Chapter III

Diosgenin extraction and characterization in

Dioscorea prazeri

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3.1 Introduction

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Consumer demand for natural products coupled with their physicochemical (surfactant) properties and mounting evidence on their biological activity (such as anticancer and anticholesterol activity) has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors. Saponins, also glycosides widely distributed in the plant kingdom, and include a diverse group of compounds characterized by their steroidal or triterpenoid aglycone structure with one or more sugar chains. Their structural diversity is reflected in their physicochemical and biological properties, which are exploited in a number of traditional (as soaps, fish poison, and molluscicides) and industrial applications (Price et al., 1987; Oakenfull, 1981; Fenwick et al., 1991; Hostettmann and Marston, 1995; Oakenfull and Sidhu, 1989). Research has established saponins as the active components in many herbal medicines (Liu and Henkel, 2002; Alice et al., 1991).

Fig. 3.1 Diosgenin

3.1.1 The steroidal sapogenin pathway

Steroidal sapogenins (spirosstanols) e.g., Diosgenin (Fig. 3.1) are synthesized from cholesterol in several plants (Mahato et al., 1982), through isoprenoid biosynthetic pathway (Fig. 3.2). Cytosolic isoprenoids are synthesized from acetyl CoA through
intermediate formation of mevalonate, isopentanyldiphosphate, dimethylallyldiphosphate, isopentenyl diphosphate, geranyldiphosphate, farnesyl diphosphate, squalene, cycloartenol and leads to steroidal sapogenin in broad view. Saponins are glycosides containing one or more sugar chains on a triterpene or steroid aglycone backbone called a sapogenin. Steroidal saponins in which the side chain is held open by glycoside formation (furostanols) are naturally occurring glucosides in several plant species (Sharma et al., 1982). These glycosides are converted \textit{in vitro} to spirostanols by the elimination of the glucose molecule at Carbon positioned ‘26’ lead to ring closure by the action of glucosidases.

Fig. 3. 2 Diosgenin Biosynthetic Pathway.
3.1.2 Diosgenin Chemistry

Steroids form an important group of compounds based on the fundamental saturated tetracyclic hydrocarbon: 1,2-cyclopentanoperhydrophenanthrene (sterane or gonane). According to their chemical structure, the wide array of steroid molecules may be divided into several groups as Sterols, Brassinosteroids and Sapogenins. All these compounds have basic structural skeleton or nucleus of four fused rings of 17-carbon atoms but they differ in chemical groups or side chain attached to the basic skeleton and double bond at specific position in the nucleus (Asolkar and Chadha., 1979). Saponins are categorised according to number of sugar chains in their structure. Bidesmosidic saponins have two sugar chains with one attached through an ether linkage at C-26 are furastanol saponins. The nature of the aglycone and the functional groups on the aglycone backbone and number and nature of the sugars can vary greatly resulting in a very diverse group of compounds (Price et al., 1987; Hostettmann and Marston, 1995).

Diosgenin has spiroketal side chain attached at positions 16 and 17 of the sterane and has a double bond at 5-6. It has a hydroxyl group at 3rd position; hydroxyl groups are mostly found combined with sugars, making the compounds water soluble and highly saponaceous. It is a steroidal sapogenin that is isolated from plants and is structurally similar to cholesterol.

Diosgenin is obtained entirely from natural sources (Yams of Dioscorea spp.) since the synthetic product is not an attractive proposition commercially. Although total synthesis of a few steroid drugs has been achieved, until recently none could compete economically feasible method of isolation of Diosgenin from naturally occurring compounds.

