. grandiflorum* at 21^0C and thus can be used to produce mini rhizomes for more rapid and alternative strategy towards conservation.

A novel 18- norspirostanol saponins along with Trillenoside A was isolated from rhizome of *T. tschonoskii*(Wang et al. 2007). Compound 1 ($C_{47}H_{70}O_{25}Na$) was a colorless powder and Compound 2 (Trillenoside A) was a white powder. Ihe inhibitory action of 1 and 2 was found against COX-2 production which are inhibitors in type of non-steroidal anti-inflammatory drug. The rhizome of *T. tschonoskii* also reported to induce apoptosis in human lung cancer cells (Huang and Zou, 2011)

Wang et al. (2013) found a way to combat with multidrug resistance (MDR) which is the major part of hepatocellular carcinoma (HCC) chemotherapy. Research found that *T. tschonoskii* (TTS) could reverse the MDR in HCC and thereby enhancing chemo-sensitization. The *in vitro* studies on rats revealed that TTS on dose dependent manner inhibited HepG2 and R-HepG2 cell survival.

TTS also down- regulate the expression of MDR genes thus becoming a potential method to overcome MDR in HCC treatment. Experiments on mice models also proved that tumors formation was significantly blocked when mice were administered by TTS. Thus, proving TTS as a candidate to reverse the MDR in HCC by enhanced chemosensitivity of MDR HCC cells and prevent tumors formation.

Sagar et al. (2017) reported the presence of various endophytes and anti-bacterial activity from *T. govanianum*. They isolated endophytes from various plant parts (viz. stem, leaves, and rhizomes) and listed according to their occurrence during summer or rainy season. Total isolated endophytes were *Alternaria sp. Aspergillus nidulans, Aspergillus niger, Aspergillus wentii, Fusarium solani, Mucor plumbeus, Phoma sp., Pythium sp. Rhizopus nigricans, Rhizopus oryzaoe, Stachybotrys atra, Trichoderma viride*. Antibacterial activity was performed of methanol, ethanol, acetone and distilled water extracts. Well diffusion method was used and screening revealed that methanol rhizome extract of the species was most effective in inhibiting the growth of *S. aureus* out of *E. coli, Yersinia pestis* (Human pathogenic bacteria).

2.5 Extraction of diosgenin

The presence of various well – established analytical methods, latest technologies are now investigated to overcome the challenges in extraction, processing and identification of new drug molecules from plant sources. In any of the extraction process the major factors which determine the process efficacy and properties of end product are the extraction solvent, properties of raw material (such as composition and particle size), extraction conditions (such as pH, solid-liquid ratio, temperature). Pretreatment of sample, extraction solvents are always the significant steps during the extraction process and always hold extra importance for pharmaceutical drug formulation.

Diosgenin is a novel multitarget drug based on chemopreventive or therapeutic agent against several chronic ailment. This valuable drug is having a huge steroidal drug market with ever increasing demand and mostly extracted from many plant species like Dioscorea, Trillium, Trigonella etc. Nowadays, *T. govanianum* is on the target and Dioscorea spp. has taken a back seat because of various disadvantages and one being that dispersal of winged seeds and the new tubers take 4-5 years before they are exploitable. This long duration of waiting for extraction has made

researcher and botanists to exploit new species containing diosgenin.

Few analytical approach for Diosgenin extraction were studied while main point being the matrix from which the diosgenin has to be extracted. Matrix are present in several plant species and most usually used in extraction processes for further analysis. However, other matrices can be involved too, including cosmeceutical/ pharmaceutical herbal formulation etc. Major protocol refer the use of pulverized plant material with around 7-8 gm fresh tuber or whole plant. Then after Soxhlet extraction can be done with HPLC grade light petroleum ether, Hydroalcholic, Ethyl acetate of isopropanol. The solvent selection is dependent upon their position in eluotropic series which measure how well the solvent pull an analyte off the adsorbent to which it is attached. Then direct acid hydrolysis/spontaneous fermentation or enzyme catalysis followed by liquid –liquid extraction or solid phase extraction are the most commonly used techniques to obtain diosgenin. These procedures are having some disadvantages like need of high volume of solvent and extraction duration being also long. For these reasons other extraction procedures like supercritical fluid extraction after acid hydrolysis, followed by high –speed counter current chromatography with evaporative light scattering detection. These conventional techniques were optimized using multienzymatic catalysis in combination with acid hydrolysis thus allowing obtaining high purity diosgenin (> 96%) from Dioscorea spp. (Zhang et al., 2007). From *Trigonella foenum graceum* leaves and root diosgenin was extracted using focused microwave- assisted extraction.

According to literature, incomplete acid hydrolysis yields saponins, while complete acid hydrolysis was found to produce the constituent aglycone. Mostly Acid hydrolysis is the techniques used for extraction of diosgenin. In this extract after soxhlet process is refluxed with sulphuric acid and isopropanol for around 10-12 hours. The extract was filtered and extracted with hexane and rinsed with sodium hydroxide for achieving neutralization point during experiment and then lastly with distilled water (Shah and Lele, 2012) to maintain pH. Then sample can be concentrated by evaporating in rotary evaporator followed by lyophilization to obtain dried powder which can be subjected for classical analytical methods for detection/ quantification of diosgenin using TLC, HPLC, etc.

2.6 Tissue culture of *T. govanianum*

Till now no publication is cited on tissue culture of *T. govanianum* but some literature are available on other Trillium species. Many researchers tried their level best for multiplication of *T.*

govanianum but found no success.

Report on trial and error with *Trillium* micropropagation was performed at the Atlanta botanical garden by Ron Gagliardo. He performed informal experiment with dozens of *Trillium* species in 2001 and after many years of laboratory trial he grew many *in vitro* propagated plants in the nursery. According to some pictures from internet he has done with clonal propagation via meristem culture and initiation was done on ½ MS with 5 mg/l BAP and 1 mg/l NAA followed by multiplication on ½ MS with 1-2 mg/l BAP. But the publication is not found supporting the study and also replicative study was done resulting in failure.

Kitto (2002) tried working on *in vitro* culture establishment through tissue culture of various Trillium species. But unfortunately only species *T. grandiflorum* was not able to establish after 4 years of work when compared to other species like *T. maculatum, T.rugelii and T.decumbens* that were established on the first try. This clearly indicate the need to efficient tissue culture technology for mass multiplication of some *trillium* species even like *T. govanianum*. But the problem in tissue culture of *T. govanianum* is the non- availability of plant material and seeds that are very small and as per gathered information from villagers that seeds are taken by ants and insects as a source of food. Local people also told that the plant is extracted from the wild even before the seed formation thus threatening habitat of species.

2.7 Biosynthesis of diosgenin in plants

It has been reported that in biosynthesis of diosgenin glycolytic, mevalonate, and steroid biosynthesis pathways play pivotal role with various intermediate steps which have not yet been completely elucidated and cholesterol being the precursor of this compound (Dewick 2002).

It is known that the mevalonate pathway or HMG-CoA reductase pathway route for isoprenoid biosynthesis is an important pathway present in all higher eukaryotes and bacteria. The reaction catalyzed by hydroxymethylglutaryl Co-A reductase (HMGR) is rate determining step on the pathway for the synthesis of cholesterol. Mehrafarin et al. (2010) have reported that diosgenin is synthesized from cholesterol in several plant species but not much information is available about the biosynthetic pathway of steroidal saponins and its precursors are yet to be exploited. Mevalonate is the key precursor for synthesis of cholesterol and related isoprenoid compounds which arise from acetyl-CoA and gets converted to isopentenyl diphosphate (IPP); the "active

isoprene". Then IPP undergoes isomerization to form dimethylallyl diphosphate. Further these two C5 molecules condense to yield geranyl diphosphate (GPP) and the addition of another IPP produces farnesyl diphosphate (FPP). FPP undergoes dimerization in head to head reaction to yield squalene, a linear isoprenoid which undergoes cyclization with O_2 to form lanosterol, a C30 sterol. Squalene, a hexaisoprenoid or triterpene is of wide occurrence in both plant and animal tissues and this is as it should be starting point for biosynthesis of steroids specially sterols.

Now in steroid pathway diosgenin might be formed from squalene-2, 3-oxide in two ways viz. either from lanosterol via formation of cholesterol or from cycloartenol via the formation of sitosterol. It has been hypothesized that UDP glucose is added to sitosterol with the enzymatic activity of sterol 3-beta-glucosyl transferase. Finally, 2, 6-beta glucosidase converts sterol 3 –beta glucosidase into diosgenin by removal of glucose molecule (Vaidya et al. 2013; Diarra et al. 2013). It is quite likely that kryptogenin is an intermediate in conversion of cholesterol to diosgenin. This compound could arise from cholesterol by oxidation at C-16, C-22 and C-27 (Bennett and Heftmann, 1966).

2.8 Medicinal properties of *T. govanianum*

Trillium species have been used in the traditional as well as in modern systems of medicine. The North-American species of *Trillium* are known to have uterine stimulant, antimicrobial, antifungal and antibacterial properties (Huang and Zou, 2011; Ono et al., 2007). *T. govanianum* rhizome which is the key material of trade is used for the treatment of various disorders like dysentery, backache, healing of wounds, inflammation, Skin boils, menstrual and sexual disorder (Rani et al. (2013); Mahmood et al. (2012) Sharma & Samant (2014). Also reports are available on powered plant used as anthelmintic (Lone et al. 2013). Plant extract also have antifungal, antimicrobial like activities. Some of the medicinal properties of *T. govanianum* are listed in Table 2.

Table 2.4 Major medicinal properties of *T. govanianum*

Medicinal Property	**References**
Anthelmintic	Bhardwaj et al., (2013) Lone et al., (2013)

Skin infection	Lone et al. (2013)
Anti-analgesic	Ur Rahman et al. (2016)
Anti-inflammatory	Ur Rahman et al. (2016)
Treating sexual disorder	Rani et al. (2013)
Antidiarrhoeal and Anti-septic	Mahmood et al. (2012) Sharma & Samant (2014)
Anti-fertility	Maliwichi-Nyirenda, C.P. and Maliwichi, L.L. (2010)

Govanoside A ($C_{56}H_{88}O_{29}$) and Borassoside E ($C_{45}H_{72}O_{16}$) are the most recent spirostane steroidal saponins isolated from *T. govanianum*. They both are white amorphous powder and are obtained from the shade dried rhizome of *T. govanianum*. The only biological report of Govanoside A from species is its positive anti-fungal nature against *Candida albicans*, and *Candida glabrata*. Borassoside E also has exhibit considerable anti-fungal activities. Borassoside E has three sugar units and shows better antifungal activity in comparison to Govanoside A which has five sugar units. This high antifungal activity of Borassoside A might be due to the high polar nature of Govanoside A which may hinder in its fungal membrane permeability. (Ur Rahman et al., 2016).