3.1.3 Medicinal Significance of Diosgenin

Diosgenin, one of the most important secondary metabolites present in *D. prazeri* tuber is a pharmacologically important steroidal sapogenin. It is a precursor of sex hormones (progesterone), corticosteroids (corticosone) and contraceptives (Onwueme, 1978; Coursey, 1967). Over 50 species have been cultivated till now for commercial extraction of the compound. Diosgenin is among the ten most important sources of steroids and is also the most often prescribed medicine of plant origin (Fowler, 1984). Diosgenin induces apoptosis in cancerous cells by cyclooxygenase up-regulation and in HeLa cells
by caspase pathway (Huo et al., 2004). Dioscin, a derivative compound from Diosgenin, has been reported to induce apoptosis in HeLa cells through caspase-9 and caspase-3 pathway (Cai et al., 2002). It causes an inhibition of growth of fibroblast-like synoviocytes in human rheumatoid arthritis with apoptosis induction associated with cylooxygenase-2 up-regulation (Liagre et al., 2004). Diosgenyl saponins induce apoptosis and mitotic arrest in human leukemia cell lines (Ming-Jie, 2004). Diosgenin has both antioxidant property and anticholesterolomic activity. It has been reported to have various effects such as hypocholesterolemic action in rat (Accatino et al., 1998), antioxidant activity in HIV patients with dementia and apoptosis through cyclooxygenase activity in osteosarcoma cells (Moalic et al., 2001). Cholesterol-lowering activity of saponins, which was demonstrated in animal (Matsuura, 2001), and human trials were attributed to inhibition of the absorption of cholesterol from the small intestine, or the re-absorption of bile acids (Oakenfull and Sidhu, 1990). Pharmaceutical applications of saponins include as raw materials for production of hormones (Blunden et al., 1975), immunological adjuvants (Kensil et al., 2004), treatment of cognitive impairment (Chuang et al., 2011) and as drugs. Saponins have also been reported to be the active ingredients in various natural health products, such as herbal extracts (Balandrin, 1996). The diverse physicochemical and biological properties of saponins have been successfully exploited in a number of commercial applications in food, cosmetics, agriculture and pharmaceutical sectors. Market trends showed increasing evidence of the use of natural ingredients for their biological activity and have increased the demand for saponins in recent years (Ozlem and Mazza, 2007). In cosmetics, due to their surface active properties, saponins are being utilized as natural surfactants in cleansing products in the personal care sector such as shower gels, shampoos, foam baths, hair conditioners and lotions, bath/shower detergents, liquid soaps, baby care products, mouth washes, and toothpastes (Indena, 2005; Brand and Brand, 2004; Olmstead, 2002). Saponins and sapogenins are also marketed as bioactive ingredients in cosmetic formulations with claims to delay the aging process of the skin (Yoo et al., 2003; Bonte et al., 1998) and prevent acne (Bombardelli et al., 2001).
3.1.4 Extraction of active component Diosgenin

In addition to well-established analytical methodologies, new technologies and approaches are also being investigated to overcome processing challenges posed by the complex nature and diversity of this unique class of compounds. While common trends can be identified, process development is carried out for each raw material as the composition of the plant material and the saponin mixture will affect the process considerably. The first step in the processing of saponins involves their extraction from the plant matrix. As in any extraction process, the extraction solvent, extraction conditions (such as temperature, time, pH, solvent to feed ratio), and the properties of the feed material (such as composition and particle size) are the main factors that determine process efficiency and the properties of the end product. Sample pretreatments, extraction methods and extraction solvents were a few significant steps during the extraction procedures and holds importance in its application and for its activity.

The processing methods and temperatures used for drying have considerable effect on the quality of the medicinal plant materials. Shade drying or drying at lower temperatures are the preferred method for drying plant material since it can maintain or minimize loss of color of the plant material; and the lower temperatures can prevent the loss of volatile substances in the plant materials (Ibanez et al., 2003, Bartram, 1995). However, plants can be dried in a number of ways like drying ovens or at room temperature, solar dryers, indirect fire, baking, lyophilization, microwave or infrared devices. Pre-selection, peeling the skins of roots and rhizomes, boiling in water, steaming, soaking, pickling, distillation, fumigation, roasting, natural fermentation, and treatment with lime and chopping are some of the common processing practices. All processed medicinal plant materials should be protected from contamination and decomposition as well as from insects, rodents, birds and other pests, and from livestock and domestic animals. Medicinal plant preparations can be prepared in several ways that usually vary based upon the plant being used, and sometimes, the condition for which it is being used. These preparations can be in the form of infusions, decoctions, tinctures, macerations, fresh juices, etc. Some other methods include hot baths, powdered plants, steam inhalation and even aromatherapy. Hence the processing of the material needs significant attention and standardisation to obtain high yield. (Bartram, 1995)
A few analytical methods for the Diosgenin estimation from plant material were mentioned as follows. These protocols refer to use pulverized plant material of approximately 8.0 gm of fresh tubers/whole plantlet. The hydrolysed sample for 4 h with hydrochloric acid were filtered using Qualigen filter paper No. 615 and washed with distilled water until the residue was acid free. The washed residue was extracted with petroleum ether (Boiling point: 60-80°C) in a soxhlet extractor for 4-6 h. The solvent was evaporated and the residue dissolved in HPLC grade light petroleum ether and isopropanol (12:1). It was then filtered into a measuring flask using a sample clarification kit (Millipore, Bedford, MA) consisting of a 10 mL syringe, filter holder and Millipore filters (0. 5 mM) (Dixit et al., 2003). Another general method consist of using fresh tubers, which were cleaned under running tap water and dried by wiping with clean cloth/tissue. The whole plantlet/tubers were chopped and dried. The dried tubers/whole plantlet were powdered and mixed with 50 mL of distilled water with simultaneous stirring for 10 minutes in round bottom flask. To the slurry add distilled water and concentrated hydrochloric acid in accord to maintain 5% of acid concentration (w/v). The flask fixed with condenser was refluxed on a boiling water bath for 2 hour 30 minutes to 3.0 hour to complete the hydrolysis. After the hydrolysis, this slurry was allowed to attain room temperature and filtered in a Buchner funnel under vacuum. The residue was washed with distilled water till the filtrate is free from acid. The acid free residue was transferred to Petri dish and dried in an oven at 100°C at 6 hours. The dried residue was extracted with n-hexane in a soxhlet apparatus for 8 hours. The extracted solvent containing Diosgenin was concentrated, chilled on ice (0°C) and filtered. The mother liquor obtained after filtering was again concentrated, chilled on ice and re-extracted. Diosgenin obtained from extractions were pooled and weighed after drying (for 2 hours at 80°C) temperature and values were expressed on dry weight basis (Nandi, 1980).