Khan et. Al (2016) reported for the first time on the cytotoxicity of methanol extract of root of *T. govanianum* and it solid phase extraction fraction against four human carcinoma cell lines: breast, liver, lung and urinary bladder using MTT assay. The MeOH extract showed differential response in level of significant cytotoxicity against breast (MCF-7), liver (HepG2), lung (A549), and urinary bladder (EJ138). MeOH extract displayed the highest level of cytotoxicity in EJ138 but considerably active in MCF-7, HepG2 and A549. The major bioactive components of the genus *Trillium* are saponins and sapogenins which are well known to exhibit cytotoxicity (Nooter and Herweijer, 1991; Yokosuka and Mimaki, 2008; Hayes et al., 2009)

Ur-Rahman et al. (2015) investigated the phytochemical screening followed by GC/MS analysis of n-hexane fraction. Phytochemical screening revealed the presence of steroids, glycosides and saponins which were previously reported in same genus *Trillium* (Akihito et al., 2008 and Jiang et al., 2014). GC/MS analysis of n-hexane fraction showed the presence of saturated and unsaturated fatty acids and thus representing biologically active compounds with antifungal, anti-bacterial and

anticancer activites (Ching, 2008; Qiong et al., 2011). So far number of anti-cancer metabolites are reported from genus *Trillium* (Jiang et al., 2014; Ono et al., 2007; Ono et al., 1986) which also included *T. erectum* and *T. tschonoskii* (Akihito et al., 2008; Zhao et al., 2011).

Thus, the review of literature has shown following research gaps:

- Lack of methodology for diosgenin extraction from *T. govanianum*
- No information on biosynthetic pathway genes for diosgenin production in *T. govanianum*
- No reports on anti-cancerous & anti-fertility potential of crude and hydroalcholic extracts of *T. govanianum*

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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3.1 Plant material

Twelve accessions of *T. govanianum* were collected from Sonmarg, Jammu & Kashmir and high altitudes of Chamba, Lahaul & Spiti areas of Himachal Pradesh in the month of August 2014.

Table 3.1 *T. govanianum* accessions from different geographical locations of H.P and J&K

Accession	Location	District	Altitude range (m asl)
TG-1	Maingal	Chamba	2,500-3,500
TG-2	Bharmour	Chamba	
TG-3	Drobi jangal	Chamba	
TG-4	Baror	Chamba	
TG-5	Hirni	Kullu	2,050- 3,050
TG-6	Gojra	Kullu	
TG-7	Allain Duhan	Kullu	
TG-8	Naggar	Kullu	
TG-9	Chandra	Lahaul & Spiti	2,320- 5,000
TG-10	Bhaga	Lahaul & Spiti	
TG-11	Pattan	Lahaul & Spiti	
TG-12	Mayar	Lahaul & Spiti	

3.2 Preprocessing of plant material and Soxhlet extraction

The experimental plant materials obtained from wild were rinsed thoroughly in running tap water for 10-15 minutes. The rhizomes were shade blot dried and incised into thin uniform slices for the temperature exposure at same pace. The dried plant material was pulverized and stored at room temperature for short term and at -20^0 C for long term. Various solvent systems used for the study were selected on the basis of eluotropic series starting from Hydroalcoholic (30:70; Water: Methanol), Isopropanol, Acetonitrile, Ethyl acetate, Chloroform, Diethyl ether and Hexane. After

completion of extraction process through soxhlet apparatus extractive yield was calculated and tabulated. The instrument used were Soxhlet extraction unit, Rotary evaporator (Heidolf, Germany), Lyophilizer, Hot air oven, HPLC (Agilent 1200 series). Extractive yields were calculated, and extracts were stored at 4°C until used further.

3.3 Diosgenin quantification

3.3.1 Hydrolysis

Extract hydrolysis was performed for all accessions in accordance to method described by Drapeau et al. (1986). 1500 mg plant extract after soxhlet extraction was hydrolyzed in 150 ml of refluxing 20% H_2SO_4 in 70% isopropanol for 12 hours of duration.

3.3.2 Neutralisation and drying

The extracts were then filtered and extracted with hexane (50 ml×3), combined and rinsed thrice with 5% Sodium hydroxide and then rinsed thrice with distilled water. The samples were concentrated by evaporating the solvent at 65°C in a rotary evaporator, and then lyophilized to obtain dried powder. Powder of each extraction was then dissolved in MeOH for HPLC analysis.

3.3.3 Standard preparation and HPLC analysis

Diosgenin standard was purchased from Chromadex Inc. (Bangalore, India). A standard stock solution of diosgenin was prepared in HPLC grade methanol and then working solution was made. Quantification was carried out with reverse phase HPLC (Agilent 1200 series) equipped with HPLC pump, DAD photodiode array detector range from 190-800 nm. Diosgenin was estimated with a Zorbax-Eclipse XBD C-18 3.5 µm (4.6 × 150mm). Mobile phase used was in gradient mode to run the samples was (0.1% - 0.5%) Formic Acid with varying ratio of HPLC grade Water and Methanol. The column was eluted in the gradient mode with a flow rate of 0.8 ml/min. Diosgenin was detected at absorbance of 230 nm wavelength. The cycle time of analysis was 30 min at 25°C with injection volume of 10 µl. At the end of each run, the column was rinsed with pure solvents. The compound was identified on the basis of retention time and comparison of UV spectra with the authentic standard from ChromaDex Inc.

3.4 Statistical optimization of diosgenin content using RSM (Response surface methodology)

T. govanianum accession with highest diosgenin content was selected for statistical optimization of diosgenin content using RSM. In RSM a total of three different extraction parameters, viz., incubation time (minutes), temperature (°C), and solid-liquid ratio (g/ml) were selected and their cumulative effect was assessed by using a statistical software package Design Expert® 8.0.7.1, 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.

3.4.1 Hydrolysis

Extract hydrolysis of highest diosgenin content accession was performed as described by Drapeau et al. (1986) with modifications. 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 with 20% H_2SO_4 in 70% isopropanol for 0-16 hrs at temperature ranging from 70-100°C.

3.4.2 Neutralisation and drying

The extract was then filtered and extracted with hexane (50 ml × 3). The three hexane extracts were combined and rinsed three 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 65°C in a rotary evaporator. Hydrolyzed extract were then fractionated with hexane in separating funnel followed by freezing them in −80°C. Samples were lyophilized and then dissolved in MeOH for HPLC analysis.

3.5 Selection of genes

Genes pertaining to primary and secondary metabolism such as HMGR of Mevalonate and FPPS, SQS, CAS and BETA of sterol pathway are the key genes for diosgenin production in plants were selected to study. These key genes are reported to have important role for diosgenin production in *T. foenum* (Vaidya et al. 2013; Diarra et al. 2013). Since no reports were on genes of *T. govanianum* so these all key genes were analyzed on the species and studied their expression pattern for plant samples of highest diosgenin content v/s lowest diosgenin content.

3.6 RNA isolation and cDNA synthesis

Leaves of high and low diosgenin content *T. govanianum* accessions were used to isolate genomic DNA as per method described by Murray and Thompson (1980). RaFlex Total RNA isolation kit (GeNei™) was used to isolate total RNA from rhizomes as per manufacturer's instructions. RNA quality was checked in 1% (w/v) ethidium bromide-stained agarose gel via absorbance spectrum at 260 nm and 280 nm wavelengths. Samples were kept at -80°C until further use.

3.7 Cloning and sequencing of diosgenin pathway genes in *T. govanianum*

Five enzymes HMG Co-A reductase (HMGR), squalene synthase (SQS), farnesyl pyrophosphate synthase (FPPS), 26-O-beta-glucosidase (BETA) and cycloartenol synthase (CAS) have been well recognized as key enzymes involved in diosgenin biosynthesis pathway (Vaidya et al. 2013; Diarra et al. 2013). Nucleotide sequences for these diosgenin biosynthesis pathway genes belonging to different plant species were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and multiple sequence alignments (MSA) were done to identify conserved sequence regions. Primer pairs were designed from these regions using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) for gene amplification (Table 3.2)

PCR amplification was performed on 30 ng of genomic DNA with primer pairs, Mg2+, dNTPs and Taq DNA polymerase. Amplification programs included 94°C for 3 min, 30 cycles of 94°C for 30 s, annealing temperature (49-54 °C) for 45s, 72°C for 2 min and a final extension of 7 min at 72°C. 10 µl of each PCR product was mixed with 2 µl of 6 X gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol) and electrophoresed in a 1.2% agarose gel prepared in 0.5 X Tris acetate-EDTA (TAE) buffer. The gels were analyzed using gel documentation system Alpha Imager EP (Alpha Innotech Corp., USA). PCR products were cloned in pGEMT vector (Promega) and sequenced. Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) was used to calculate sequence similarities with NR database of NCBI. Further, the sequenced products for respective genes were used to design gene specific primer pairs for expression analysis (Table 3.3).

Table 4.2 Primer sequences used to clone five genes of diosgenin biosynthetic pathway in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature (°C)
HMGR	CTTCCGATGTTTTACCTTTG	GCAGGCTTCTCTATGATGAC	52
SQS	TTGAACGACATGGTCACTAA	CATTGTAGATGTTGGTGCAG	54
FPPS	AATGTCTGGTGAAGATTTGG	AAGAGATCAGCTGCTCGTAG	52
BETA	AGTCTGAGCTGATCAAAGGA	TGAACCACTTGGTAGAATCC	50
CAS	GGTTTACATATCGAGGGTCA	GAGCAAGGTTCCAGTCTATG	49

Table 4.3 Gene specific primers used for qRT-PCR analysis in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature (°C)
26S rRNA	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56
HMGR	AACGTCCTGGATTTCCTTCA	TCTTCACCACCTCCTCCTTG	55
SQS	ACTCAGTCAAGGCAGTGCAA	GCAGTAAGACCACGCCTCAT	59
FPPS	GGTTTAGGGTGCCTCAGGTT	GCCCTGAAGCTGTCTTGAAC	55
BETA	GAATTCGGGGACAGAGTGAA	GAGGTTGTGGGTGACTTCGT	55
CAS	AGTGCACCTCGGCATCTATC	TACAGCCCAAGAGCCATACC	55

3.8 Expression analysis of diosgenin biosynthesis pathway genes through quantitative real-time PCR (RT-qPCR)

cDNA was prepared from 5 µg of RNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (GeNei™) with oligo-dT primer. Equal sample quantities were verified by measuring the amount of RNA with a spectrophotometer. The qPCR was performed using gene specific primers in triplicate on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with the iScript one step RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 55–59 °C, followed by one elongation step for 20 s at 72°C. 26S rRNA and GAPDH were used as internal controls for normalization of gene expression. The significant differences between treatments were statistically evaluated by standard deviation in the form of error bars as mean ± SD for data recorded in triplicates (repeated thrice). The fold expression variation for pathway genes was calculated for high diosgenin content sample versus low content sample.

3.9 Statistical analysis

Data were recorded in triplicates and repeated thrice for *T. govanianum*. Descriptive analysis of the data was performed using SPSS 17.0. Analysis of variance (ANOVA) with comparative Duncan‟s multiple range tests at 5% was used to determine the significance of differences between replicates.

3.10 Anti-cancer study of *T. govanianum* extract (TG)

3.10.1 Cell lines for studying anti- cancerous effect of *T. govanianum* extract

Cytotoxic effect of TG extract was evaluated on three cell lines viz. Madin-Darby Canine Kidney Epithelial Cells (MDCK), Michigan Cancer Foundation-7 (MCF- 7) and MDA-MB-231. All three cell lines were obtained from the NCCS (National Centre for Cell Science), Pune. All three cell

lines were maintained at 37^0C inside humidified incubator. Cell lines were cryopreserved in vaporized liquid nitrogen for future use.

3.10.2 Cell Culture

Cells were grown in T25 culture flasks containing Dulbecco's modified Eagle medium (DMEM) for MDCK and MCF-7. Media was supplemented with 2.0 mM L- glutamine, adjusted to 3.7 g/L sodium bicarbonate, 4.5 g/L glucose. Likewise MDA-MB-231 cell were cultured in Leibovitz (L-15) media. Both DMEM and L-15 were supplemented with 10% FBS 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. Upon reaching confluency, the cells were trypsinized and passaged.

3.10.3 MTT assay

Principle:

Determination of cell cytotoxicity by spectrophotometric method was done by performing MTT assay. The underlying principle behind this technique is reduction of 3-)4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenase enzyme into purple colored formazan products in case of normal healthy cells. These products are solubilized by dissolving them with Dimethyl sulfoxide (DMSO). Since MTT is only reduced in metabolically active cells, therefore the level of metabolically active and metabolically inactive cells can be estimated through this assay.

Reagents:

1. MTT (5 mg/mL dissolved in 1X PBS)
2. DMSO
3. Test sample
4. Cystone
5. 2.0 mM Oxalate

Methodology:

The cytotoxicity studies were performed by MTT assay, according to well established method with slight modification at laboratory conditions (Baydoun et al. 2013; Mosmann, 1983) on three cell lines (MDCK, MCF-7 and MDA-MB-231). 1 x 10^4 cells were seeded into each well of a 96-well microplate and incubated for 24 hours at 37°C and 5% CO_2 in humidified chambers. The cells were then treated with different concentrations of crude extract and hydrolyzed extract (HE) (1ng/ml, 500ng/ml, 1µg/ml, 500µg/ml, 1mg/ml) were incubated for 24 h. 40µl MTT 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. 100 µl DMSO 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 630nm as reference wavelength to evaluate cell cytotoxicity by using microplate reader (Model 680, Bio-Rad). Triplicate wells were assayed for each condition and standard deviations were determined.

Calculations:

An increase in absorbance in this assay measures the extent of increase in the number of viable cells on exposure to oxalate in the presence of test sample. The cell viability was calculated by using the following formula:

% Cell viability = $(A_{sample} - A_{blank}/A_{control} - A_{blank}) \times 100$

where $A_{control}$ is the absorbance of untreated cells; A_{sample} is the absorbance of extract treated cells and A_{blank} is the absorbance of media only. Here, DMEM was used as blank.

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 by using the following formula:

$$\% \text{ Cell cytotoxicity} = \left[\frac{\text{Abs test}}{\text{Abs control}} \times 100\right]$$

3.10.4 Acridine orange (AO) and ethidium bromide (EtBr) for live/dead cells staining

Further, the best selected concentration was analyzed for cell morphological changes in MDCK, MCF-7 and MDA-MB-231 cell lines by dual AO and ETBR staining. Briefly, cells were seeded at a density of 2 $\times 10^5$ cells/well in 6 well plate comprising 1ml 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 24h. After desired time period the cells were harvested, washed with ice-cold PBS and fixed with 4% of para-formaldehyde for 30 min.

min. Afterwards, the supernatant was discarded and cells were treated with 1% triton X for 15min followed by washing of cells with PBS. Cells were stained with dye mixture; comprising 5µL of acridine orange (1 mg/mL) and 5µL of ETBR (1 mg/mL) for 5 min and then washed thrice with PBS. The stained cell with morphological changes were observed in fluorescence microscope at 200 X (Nikon Eclipse-80i, Japan).

3.11 *In vivo* anti-implantation activity of crude *T. govanianum* extract

3.11.1 Animals under study

Wistar rats (200-250 g) were procured from the Central Animal Facility, National Institute of Nutrition, Hyderabad, India, and were housed in the polystyrene cages inside animal house of Jaypee University of Information Technology, Solan, Himachal Pradesh, India. The animals were maintained under standard conditions with 21.5±2ºC temperature, 60±1% humidity and 12 h light/dark cycle. Animal were fed standard rat pellets and were having free access to water. Entire experimental procedures were performed after approval from the Institutional Animal Ethical Committee and all the guidelines of CPCSEA were strictly followed. All efforts were made to minimize suffering to the animals.

3.11.2 Experimental Design

The method described by Williamson et al. (1996) and Tafesse et al. (2005) was used. The rats exhibiting the copulation plug or thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day 0.5 of pregnancy, and those rats were divided into three groups:

$$\% \text{ Anti-implantation activity} = \left[\frac{\text{No.of implants in control} - \text{No.of implants in test group}}{\text{No.of implants in control group}} \times 100 \right]$$

Table 3.4 Experimental design for Anti- implantation activity study

Group	**Dose**
Group I	Served as control and received only vehicle (CMC, 0.3% w/w in saline)
Groups II	Received the extract TG orally at the dose level of 125 mg/kg from day 1 to day 10 of pregnancy
Group III	Received the extract TG orally at the dose level of 250 mg/kg from day 1 to day 10 of pregnancy

Esterous cycle in female Wistar rats was regularly monitored and two female rats were caged with one male rat (2:1) on the evening of proestrous stage. Female animals were examined after 12 h next day morning for the evidence of copulation by taking vaginal smears. The rats tested positive for the copulation plug or thick clumps of spermatozoa in vaginal smears were separated and that day was designated as the day 0.5 of the pregnancy. Animals were then divided into three groups as mentioned in Table 3.4. All the treatments were continued for 11 days and on day 12 animals were sacrificed under anesthesia (90 mg/kg ketamine and 5 mg/kg xylazine), dissected, uterus was weighed and examined to determine the number of implantation sites. Besides, changes in body weight was measured regularly (Khanna and Chaudhary, 1968; Mohamed et al., 2012).

3.11.3 Histopathological examination

To determine the effect of TG extract on uterus morphology, uterus along with fallopian tube and ovaries were dissected and fixed with 4% formalin solution. Fixed tissue was subjected to microtome sectioning and 5 µm thick sections were prepared. Sections were subjected to hematoxylin- eosin staining to visualize cellular morphology. Nucleus was stained blue and cytosol stained pink. Images were captured at 400X magnification using light microscope (Nikon) and were analyzed for any signs of damage.

3.11.4 Statistical Analysis

Results are depicted as mean ± SD. Statistical significance was determined using one way ANOVA followed by Dunnett's multiple comparison test test, by using Graph pad prizm 6 software. All ten results were compared to control and statistical significance was determined at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extractive yield from different accessions of *T. govanianum*

Extraction is the first step for any of the plant species of high value medicinal value. Extraction methodology for various plant materials can be done with vast development taking in modern chromatographic and spectrometric data analysis. Thing that should always be taken care of the environment, less use of any synthetic and organic chemicals, reduce operational time and finally getting better yield and quality of extract (Wijngaard et al. 2012; Azmir et al. 2013).

In nature the great variation is seen among all the plant secondary metabolites which are always being further extracted from complex plant sample, modifying the efficacy and lastly preparation of herbal formulations (Azmir et al. 2013). Out of various classical existing extraction methods like Soxhlet extraction, Maceration and Hydrodistillation, Soxhlet which was first proposed by German chemist Franz Ritter Von Soxhlet (1879) was the best as the process runs repeatedly until the extraction is completed. Comparing to Maceration which is a homemade method for preparation of tonic and disadvantage being occasional shaking and use of more solvent. In Hydrodistillation method the major disadvantage is that at a high temperature some volatile components may be lost thus limits the use of thermos labile compound extraction.

In our study we have done Soxhlet extraction methodology with all the solvents according to eluotropic series starting from Hydroalcholic (30:70; Water: Methanol), Isopropanol, Acetonitrile, Ethyl acetate, Chloroform, Diethyl ether, Hexane was carried out at room temperature and lastly extractive yield was calculated. Extractive yield was found to be in the range of 33.26-43.56%, 22.41-43.82% and 26.37-47.21%, respectively for Hydroalcholic, Hexane and Diethyl ether extract (Fig. 4). The extractive yield of other were very close depending upon their polarity and eluotropic values.

It should be noted that extractive yield percentage has no relevance to Diosgenin percentage in all the accessions from TG-1 to TG-12. Later the extract was stored at -4^0C until further use.

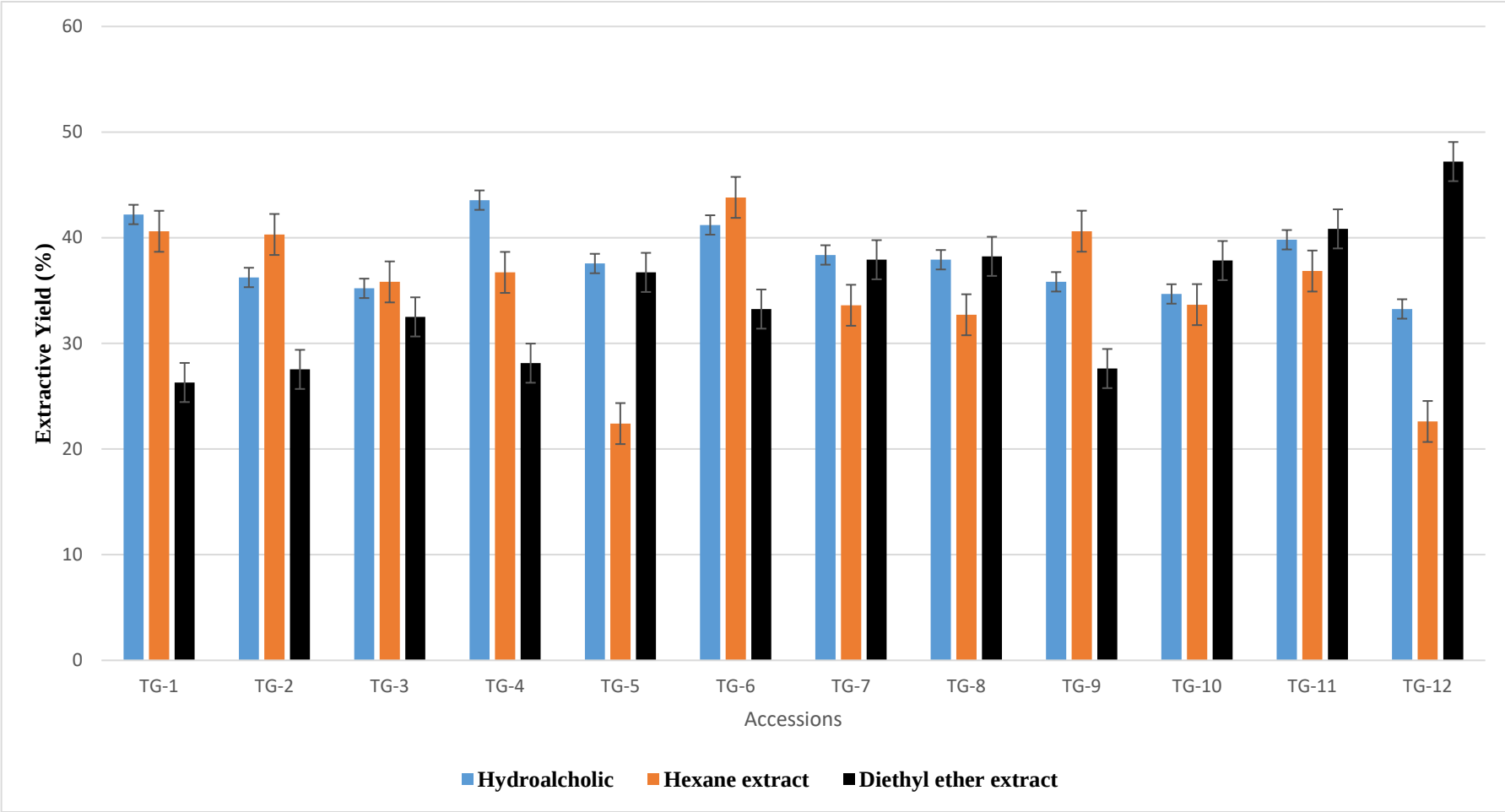

Fig 4.1 Percent extractive yield in different accessions of *T. govanianum*. These include extractive yield for Hydroalcoholic solvent (30:70; Water: Methanol) in the range of 33.26-43.56%, for Hexane in the range of 22.41-43.82% and for Diethyl ether in the range of 26.37-47.21%.