Solubility enhancement has important implications for the bioactivity and processing of saponins. Solubility of saponins is also affected by the properties of the solvent (as affected by temperature, composition and pH). While water, alcohols (methanol, ethanol) and aqueous alcohols are the most common extraction solvents for saponins, solubility of some saponins in ether, chloroform, benzene, ethyl acetate, or glacial acetic acid has also been reported. The complex structure of saponins may
undergo chemical transformations during storage or processing which in turn may modify their properties/activity. The glycosidic bond (between the sugar chain and the aglycone), and the interglycosidic bonds between the sugar residues can undergo hydrolysis in the presence of acids/alkali, due to hydrothermolysis (heating in presence of water) or enzymatic/microbial activity resulting in the formation of aglycones, prosapogenins, sugar residues or monosaccharides depending on the hydrolysis method and conditions (Hostettmann and Marston, 1995). Incomplete acid hydrolysis yields saponins, while complete acid hydrolysis was found to produce the constituent aglycone.

The saponin content of plant materials is affected by the plant species, genetic origin, the part of the plant being examined, the environmental and agronomic factors associated with growth of the plant, and post-harvest treatments such as storage and processing (Fenwick et al., 1991), hence all these parameters require optimization and standardization to obtain high yield.

3.1.5 Characterisation of active component Diosgenin

i. Thin Layer Chromatography analysis with Dioscorea prazeri extracts (Dp Extract)

TLC is a simple, quick procedure that gives the chemist a quick answer as to how many components are in a mixture. It supports the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound. It is particularly valuable for qualitative determination of small amounts of impurities. The finger print profile of the plant is easier to study based on the separation of colored bands, number and Rf values. Here the chromatographic analyses were used for the qualitative analysis of Diosgenin in D. prazeri.

ii. Diosgenin analysis by HPLC of Dioscorea prazeri extracts (Dp Extract)

The active principle compound Diosgenin was necessarily to be characterized on HPLC/Mass spec to evaluate its status in cryopreserved and transgenic samples. A comparative study of the Diosgenin content of these plants along with control plants need to be assessed for biochemical stability. Diosgenin estimation was carried out with Dioscorea prazeri whole plantlet, leaf, root and tuber extracts. For plotting standard
The Diosgenin curve, Diosgenin (standard) was procured from Sigma, USA (~98% pure). The Extraction and HPLC conditions were critical and essentially to be standardised with various solvent systems, isocratic or gradient to obtain better yield from the plant, *D. prazeri*. The diosgenin content of tubers of *Dioscorea prazeri* obtained from diverse provinces and from various plant species showed various concentration of Disogenin as per literature review (Nandi, 1980).

*Dioscorea prazeri* obtained from different region were found to have various level of Diosgenin content and maximum at reproductive stage as mentioned along the region as Jammu & Kashmir, Meghalaya, Assam, West Bengal, Tamil Nadu and U.P, was reported with 1.6 %, 1.8%, 2.0%, 2.2%, 1.6% and 1.9% as respectively. The sapogenin content from various sources as mentioned as *Dioscorea prazeri* (Diosgenin) with 1-2.0%, *Costus speciosus* (Diosgenin) with 0.8-1.2%, *Kallastroemia sp* (Diosgenin) with 0.5-0.6%, *Trigonella foenum* (Hecogenin) with 0.02-0.04%, *Agave cantala* (Hecogenin) with 1.10-1.20%, *Solanum khasianum* (Solasodine) with 1.5-2.5%, *Soyabeen* (Stigmasterol) with 0.02-0.05% and *Smilax* (Smilagenin) with trace amount.

### 3.2 Materials and Method

#### 3.2.1 Extraction and characterisation of *D. prazeri*

The experimental plant materials used for extraction and characterisation studies of the compound, Diosgenin of *Dioscorea prazeri* were obtained from plants that were transferred to green house and grown in control environmental conditions. Tubers of *D. prazeri* obtained from *In vitro* grown, wild plant, cryopreserved plants, transgenic plants; whole plantlet of *D. prazeri*; leaves of *D. prazeri*; stems of *D. prazeri*; roots of *D. prazeri*; tubers of the *Dioscorea alata*; whole plantlet of *D. alata* and the leaves of *D. alata* were used for the analysis f the active secondary metabolite. The tubers obtained from various stages of growth phases of *D. prazeri* were analysed for the study on Diosgenin content. Various solvent system were used for the study like Ethanol, Methanol, Acetonitrile, Chloroform, Petroleum ether, n-Hexane, 2-Isopropanol, Analytical grade water, Hydrochloric acid (2.5 N) and Sodium hydroxide for achieving neutralisation point during the experiment.
The instruments used were Soxhlet extraction unit, Roto evaporator (BUCHI), Nitrogen evaporator, Lyophilizer (Maxi Lyo), Vacuum Centrifuge (Eppendorf), Hot air vacuum oven (Scientek, Alpha systems), TLC apparatus, HPLC system (Schimadzu) and Soxhlet apparatus (Biosox).