4.2 Diosgenin content from different accessions of *T. govanianum*

Diosgenin which is widely used starting material for semi-synthetic production of steroid drugs can be obtained by acid hydrolysis from the plants. Plants contain spirostanol and furostanol saponins which upon hydrolysis lose their sugar moieties to become sapogenin and mostly diosgenin.

The extract after Soxhlet extraction which was stored in -4^{0}C, and further used for hydrolysis as per method described by Drapeau et al. (1986). For diosgenin extraction from the plant extract 1500 mg of plant extract was weighed and kept in round bottom flask and refluxing was done with 150 ml of 20% H_2SO_4 in 70% isopropanol for 12 hours of duration.

Extract was subjected to HPLC analysis at cycle of 30 mins at 25^{0}C with injection volume of 10µl. Upon successful completion of HPLC analysis the observations revealed that diosgenin content was highest in hydroalcholic extract of TG-1 sample (2.4%), which was collected from Chamba region of Himachal Pradesh. Comparatively hydroalcholic extract showed higher levels of diosgenin content as compared to hexane and diethyl ether extracts. Diosgenin content was found to be in the order, hydroalcholic extract > hexane > diethyl ether (Fig. 4.2, 4.3). This can be explained on the basis of the polarity of solvents used in extraction. These results are in agreement to the results of diosgenin content from *Elephantopus scaber* where many solvents of different eluotropic series were used and the best result with highest diosgenin content was seen in aqueous solvent (Kharat et al. 2015). The level of diosgenin was observed to be higher in hexane extract as compared to diethyl ether (having higher polarity). Previous reports on diosgenin content and relation to solvent used was reported by Thankappan (2014) showing that solvent with highest polarity methanol has highest diosgenin content (0.7 %) and minimum in least polar petroleum ether (0.4 % diosgenin). Hydroalcholic extraction is a well-established method to extract compounds of all polarity from the plant samples (approx. 95% extraction) which might have resulted in highest amount of diosgenin in this extract (Gupta et al. 2012).

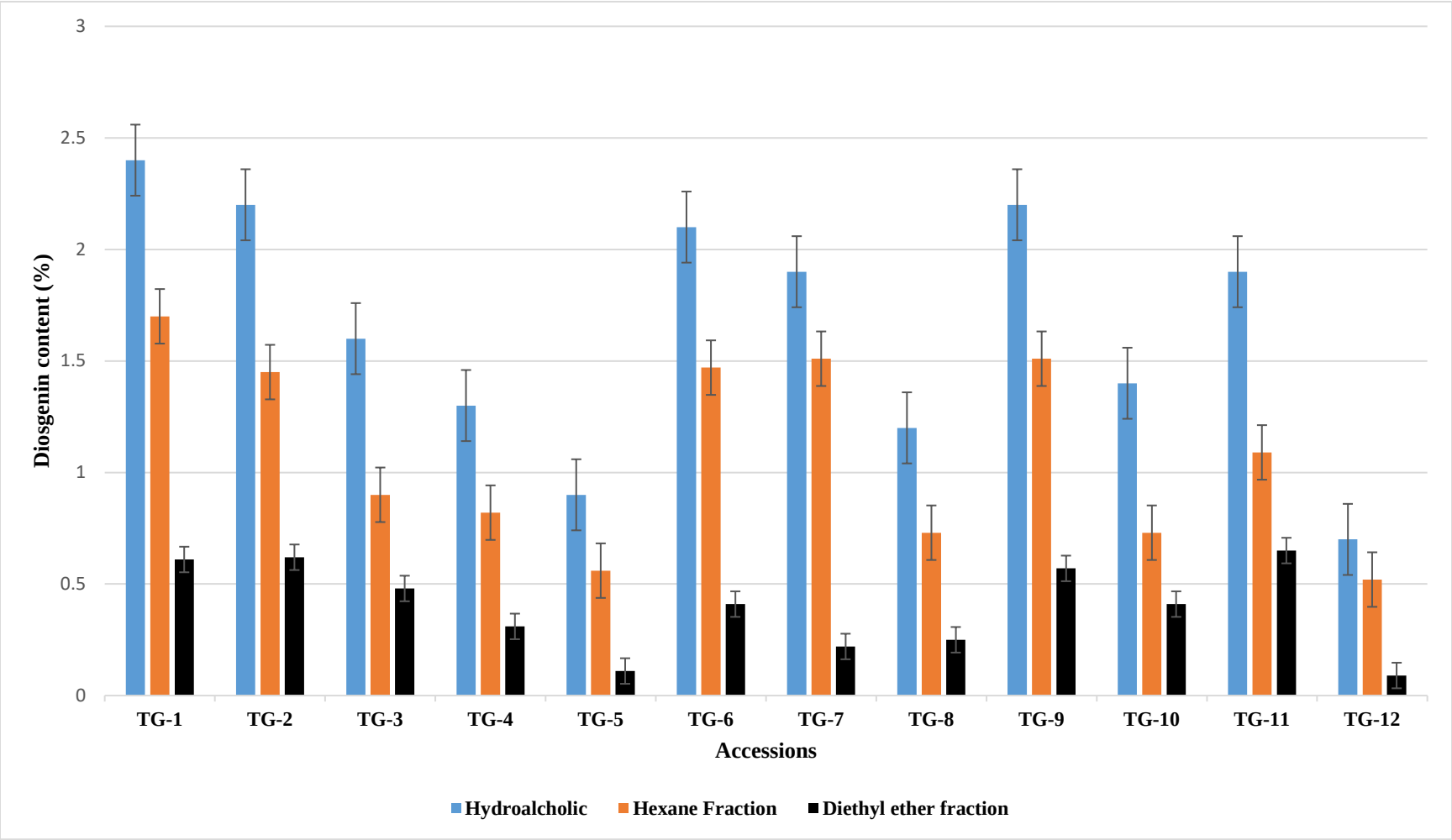

Fig 4.2 Percent diosgenin content in the Hydrolyzed extract from Hydroalcholic, Hexane and Diethyl ether soxhlet extracts of different accessions of *T. govanianum* from TG-1 to TG-12. TG-1 was sample from Chamba region of H.P and found to be having 2.4% of diosgenin as compared to lowest content 0.7 % in Diethyl ether extract.

It was clear from graphical representation (Fig 4.2) that Hydroalcoholic method of extraction was found to be the best for eluting all major phytochemicals from rhizomes of *T. govanianum* and later on that this extract only should be used for hydrolysis as it contains maximum diosgenin when compared between all accessions from TG-1 to TG-12. HPLC chromatogram (Fig 4.3) clearly depicts that diosgenin content is extracted to maximum through Hydroalcholic (30:70; Water: MeoH) soxhlet extraction, when compared to Hexane extract and Diethyl ether extract.

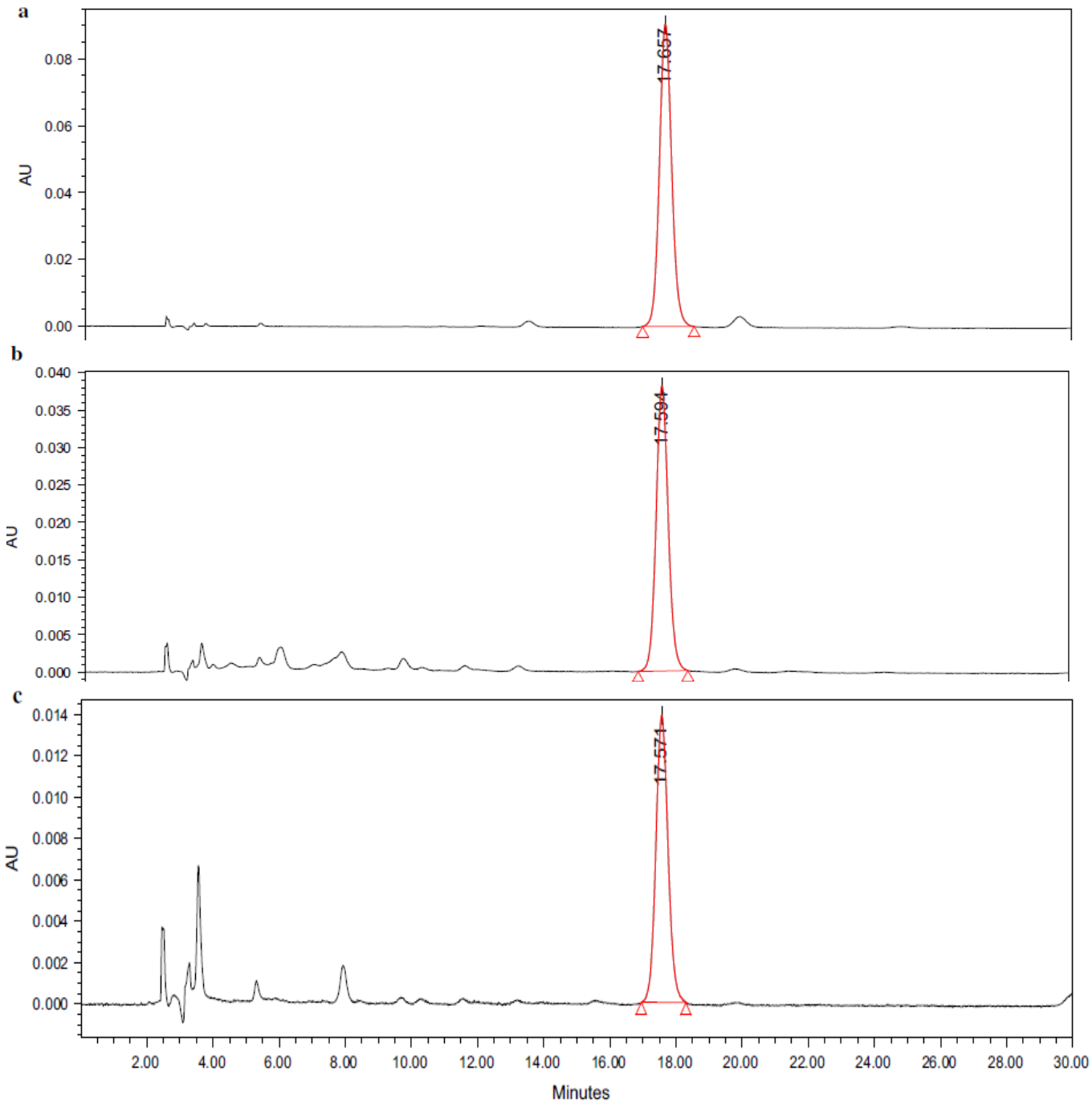

Fig. 4.3 HPLC chromatograms of (a) Diosgenin standard (b) Rhizome samples of *T. govanianum* from TG-1 (Chamba region) with maximum content (2.4 % diosgenin) and (c) Rhizome samples of *T. govanianum* from TG-12 (Lahaul & Spiti region) (0.7% diosgenin).

4.3 Statistical optimization for maximum diosgenin content by Response surface methodology and Diosgenin extraction: Hydrolysis and neutralization

Mostly the content of phytopharmaceuticals from plants are very minimum thus requiring optimization process which can be achieved either by classical or statistical methods. Classical method always being very tedious one as it involves variation of all factors, time consuming and incapable to detect true optimum conditions (Liu and Tzeng 1998). Statistical method being easy, effective are used to evaluate the role of multiple factors and their interaction with one or more variables (Myers and Montgomery 2002). We used Response surface methodology(RSM) which can take into consideration all the variables of effective hydrolysis like solvent concentration, extraction time, temperature (Wettasinghe and Shahidi 1999; Xu et al. 2013), and solvent to solid ratio (Cacace and Mazza 2003) with highest diosgenin content from the sample.

Out of all twelve samples i.e TG-1 to TG-12, TG-1 was having highest diosgenin content (2.4 %) and taken into consideration for RSM optimization using a statistical software package Design Expert® 8.0.7.1, Stat-Ease. To validate the statistical experimental strategies, 20 set of experiments were carried out in triplicates under selected conditions along with one control sample processed under the same conditions as mentioned by Drapeau et al. (1986). The three factors and lower, middle and upper design points for RSM along with the experimental results of diosgenin content (%) are shown in Table 4.1. 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 (%) = $4.97+2.27\,A- 0.22\,B + 0.70\,C- 0.14\,AB + 0.14\,AC + 0.061\,BC - 1.89A^2 -10.94\,B^2 -0.25\,C^2$ where A is incubation time (hr), B is temperature (^{0}C) and C is solid-liquid ratio (gm/mL).

After getting experimental values as shown in Table 4.1 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. The R^2 value explains the level of variability in the model, also Le et al. (2010) and Chauhan and Gupta

(2004) have emphasized that R^2 should be > 0.75 but in contrast our R^2 was 0.87 thus proving the significance of our model. The model equation was statistically significant as determined by Analysis of Variance (ANOVA).

The cumulative effect of the selected variables i.e. incubation time, temperature and solid-liquid ratio are represented as contour plots (2D) as shown in Figure 4.4 which 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 (Fig. 4.4a). In contrast to this Fig. 4.4b showed maximum diosgenin content at high levels of solid-liquid ratio while minimum at low and high levels of incubation time. From Fig. 4.4c 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: 12hrs, temperature: 85°C and solid-liquid ratio: 1:234 g/mL), the maximum diosgenin content was 5.99% which was highest reported till date.

Finally, RSM was successfully used to determine the optimum extraction conditions for diosgenin after hydrolysis, and the results are in congruence with the previous reports, which showed a significant increase in metabolite content using RSM for getting maximum yield of picrosides in *Picrorhiza kurrroa* (Kumar et al. 2015; Pandit et al. 2012; Bhandari et al. 2008; Singh et al. 2011)

Table 4.1: Factors and levels in the response surface central composite design arrangement along with the experimental and predicted results for Diosgenin content (%)

X1- Incubation time (Hr), X2- Temperature (°C) and X3- Solid: Liquid ratio (g/mL)

No.	X1	X2	X3	Experimental Diosgenin %	Predicted Diosgenin %
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

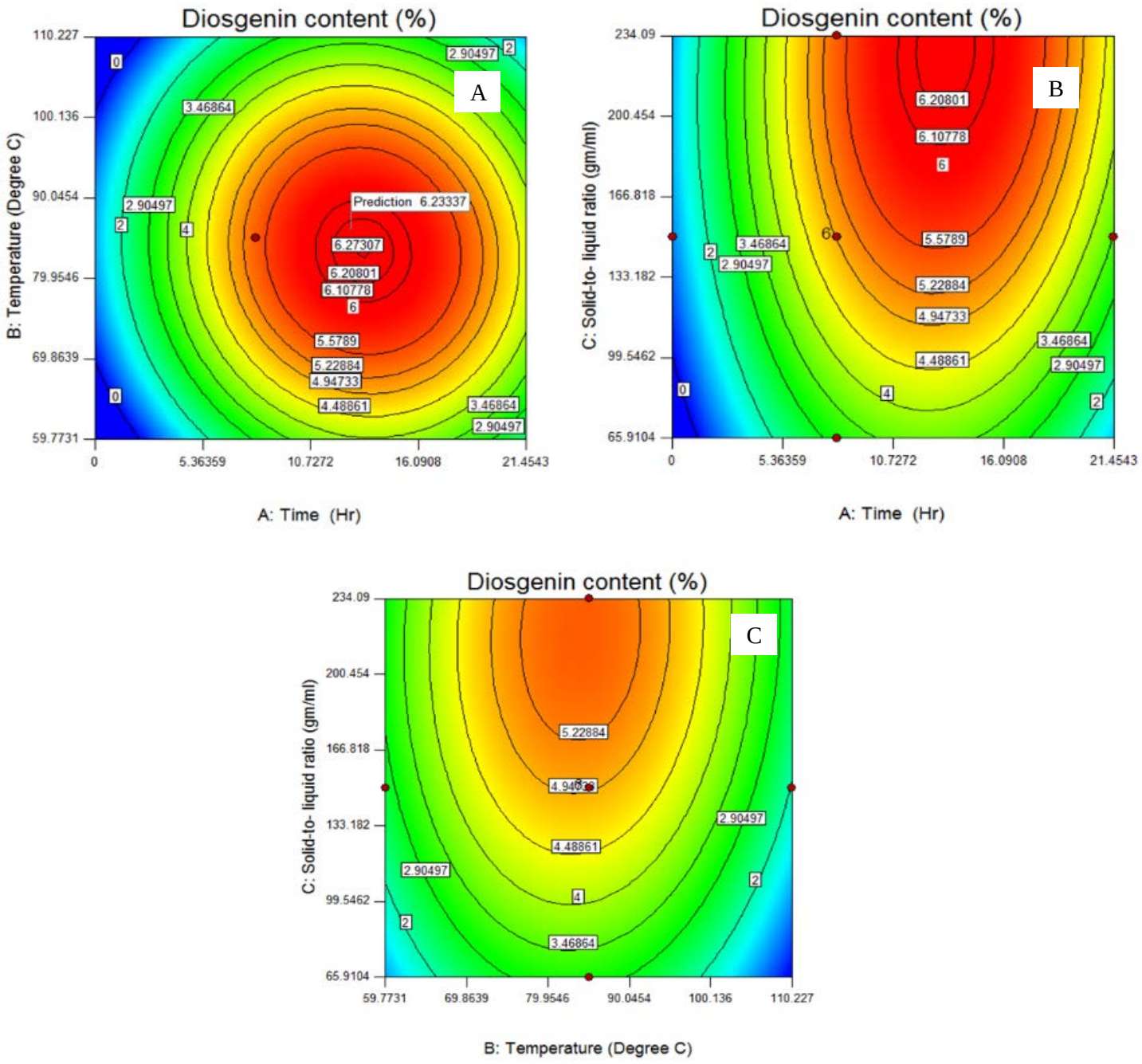

Fig. 4.4. (a) Contour plot (2D) showing the effect of Time and Temperature on the yield of Diosgenin

(b) Contour plot (2D) showing the effect of Time and Solid-Liquid ratio on the yield of Diosgenin

(c) Contour plot (2D) showing the effect of Temperature and Solid-Liquid ratio on the yield of Diosgenin.

4.4 Discerning the expression status of steroidal pathway genes vis-a-vis diosgenin content in different accessions of *T. govanianum*

4.4.1 Cloning steroid pathway genes in *T. govanianum*

Partial gene sequences of 5 genes encoding key enzymes viz. HMG Co-A reductase (HMGR) squalene synthase (SQS), farnesyl pyrophosphate synthase (FPPS), 26-O-beta-glucosidase (BETA) and cycloartenol synthase (CAS), involved in diosgenin biosynthesis pathway were cloned from *T. govanianum* through comparative genomics using primer pairs designed from gene regions conserved across several plant species (Table 4.2). The amplicons for all gene fragments ranging from 300-600 bp, were sequenced and annotated for biological function. These sequences were further utilized for designing species specific primers for gene expression analysis (Table 4.3).

Table 4.2 Primer sequences used to clone five genes of diosgenin biosynthetic pathway in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature (°C)
HMGR	CTTCCGATGTTTTACCTTTG	GCAGGCTTCTCTATGATGAC	52
SQS	TTGAACGACATGGTCACTAA	CATTGTAGATGTTGGTGCAG	54
FPPS	AATGTCTGGTGAAGATTTGG	AAGAGATCAGCTGCTCGTAG	52
BETA	AGTCTGAGCTGATCAAAGGA	TGAACCACTTGGTAGAATCC	50
CAS	GGTTTACATATCGAGGGTCA	GAGCAAGGTTCCAGTCTATG	49

Table 4.3 Gene specific primers used for qRT-PCR analysis in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature (°C)
26S rRNA	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56
HMGR	AACGTCCTGGATTTCCTTCA	TCTTCACCACCTCCTCCTTG	55
SQS	ACTCAGTCAAGGCAGTGCAA	GCAGTAAGACCACGCCTCAT	59
FPPS	GGTTTAGGGTGCCTCAGGTT	GCCCTGAAGCTGTCTTGAAC	55
BETA	GAATTCGGGGACAGAGTGAA	GAGGTTGTGGGTGACTTCGT	55
CAS	AGTGCACCTCGGCATCTATC	TACAGCCCAAGAGCCATACC	55

4.4.2 Expression analysis of diosgenin biosynthetic pathway genes

The qRT-PCR analysis revealed that all five genes viz. HMGR, FPPS, SQS, BETA and CAS involved in diosgenin biosynthesis had relatively higher expression in the rhizomes of Chamba (2.4%) (up to 3.2 folds) ($p<0.05$) as compared to Lahaul and Spiti (0.7%) (Fig. 3). These results could be positively correlated to the findings of Mehrafarin et al. (2010) and Vaidya et al. (2013) which suggested the significance of these genes for diosgenin production in *Trigonella foenum-graecum*. Previous reports on aconites biosynthesis in *A. heterophyllum* (Malhotra et al. 2014, 2016a, b) and picrosides biosynthesis in *Picrorhiza kurroa* (Kumar et al. 2015) also demonstrate this scenario very well. As HMGR catalyzes the first rate-determining step of isoprenoid biosynthesis in MVA pathway (Wang et al. 2007), it showed highest expression of 3.2-fold ($p<0.05$) compared to all other genes in *T. govanianum*. Relatively higher expression of HMGR gene was positively associated with its role in shikonin and aconites biosynthesis in *Arnebia euchroma* and *A. heterophyllum*, respectively (Singh et al. 2010; Pal et al. 2015). It has also been shown to regulate mevalonate pathway by acting as a feeder to GPP, thereby influencing phytosterol biosynthesis (Nogue´s et al. 2006). The enzyme coding for CAS was upregulated by 2.5-fold (p\0.05). CAS has been known to play a vital role in plant cell viability and regulation of triterpenoid production. The biosynthesis of sterols in *Nicotiana benthamiana* is reported to be controlled by CAS (Gas-Pascual et al. 2014), while it's essential role in plant development has been widely characterized in *Arabidopsis thaliana* (Babiychuk et al. 2008). Further, enzymes coding for SQS, FPP and BETA were upregulated by 1.3-fold ($p<0.05$), 2.1-fold ($p<0.05$) and 1.7-fold ($p<0.05$), respectively.