### 3.2.2 Standard Stock Solution and Calibration curve

A standard stock solution of Diosgenin was prepared in HPLC grade Methanol. Working standard solutions in a range 1-5µg were prepared by dilution from this stock solution. Calibration curve was prepared based on peak areas of 5 concentration runs. Linearity was obtained in the concentration range of 1-5 µg. The equation and the good to fitness (R2) were calculated. All data were processed using LC-Solution software (Shimadzu, Japan).

### 3.2.3 Chromatographic Conditions

Chromatographic analysis was carried out on Shimadzu Series LC-20 AT liquid chromatographic system, equipped with a diode array detector SPD-M20A, and a pump of LC-20AT. Chromatographic separations were performed on C18 Column (Atlantis\textsuperscript{R} d C18 5µm 4.6x250mm column) and standardized the column temperature for the Diosgenin estimation. The peaks were resolved at a range of wavelength from 190 to 235 nm. Isocratic and gradient methods were used for the standardisation of method for analyzing the Diosgenin content. The flow rate was adjusted to 1 mL min\textsuperscript{-1}. At the end of each run, the column was rinsed with pure solvents.

### 3.2.4 Pre-processing of plant material

The experimental plant materials obtained from wild plants and the acclimatised in vitro grown plants were rinsed thoroughly in running tap water for 15 minutes. The tubers were blot dried and incised into thin uniform slices for the temperature exposure in same pace. The tubers were weighed to obtain the fresh weight. The complete drying of the plant material was performed with various temperature conditions range from 25 °C to 100 °C for optimisation. The plant material was dried for various durations ranging from 2 hours to two days. The dried plant materials was pulverized and stored at room temperature for short term and at –20 °C for long term. Pre-processing showed great impact on attaining high yield of Diosgenin.
1.2.5 Diosgenin extraction: Hydrolysis and neutralisation - Method I

i. Hydrolysis
The powdered materials were hydrolyzed with 2.0N to 2.5N Hydrochloric acid (Merck). Hydrolysis was carried out in various temperature conditions ranging from 75 ºC to 100 ºC for 2 to 6 hours in water bath for the optimization. The conical flask was loosely covered with sterile cotton plug). The extract was centrifuged at 25 ºC in 4000 rpm for 25 minutes. The extracts were filtered with a suction pump in Whatman filter paper (No. 42; 0.42-micron size).

ii. Neutrilisation and Drying
The slurry was washed with HPLC grade water to make it acid free (Nb. The slurry was washed for 7 times and centrifuged at centrifuged at 4000 rpm for 15 minutes each time and filtered to get the left out residue in the supernatant) each time the filtrate was kept for a period of 40 minutes in the water after mixing it thoroughly). The pH was neutralised from pH1.69 to pH7.0. The slurry was kept for drying in sterile petriplate at various temperatures ranging from 37 ºC to 78 ºC for a period of 2 hours to 4 days for complete removal of water.

1.2.6 Diosgenin extraction; Hydrolysis and neutralisation; Method II

i. Hydrolysis
The plantlets were dried at 45ºC to 55 ºC for two days and were were pulverised and weighed. These materials were hydrolysed at 95ºC for 3 hours 30 minutes in 2.0N hydrochloric acid, in water bath.

ii. Neutrilisation and Drying
The extracts were allowed to cool. The pH on post hydrolysis (1.69) was neutralized (7.0) with 2N sodium hydroxide and was centrifuged at 4000 rpm for 25 minutes at 25 ºC. These extracts were centrifuged at 4000 rpm for 15 minutes at room temperature and the supernatant was decanted. The residue was dried at 55-78 ºC for optimising the conditions on post-hydrolysis.
1.2.7 Diosgenin Extraction

The extraction was carried out at room temperature using n-Hexane, Acetonitrile, petroleum ether and methanol for 4 to 6 hours with the hydrolysed plant extracts and with the non-hydrolysed plant extracts. Soxhlet extraction was carried out with the same solvents at various temperatures below the melting point of the active component. The temperature for the HPLC analysis ranged from 23°C to 40°C. The extract obtained was dried and re-dissolved in various solvents as petroleum ether, chloroform: isopropanol (12:1) and methanol for HPLC analysis to obtain Diosgenin. The experiments were conducted to obtain the consistent yield and to obtain the active component. Detailed experiments were carried out with two different solvents petroleum ether and methanol.