They are also known to be involved in steroid biosynthesis (Vaidya et al. 2013). SQS is a bifunctional enzyme which catalyzes the condensation of two molecules of FPP to squalene, the main precursor for sterol and triterpene biosynthesis in plant cells (Huang et al. 2007; Lee and Poulter 2008). A study by Devarenne et al. (2002) has shown that it regulates sterol biosynthesis in *N. tabacum*. FPPS has been found to be involved in regulating the supply of precursors for the production of carotenoids, dolichols, with anolides besides helping in farnesylation of proteins (Dhar et al. 2013). It also acts as an essential enzyme for organ growth in plants, although it has not been, earlier, recognized as a key regulatory enzyme in triterpene biosynthesis (Kim et al. 2010). BETA is hypothesized to be involved in the last stage of diosgenin synthesis by removing

the glucose molecule and conversion of sterol 3-beta-D glucoside to diosgenin (Vaidya et al. 2013). It may also act in defense mechanism in the form of torvoside in Solanum torvum by releasing aglycone to dissuade pathogens, although this requires advanced studies for corroboration (Arthan et al. 2006). Overall, the current study implied the role of key genes which might influence the diosgenin biosynthesis in *T. govanianum*. For biosynthetic pathways, molecular dissection of genes/enzymes is required which necessitates the metabolic engineering of pathways to meet the growing requirements of required secondary moieties in target plant species. This study, hence, offers a dais for developing an appropriate genetic intervention strategy to raise the production of diosgenin in *T. govanianum*.

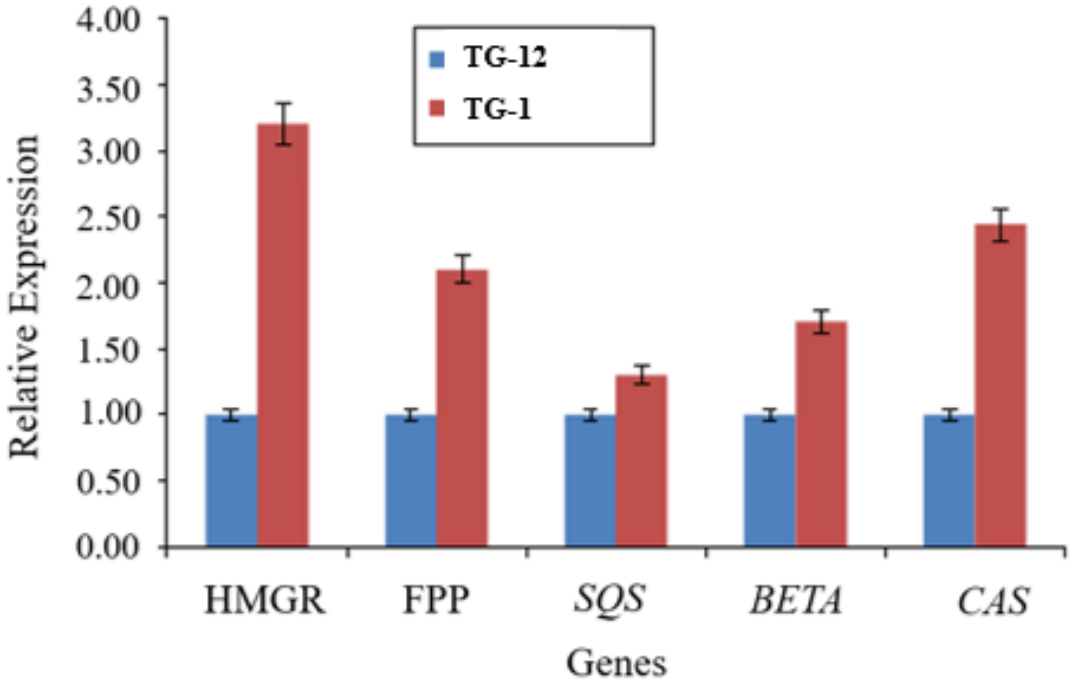

Fig. 4.5 Relative expression of key genes involved in diosgenin biosynthesis pathway in rhizomes of *T. govanianum* from Chamba (2.4%) and Lahaul & Spiti (0.7%). Error bars represent mean ± SD for data recorded in triplicates (repeated thrice). Significance was evaluated for each gene between both samples ($*p < 0.05$).

4.5 *In vitro* cell line study of crude extract from *T. govanianum*

4.5.1 Anti- cancerous effect of *T. govanianum* extract (MTT Assay)

Crude rhizome extract of *T.govanianum* showed less toxicity on MDCK cells (Fig. 4.6a) with IC_{50} value of 2.1 µg/ml, compared to cell viability of MCF-7 and MDA-MB-231 cells, which showed IC_{50} values of 2.03 and 1.8 µg/ml, respectively (Fig 4.6d). Likewise, hydrolyzed extract of *T. govanianum* was observed to be less toxic towards normal MDCK cells (Fig 4.6a), with IC_{50} value of 1.7 µg/ml. A significant reduction in the proliferation of MCF-7 (Fig. 4.6b) and MDA-MB-231 cells (Fig. 4.6c) was observed with IC_{50} values of 1.11 and 0.495 µg/ml, respectively. These results suggest that extract is less toxic against normal cells but showed a significant anti-proliferative activity on cancer cells (MCF-7 and MDA-MB-231). On the contrary, hydrolyzed extract had less toxicity towards normal cells, whereas considerably reduced cancer cell viability, suggesting that anti-cancerous bioactive moiety might be generated after hydrolysis process of the extract. Cytotoxity of diosgenin has been previously established on many cell lines like V79 fibroblast and K562 cells (Liu et al. 2005; Melo et al. 2004). 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 cell lines. This may be due to the presence of diosgenin and other steroidal saponins compounds in *T. govanianum* as those have been reported for their anticancer activity (Ur Rahman et al. 2015).

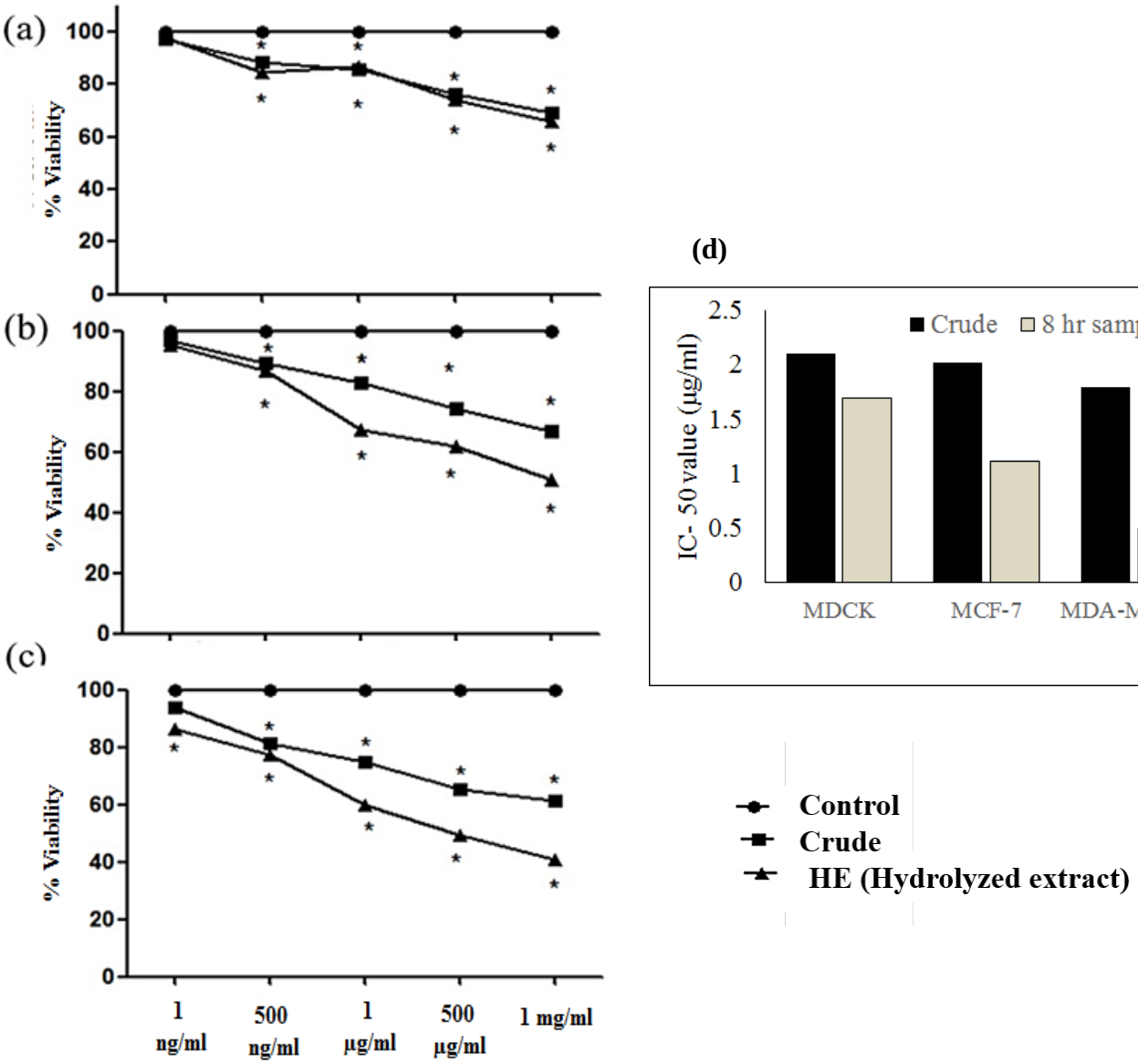

Fig. 4.6. Inhibition of proliferation in MDCK (a), MCF-7 (b) and MDA-MB-231(c) cells after treatment. Results are presented as percentage of control (Untreated cells) and values are expressed as means ± SD of six experiments (* p-value relative to control group, $p<0.05$) (d) IC_{50} values of crude extract and 8 hr hydrolyzed extract are depicted in graph above

4.5.2 Cell and nuclear morphology (Acridine orange (AO) and ethidium bromide (ETBR) for live/dead cells staining)

AO/ EtBr staining (Live dead staining) is widely used technique to evaluate cellular morphology and to demonstrate cell viability (Kasibhatla et al. 2006). We used this method to evaluate effect of crude and hydrolyzed TG extract on the morphology of MDCK (Normal cells), MCF-7 and MDA-MB-231 (Breast cancer cells) (Fig. 4.7, 4.8, 4.9), besides evaluating cell viability in term of EtBr stain (dead cells) and AO stain (alive cells). We observed that crude and hydrolyzed extract were non-toxic towards MDCK cells, as number of viable cells (AO fluorescence) in extract treated and control wells did not differed significantly. Hydrolyzed extract resulted in higher cell death when compared to crude extract treated cells, however, number of dead cells were non-significantly different from control well. These results suggest that neither of the extract is toxic towards normal cells at the tested concentration (Fig. 4.7).