i. Extraction of Diosgenin with aqueous Acetonitrile

a. Procedure I

The hydrolysed plant materials were extracted at room temperature with aqueous acetonitrile (50% v/v; Analytical grade), and sonicated for 10 minutes at amplitude of 60% (digital Branson sonifier). The sonicated sample was centrifuged at 4000 rpm for 15 minutes at room temperature (20 ºC). The supernatant was taken and roto-evaporated at 145 mbar, 95 rpm at 63°C. The dried sample was stored at –20ºC and for analysis it was re-dissolved in aqueous acetonitrile and made up to 10 mL using a volumetric flask. The extract was filtered using a 0.2-micron filter and the sample was analysed on HPLC with various gradient and isocratic methods to resolve the peak of Diosgenin from the plant extract.

b. Procedure II

The extraction was carried out using soxhlet apparatus with solvent aqueous acetonitrile for Diosgenin. The plant materials were extracted with aqueous acetonitrile at 80 ºC for 2 hours using Biosox apparatus. The extraction was carried out subsequent to acid hydrolysis for 2 to 4 hours at 75°C in a water bath. The soxhlet extracts of the plant were further used for biochemical analysis.
ii. Extraction of Diosgenin with Petroleum Ether

a. Procedure I
Diosgenin was extracted from dried sample with petroleum ether for 4 hours at room temperature. The petroleum ether phase was separated after centrifugation. The solvent was rotoevaporated at 50ºC at a pressure of 145 mbar and 100 rpm. The dried plant extract was stored at -20 ºC. The extract was dissolved in 1 mL of chloroform and analysed for Diosgenin on High Performance Liquid Chromatographic system.

b. Procedure II
The dried plant materials of *D. prazeri* were subjected to soxhlet extraction with petroleum ether at 60 ºC for 5 hours and subsequently concentrated at 75ºC to 80ºC for 1 to 2 hours in Biosox Unit (Techno Reach). On post-distillation the sample volume was around 5mL, which was then roto-evaporated in water bath at 50ºC for 20 minutes with pressure of 300PSi and cooled. The completely dried extracts were re-dissolved prior to HPLC analysis and sonicated for 15 minutes. The extract was analysed on HPLC. The tuber extract of *Dioscorea alata* was used as a negative control. The samples were analysed for Diosgenin content on HPLC. The chromatographic peaks were compared with the standard (Diosgenin) peak obtained. The yield of Diosgenin was calculated.

iii. Extraction of Diosgenin with n-Hexane

a. Procedure I
The hydrolysed residue was extracted with normal hexane (Boiling point: 50 ºC) in a soxhlet extractor for 4-6 h. The solvent was evaporated. The extracts obtained was HPLC analysed

iv. Extraction of Diosgenin with Absolute Methanol

a. Procedure I
*D. prazeri* plant materials were dried, pulversized and subjected to soxhlet extraction with absolute methanol (HPLC grade) at 80 ºC for 4 hours and then re-distilled at 80 ºC to 100 ºC in Biosox Unit (Techno Reach). On post-distillation the sample volume was around 10mL, which was then roto-evaporated in water bath at 50ºC for 20 minutes with
pressure of 300PSi and cooled. The sample was completely dried using nitrogen evaporator and was re-dissolved in various solvents and sonicated. The re-dissolved sample was filtered using 0.2 µm Filters. The extract was analysed on HPLC. The tuber extract of *Dioscorea alata* was used as a negative control. The samples were showed steroidal sapogenin peak with the mobile phase. The retention time and peak of the samples were compared with the standard (Diosgenin). The yield of the extract was calculated.

### 1.2.8 Isolation of Diosgenin Pure Fraction from *D. prazeri*

The fraction of pure Diosgenin compound was isolated from *D. prazeri* plant extracts using extraction methods standardised here with High Performance Liquid Chromatographic analysis (Shimadzu). The chromatographic separations were performed using C18 Column (Atlantis® d C18 5µm; 4.6x250mm column) at specific optimised temperature, 35ºC. The filtered (0.2 µm) extracts enriched with Diosgenin was analysed with a flow rate of 1 mL min⁻¹ on HPLC. The fractions of Diosgenin were collected according to the retention time from calibrated curve with standard (Diosgenin; SIGMA)). The fractions were spiked with the standard for the confirmation of *D. prazeri* diosgenin fractions collected. The pure fractions were collected in sterile vials and vacuumfuged (Centrifuge+Vacuum) and the white powdery compound of Diosgenin isolated was further studied for its activity.

### 1.2.9 TLC for Diosgenin assessment with *D. prazeri* plant extract

#### i. Procedure 1

Weighed 1.5 g of plant material of *D. prazeri* and added 25 mL of Petroleum ether (Merck, boiling point 60-80ºC) and extracted the metabolites using reflux condenser (Servell instruments, Bangalore) for about 1.5 hours. The volume of the extract was reduced to 1/20th of its original volume and was filtered. The plant extract (5 µL) along with Standard (Diosgenin) was spotted on precoated preparative TLC plate (coated with solid adsorbent of Silica gel 60 F 254 10cm x5 cm size).
**ii. Procedure 2**

Weighed 1.5 g of plant material of *D. prazeri* and to this add 12.5 mL of Chloroform instead of Petroleum ether (Merck, boiling point 62°C). The plant materials were extracted for the metabolite Diosgenin using reflux condenser (Servell instruments, Bangalore) for about 1.5 hour. The extracts obtained were filtered and reduced the volume of the extract to 1/5th of its original volume by evaporating the extract on the water bath. 5 µL of extract was spotted near the bottom of the pre-coated preparative TLC plate made up of solid adsorbent of Silica gel 60 F 254 10 cm x 5 cm size along with standard (Diosgenin).

**iii. Development of Chromatogram**

The solvent was transferred to chromatographic chamber to form a shallow pool of homogenous mobile phase, of 5-6 mm depth so that only the bottom of the plate touches the liquid. The chamber was closed and allowed to stand at constant temperature, protected from direct sunlight for 15 minutes so that it would be saturated. The spots were allowed to dry in plates and placed vertically in the chamber, ensuring that the points of application were above the surface of the mobile phase. The chamber was closed and allowed to develop chromatogram at room temperature. The mobile phase slowly rose through the TLC plate by capillary action till the specified distance. Chromatographic bands appeared when the equilibrium was attained between the molecules of the component and the molecules of the solution, with the solvent system. The separation of the components was based on the solubility and the strength of the adsorption to the adsorbent on plate. The plate was removed from the developing chamber, marked the solvent front and was dried. The components of the mixture were observed. The compounds that were not intrinsically colored were observed under UV (The plate fluoresces under UV lamp except the region in which the organic compound are existing). An alternative method of detection was using anisaldehyde as a detection reagent. The Rf values were calculated based on the distance travelled by the solute and the solvent. As an additional step of visualisation the chromatogram was developed with Iodine vapour. It was further treated with detective reagent, anisaldehyde for further observation. Mobile phase used for TLC analysis was Chloroform: and Petroleum ether to the ratio of 60:40 (v/v).
3.3 Results

3.3.1 Preprocessing
The plant materials of *Dioscorea prazeri* were dried at various temperature conditions in pre-processing of the extraction of steroidal sapogenin (Fig. 3.3). The sun drying was observed to decrease the yield of Diosgenin when compared to vacuum drying. Diosgenin yield was observed as 0.6±0.1% with this processing. The Diosgenin yield was reduced to 0.5-0.8% in comparison with the natural content of 2.2-2.4% of this secondary metabolite when the temperature for drying was set higher than 75°C, according to the data obtained on HPLC analysis. The sliced tubers were dried at 55 °C for 36±3 hours in hot air oven, which exhibited consistent value on Diogenin analysis. The water content of the plant material was calculated to an average of 88.5±1% from 25 sets of experiments for extraction and characterisation for biochemical stability.

3.3.2 Hydrolysis
Diosgenin from the plant material was extracted at room temperature using Acetonitrile, n-Hexane, Petroleum ether, Chloroform and Methanol. However the yield of Diosgenin obtained from extraction in room temperature was as low as 0.4± 0.1% with petroleum ether and normal hexane to maximum level of 0.7% with methanol. Chloroform was found to interfere with C-18 column and with the mobile phase during HPLC analysis, and was subsequently not preferred as a solvent system during further extraction procedures. The dried plant materials were pulverized and followed by hydrolysis with 2.0N hydrochloric acid for 3 hours 30 minutes. 1 to 2 hours of treatment were not found to be sufficient to hydrolyse the compound completely. 3.30 hours to 6.0 hours were yielded the same content of Diosgenin. So the optimised temperature studied was 3.30 minutes. Neutralized the hydrolysed plant material with sodium hydroxide (2.0 N) and these were soxhlet extracted for Diosgenin (Fig. 3.4). The extracts were given prominent peak of the compound of interest.
Fig. 3. 3 The various stages of Diosgenin extraction and characterisation of *D. prazeri* (Whole plant materials) and *D. alata* (tubers).

(A) *In vitro* raised *D. prazeri*; (B) Tubers of *D. alata* and *D. prazeri* (*In vitro* plants and Wild plants), Roots, and Whole Plantlet f *D. prazeri* (The plant material was dried under controlled temperature conditions); (C) Pulverised plant material; (D) Plant material for hydrolysis in hydrochloric acid (2.0 N).

Fig. 3. 4 The pictorial representation of various stages of extraction from *D. prazeri* plant materials.
The neutralisation of hydrolysed plant material; The plant material dried at 55 °C; Soxhlet extraction of steroidal sapogenin of D. prazeri in BIO SOX unit (Techno reach)

3.3.3 **Neutrilisation**

The neutralisation with sodium hydroxide (2.0 N) was an appropriate method for neutralisation of the hydrolysed material. The slurry was turned black with higher concentration of NaOH. The wastage of hydrolysed material was minimised with the procedure of neutralisation with restricted amount of NaOH. Continuous rinsing of the D. prazeri extract with water for neutralising pH after hydrolysis lead to loss of the active component to a greater extent, up to 1.2% compared to alternative methods used.