To evaluate anticancer effect of crude and hydrolyzed extract we used MCF-7 and MDA-MB-231 cell lines and subjected them to AO/ EtBr staining. It was observed that both, crude and hydrolyzed extract, were highly toxic towards cancerous cells, as number of viable cells (AO fluorescence) were significantly lower than that of control. These results were confirmed from EtBr staining were number of dead cells (EtBr fluorescence) were observed to be significantly higher in extract treated cells when compared to control. Further, hydrolyzed extract showed higher cytotoxicity when compared to crude extract. Both the extract showed higher toxicity towards MDA-MB-231(Fig 4.8) cells as compared to MCF-7 cells (Fig 4.9). These results suggest that TG extract is having high anti-cancerous potential, which was significantly enhanced by subjecting crude extract to hydrolysis, indicating that hydrolysis process result in production of some anti-cancerous bioactive moiety which is having higher specificity towards MDA-MB-231 cells as compared to MCF-7. Previous reports by Sundarraj et al. (2012) also used AO/EtBr staining to investigate the inhibitory effect of *Acacia nilotica* leaves extract on cell proliferation and studying the apoptotic effect and cell cycle arrest in MCF-7 and A549 cell lines. Experimental results were found suggesting sitosterol from leaves extract to be one of the potential anticancer agent and thus proving AO/EtBr one of the best method for anti-cancer studies.

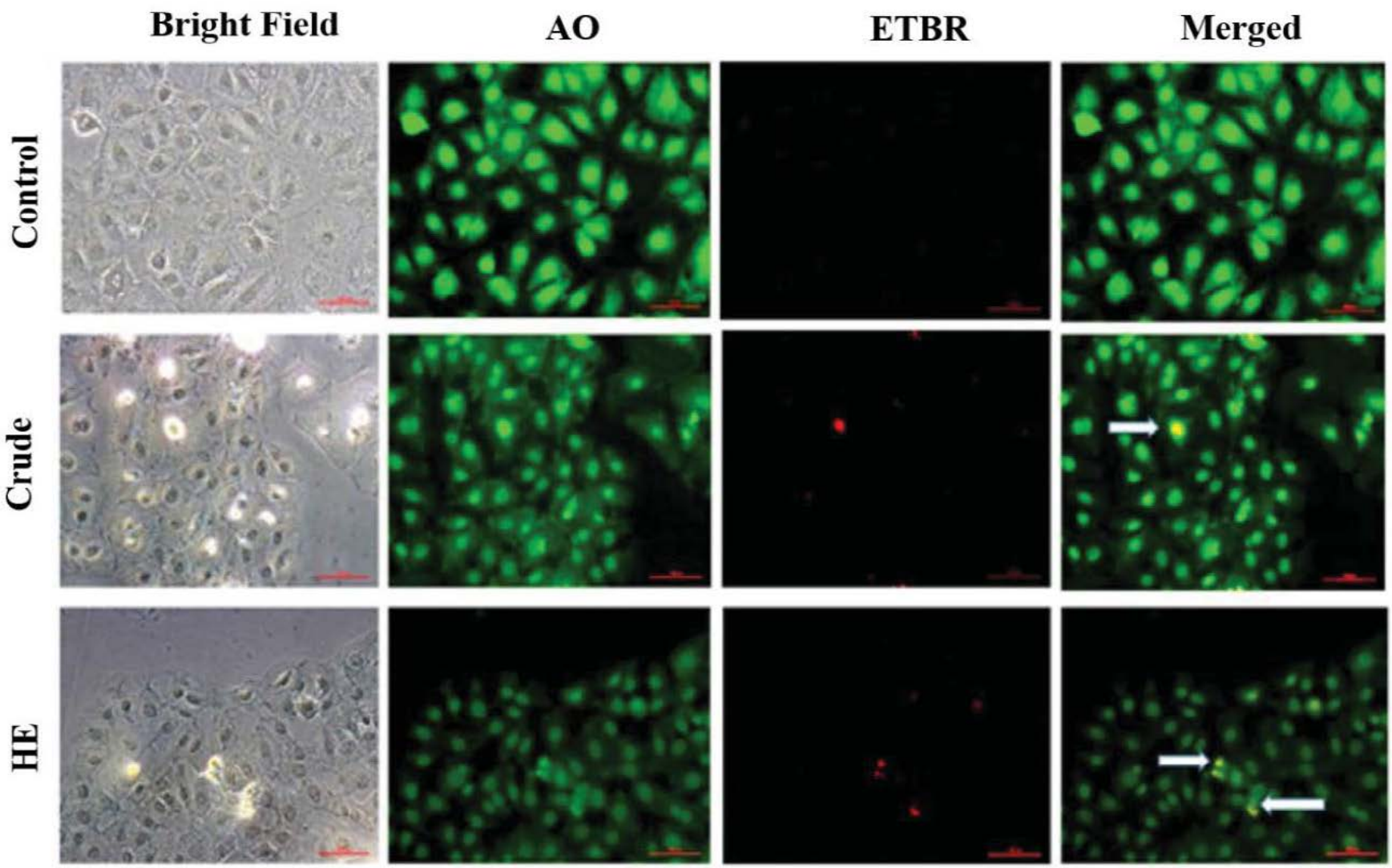

Fig. 4.7 Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDCK cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X).

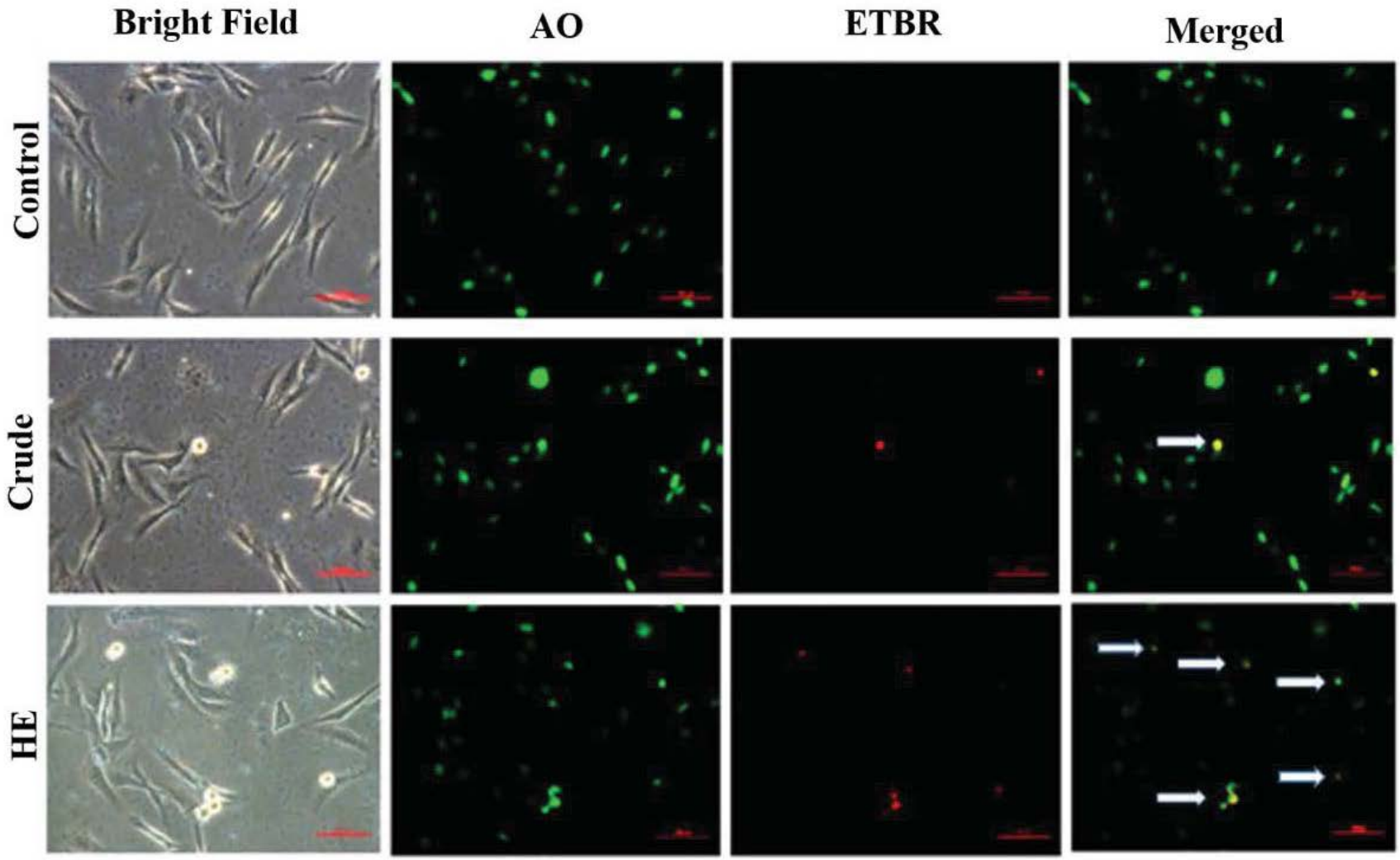

Fig. 4.8 Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDA-MB-231 cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X)

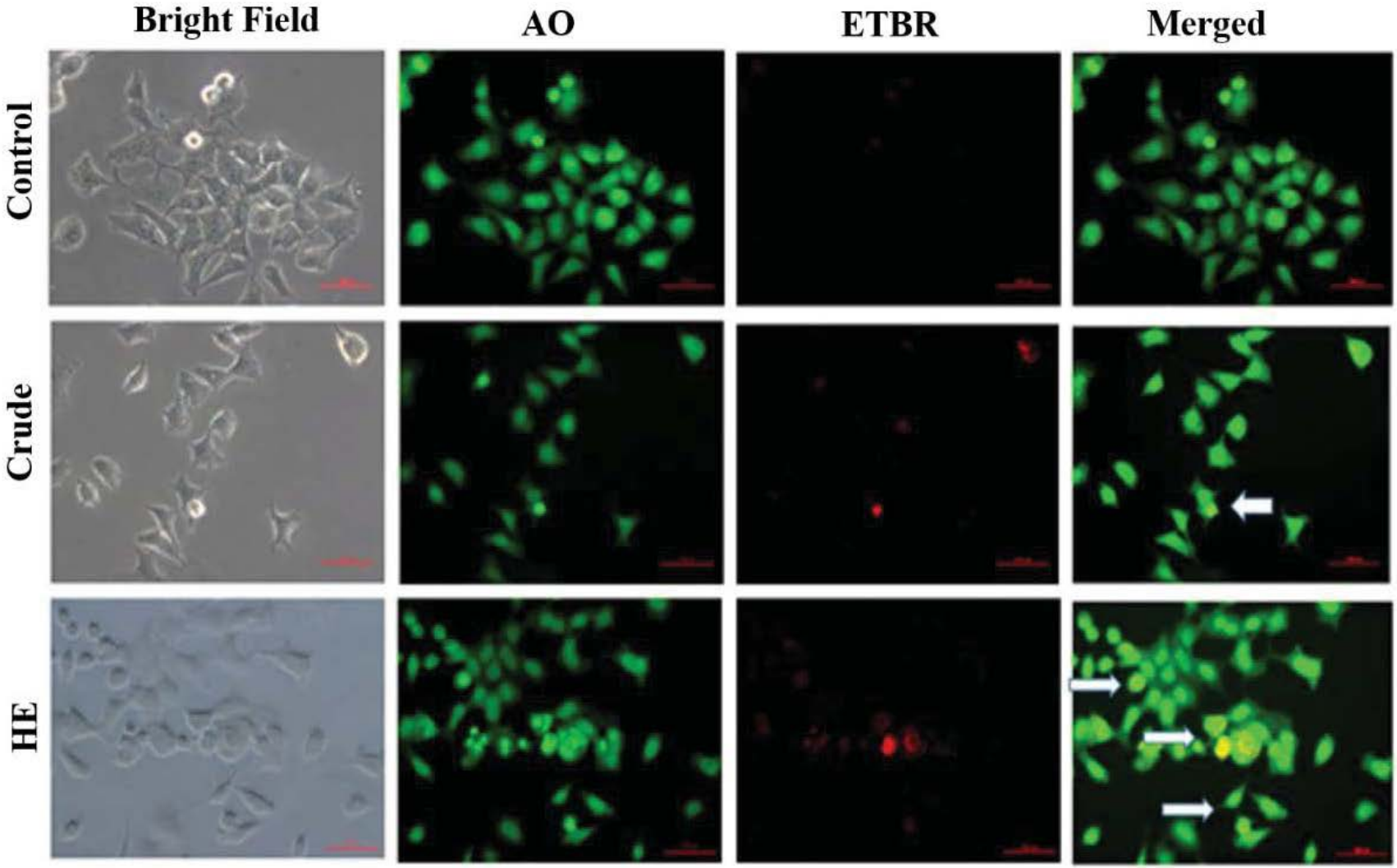

Fig. 4.9 Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MCF-7 cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X).