3.3.4 **Soxhlet extraction**

The Steroidal sapogenin, Diosgenin was extracted with BIOSOX unit with various solvent systems. Following extraction the solvent was redistilled to obtain the concentrated volumes of compound of interest. The extract was lyophilised completely to obtain the dried fraction enriched with active compound of interest. The acetonitrile extract did not give prominent peak of Diosgenin on analysis. The extraction was carried out with petroleum ether and methanol (Analytical grade; Merck) for the studies due to the high yield of Diosgenin and the consistency of data. So the procedure was standardised with petroleum ether and methanol to extract the active component of D. prazeri. The yield obtained was as high as 2.4% to 2.8% with absolute methanol and 2.0% to 2.4% with petroleum ether.

The standard curve was plotted with Diosgenin (standard) procured from Sigma, USA (~98% pure). As a part of the study the control plantlets micropropagated and the tubers obtained from it were compared with the wild plants to find out the variation and the data assessed indicated fidelity at biochemical level.

The protocol for extraction of active component of the plant, steroidal sapogenin (Diosgenin) with Methanol and an alternative procedure with Petroleum Ether were standardized in this study as follows.
i. Extraction of Diosgenin with HPLC grade Petroleum ether

The plantlets were dried at 45°C for two days and hydrolysed with 2N hydrochloric acid at 95°C for 3½ hours were given high yield of Diosgenin. The pH on post hydrolysis (1.0) was neutralized (7.0) with 2N sodium hydroxide. The extract was centrifuged and the residue was dried at 55°C in hot air oven. The dried sample was subjected to soxhlet extraction with petroleum ether at 60 °C for 5 hours and then re-distilled at 75°C to 80°C for 1 hour in Biosox Unit (Techno Reach). On post- distillation the sample volume was around 5mL, which was then roto-evaporated in water bath at 50°C for 20 minutes with pressure of 300Psi. The sonication for 15 minutes improved the yield.

The extract was analysed on HPLC. The tuber extract of *D. alata* was used as a negative control that did not give any fraction of Diosgenin. The analysed samples were exhibited conspicuous peak of Diosgenin with the mobile phase. The Diosgenin peak of the samples was compared with the standard (Diosgenin) peak obtained. The yield of the extract was calculated and found to be 2.2 ±0.2%.

ii. Extraction of Diosgenin with HPLC grade Methanol

The plantlets were dried at 45°C for two days and hydrolysed at 95°C for 3½ hours in 2N hydrochloric acid. The pH on post hydrolysis (1.0) was neutralized (7.0) with 2N sodium hydroxide. The extract was centrifuged and the residue was dried at 55°C for 36 hours in hot air oven showed consistent yield. The dried extracts were subjected to soxhlet extraction with methanol at 80 °C for 6 hours (Biosox Unit Techno Reach) and the concentrated extracts enriched in diosgenin were obtained. The temperature above 100 °C during soxhlet extraction showed lower level of Diosgenin in comparison with 60°C to 80 °C. On post- distillation the sample was completely dried by roto-evaporation apparatus in water bath at 50°C with pressure of 300PSi and cooled. The dried plant extract was dissolved in HPLC grade methanol. The yield of the extract was calculated. The extract was analysed on HPLC with methanol as a mobile phase. The samples injected were showed prominent peak with the yield of 2.4% of Diosgenin. The chromatogram of the sample was confirmed with that of the standard peak.

Extraction with methanol showed consistency in results and 0.4% higher yeild than petroleum ether. When the extract was dissolved in chloroform than methanol or
petroleum ether, the Diosgenin peak obtained was interrupted by chloroform peak on analysis.

3.3.5 HPLC analysis and the chromatographic conditions standardized for Diosgenin

i. Standard Stock Solution

A standard stock solution of Diosgenin (1,000μg mL⁻¹) was prepared in HPLC grade methanol, which was found to be completely miscible and diosgenin peaks were obtained on analysis. Working standard solutions range from 1 to 5μg was prepared by serial dilution of the stock solution and it showed consistency in data on analysis (Fig. 3.5).
Chromatographic conditions were optimized for the separation and quantification of standard Diosgenin.

1) Method 1, Isocratic method with (A) Acetonitrile: Water (95:5) for 30min Diosgenin dissolved in Chloroform/Methanol was injected; peak was interrupted with chloroform and low detection level.

2) Method 2, Isocratic method with (A) Acetonitrile: Water (90:10) for 30min Diosgenin dissolved in Chloroform was injected; peak was not detected.