4.6 *In vivo* anti-implantation activity of TG extract

4.6.1 Anti-implantation activity

Anti-implantation effect of TG extract was evaluated in fertile and healthy female Wistar rats at 125 and 250 mg/kg oral extract treatment and results are depicted in Table 4.4. Control animals demonstrated fertility and were observed to have 7.8 ± 0.84 implantation sites. TG extract showed very strong dose dependent anti-implantation effect. Number of implantation sites were significantly reduced in animals treated with 125 mg/kg extract and none of the female was observed to have any implants in the uterus when treated with 250 mg/kg extract. Extract treatment resulted in 94.87% and 100% inhibition of implantation at 125 and 250 mg/kg dose respectively. Strong anti-implantation effect of the plant extract may be attributed to its potential to significantly elevate serum levels of estrogen, which were observed to be 106.72 and 216 pg/ml at 125 and 250 mg/kg extract treatment respectively, when compared to control (100.84 pg/ml). Further, animals did not showed any significant body weight change, however, animals treated with plant extract showed significant increase in uterus weight and uterus weight was observed to be 62.3 ± 5.81, 89.51 ± 8.11 and 112.21 ± 12.11 mg in control, 125 mg/kg and 250 mg/kg extract treated groups respectively. These results suggest that plant extract possesses strong anti-implantation effect, which may be attributed to its potential to elevate serum estrogen levels.

Table 4.4. Effect of plant extract on implantation in female rats.

Treatment	**Dose (mg/kg)**	**Anti-implantation effect on Group 1- Group 3**				
		Body weight change (%)(mean)	No. of implantation sites (mean)	% Inhibition of implants on day 12.5	Estrogen (pg/ml)	Uterus weight (mg)
Control	-	5.48±0.81	7.8±0.84	0%	100.84±5.21	62.3±5.81
Plant Extract (TG)	125	1.64±0.3	0.4±0.55	94.87%	106.72±6.11	89.51±8.11
	250	8.09±0.61	0	100%	216±15.81	112.21±12.11

4.6.1.1 Histopathological examination of ovaries and uterus

We performed histopathological examination of uterus and ovaries of extract treated and control animals to evaluate the effect of various treatment on morphological alterations. Results of the histopathology are depicted in Figures 4.10 and 4.11. Histopathological examination revealed healthy morphology of uterus and ovaries in control animals. However, treating animals with extract resulted in thickening of endometrium in uterus in dose dependent manner, which might have interfered with the implantation of fetus on to the endometrium wall. Morphology of ovaries were normal in extract treated animals and showed no signs of any damage.

In TG extract 100% implantation inhibition was observed at 250 mg/kg dose, which is also a property of other medicinal plant like flower extract of *Leucas cephalotes*, leaves extracts of *Michelia champaca* (Taprial et al. 2013) etc. However, 94.87% inhibition was also observed in animals treated with 125 mg/kg extract. TG extract was better in inhibiting implantation than other reported medicinal plants as its dose was significantly lower (125 mg/kg) than that reported for other plants (upto 400mg/kg). The loss of implantation at higher dose of prepared extract may be due to anti-zygotic or blastocytotoxic activity. (Taprial et al. 2013; Bhoria et al. 2013).

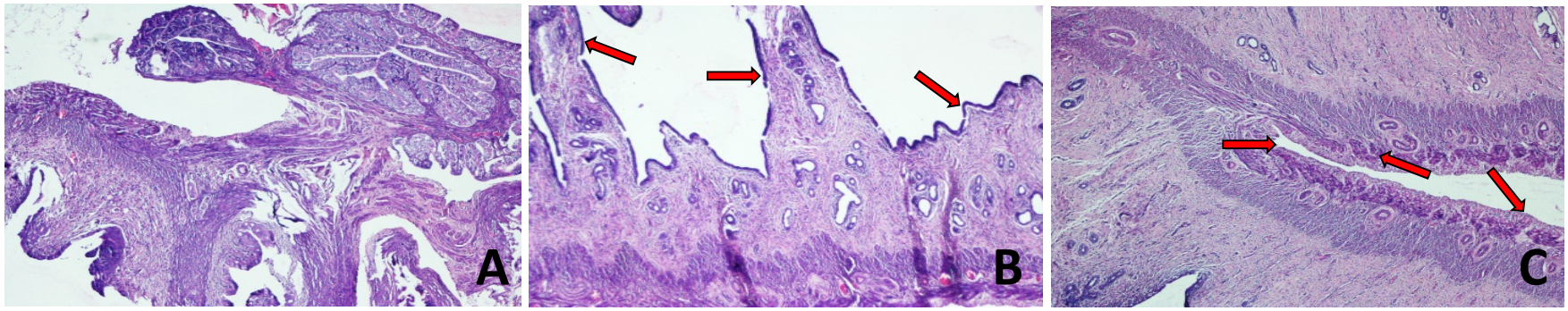

Fig. 4.10 Histological sections of uterus of the Control (A), TG extract 125 mg/kg (B) and TG extract 250 mg/kg (C). It was clear from the above picture that walls of uterus in control female rats were normal but in treated thickening of endometrium was observed.

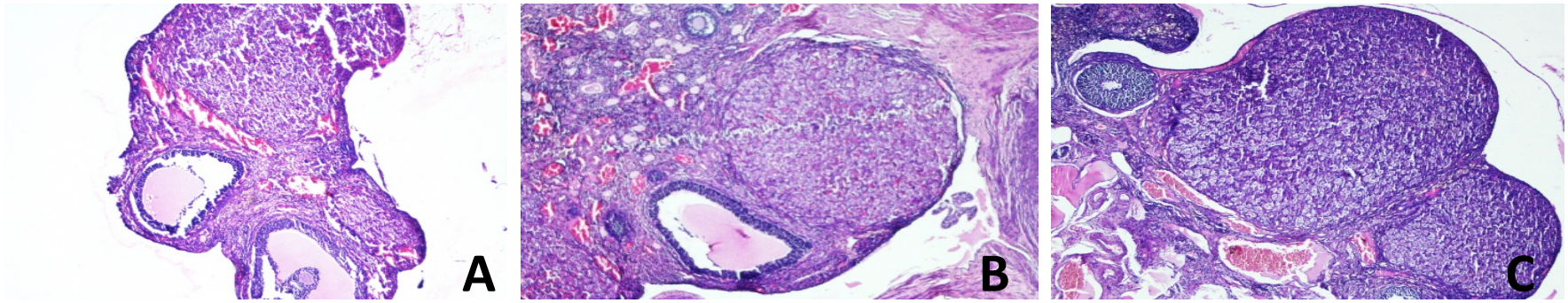

Fig. 4.11 Histological sections of ovary of the Control (A), extract 125 mg/kg (B) and extract 250 mg/kg (C). Ovary were normal and nor damage by TG extract.

For proper implantation and sustenance of pregnancy, exact equilibrium of progesterone and estrogen is necessary. Most of the cases anti-implantation and abortion are the reasons due to imbalance in progesterone and estrogen level (Psychoyos, 1996). There are many reports which proves that phenolics, alkaloids, flavonoids including steroids (Natraj et al. 2007) from variety of plant species show anti-fertility in laboratory animals (Anderson et al. 1972; Khushalani et al. 2006). Talking precisely about diosgenin, reports are available which proves that ethanol extract of *Dioscorea esculanta* resulted in depletion in sperm count when administered to male rats. This study revealed decrease in serum level of testosterone and thus reduction in testosterone level may impair spermatogenesis and caused male infertility (Udoh et al. 2005; Shajeela et al. 2011; Allaw et al. 2016; Anon, 2002). Altogether it should be noted that rhizome of *T. govanianum* does not contain diosgenin neither any plant species but after hydrolysis it is produced so it may be predicted that after administration of crude TG extract in animals the liver enzymes and gut enzymes might be responsible for conversion of natural molecule to diosgenin by breaking the sugar moiety in hydrolysis.

Since no such study has yet been carried out, it is pretty clear that *T. govanianum* can be a promising candidate for herbal drug market.

CONCLUSION
AND
FUTURE PROSPECTS

CONCLUSION AND FUTURE PROSPECTS

Optimization of extraction process for diosgenin might help pharmaceutical sector for large-scale purification and herbal formulations. Elevated expression of multiple genes indicated their role in diosgenin biosynthesis, which could be helpful in further experiments. These key genes for diosgenin biosynthesis could be utilized for genetic modifications or any other interventions for the commercial production of diosgenin. Further, trillium extract was found anti-cancerous against breast cancer cell lines which could be extended for other cancer types like cervical and ovarian. Anti-cancerous and Anti- implantation activity was enhanced by subjecting crude extract to hydrolysis process. The potentiality of herbal extract for anti-fertility was tested by using anti-implantation studies on wistar rats which could be validated by using other parameters like estrogenic/anti-estrogenic activity. The current studies on the extract of *Trillium govanianum* showed promising leads as an effective herbal contraceptive supplemented with anti-cancerous effect. Overall the study has provided several leads on multiple front which can be further explored to reach at precise conclusions.

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LIST OF PUBLICATIONS

Research Publications

1. **Shivam Sharma**, Nikhil Malhotra and Hemant Sood (2016). Expression Analysis of Steroid Pathway Genes Revealed Positive Correlation with Diosgenin Biosynthesis in *Trillium govanianum*. Acta Physiologiae Plantarum 38(11): 1-8 [IF: 1.69]. **[SCOPUS]**
2. **Shivam Sharma**, Arun Sharma, Vineet Mehta, Rajinder S. Chauhan, Udayabanu M, Hemant Sood (2016). Efficient Hydroalcoholic Extraction for Highest Diosgenin Content from *Trillium Govanianum* (Nag chhatri) and its *in vitro* anticancerous activity. Asian Journal of Pharmaceutical and Clinical Research, 9 (4): 386-392. [IF : 0.5] **[SCOPUS]**
3. **Shivam Sharma,** Vineet Mehta, Udaybanu and Hemant Sood (2016). Antifertility effect of plant extract from *Trillium govanianum.* Journal of Ethnopharmacology. [IF-2.998] [**SCOPUS]** Communicated

Conference Publications

1. **Shivam Sharma**, Varun Kumar and Hemant Sood (2016) "Optimization of extraction protocol for diosgenin by using response surface methodology (RSM) from Trillium govanianum" at International conference on "Biodiversity: Current Scenario and Future Strategies" sponsored by "State Council for Science, Technology and Environment Himachal Pradesh" held at St. Bede's College, Shimla, October 6-8