3) Method 3, Gradient method with (A) Acetonitrile: (B) Water with: linear gradient for 15th min at A of 20% and B of 80% and at 25th min A of 80% and B of 20% and reached 100% acetonitrile at 30th min. Diosgenin detection levels were less in comparison with other solvents.
4) Method 4, Isocratic method with (A) Acetonitrile: Water (80:20) for 25 min flow rate at 1mLmin\(^{-1}\). Peak was detected at RT 17-18\(^{th}\) min at the wavelength 190 and 210 nm.

5) Method 5, Isocratic method with Methanol (100\%) was done for standard Diosgenin dissolved in Methanol and also maintaining the column temperature to 35°C. The better resolution resolved chromatographic peak for Diosgenin with absorption maxima was detected at 205 nm. The retention time was observed to be 6.9 to 7.1 minute.

Calibration curve was prepared based on peak areas of 5 concentration runs in triplicates. The chromatographic analysis data on statistical analysis showed the significance with good to fitness (R\(^2\)) value, 0.999 (Fig. 3.6). The chromatographic peaks of diosgenin showed maximum absorption at 205nm when compared to the chromatogram at wavelength ranging from 190 to 235 nm (Fig. 3.7). The peak was resolved with an isocratic mobile phase of absolute methanol.

ii. Chromatographic Conditions

Chromatographic analysis was carried out on Shimadzu Series LC-20 AT liquid chromatographic system, equipped with a diode array detector SPD-M20A, and a pump of LC-20AT. Chromatographic separations were performed on C18 Column (Atlantis\textsuperscript{R} d C18 5\(\mu\)m 4.6x250mm column) at optimised temperature at 35°C, for Diosgenin extraction. The analysis at room temperature (25°C) was giving inconspicuous chromatographic peak of Diosgenin. When the temperature was higher than optimum level, at 40°C the yield obtained was showing a depletion of 0.6 to 1.0\% in Diosgenin content in detection level. The wavelength selected was 205 nm. An isocratic flow of 100\% Methanol (HPLC grade) was resolved Diosgenin peak from the extract. The flow rate was adjusted to 1mL min\(^{-1}\). At the end of each run, the column was rinsed with pure Methanol. All data were processed using LC-Solution software (Shimadzu, Japan). Comparative overlay of the sample and the standard showed the integrity of the sample analyzed (Fig. 3.8).
Fig. 3. Comparative overlay of chromatogram of Diosgenin with different concentration (1, 2, 3, 4 & 5µg) with an injection volume of 20µL.

(A) Chromatographic representation; (B) Calibration curve
The various absorbance spectra of Diosgenin and 205 nm showed the maximum absorbance.

HPLC profile depicting variation in concentration of Diosgenin according to the age of *in vitro* propagated plants.

(A) Tubers of 1.0 year old plant; (B) Tubers of 1 year 6 months old plant; (C) Tubers of 2.0 years old plant; (D) Tubers of 3.0 years old plant.
3.3.6 Diosgenin assay

The Diosgenin content was analysed from different parts of *D. prazeri* like leaves, roots, stems and tubers. The stems of *D. prazeri* were observed with negligible amount of Diosgenin. The roots exhibited lesser amount of Diosgenin, 0.4\% when compared to leaves with 1.2\% and the tubers showed the maximum content of Diosgenin of 2.8\%. Tubers of a few plants in reproductive stages showed as high as 2.9\% of Diosgenin content on HPLC analysis (Table 3.1) (Fig. 3.9).

Even though the plants produced Diosgenin at younger stages, the yield of active component was very low in comparison with the older plants (Fig. 3.10). The yield of Diosgenin was ranging from 0.7±0.2\% to 2.6±0.2\% from the age of 12 weeks to 3 years. The results obtained from the average of five replicates (Table 3.2). The plants were observed to have lesser amount of Diosgenin at its early growth phases and reached a maximum level at 3 years.

The purified fraction of Diosgenin was obtained by HPLC analysis and the fractions were collected and verified by spiking with the standard. The pure fractions obtained were completely dried and stored at -20 °C. The fraction was used for the further studies on its activity along with the soxhlet extract of *D. prazeri*.

The extraction and characterisation of the active component, Diosgenin from *D. prazeri* was optimised. The study was conducted with various plant parts, in which the tubers were found to have the highest content of steroidal sapogenins. The active component at various growth phases was studied, wherein 2.5 to 3 years old plants resulted in the highest yield. An optimised isocratic solvent system of absolute methanol for resolving the chromatogram of Diosgenin (Fig. 3.11) was employed. The biochemical stability was analysed with the wild grown donor plant and micropropagated plants during *in vitro* propagation studies, and fidelity assessment for recovered and regenerated plants on cryopreservation (Table 3.3 & 3.4). Enhancement of the compound was monitored during the genetic transformation studies on *D. prazeri*. 
The graphical representation of content of Diosgenin in *D. prazeri* based on HPLC analysis. Tubers were having high content of Diosgenin. The maximum quantity of Diosgenin observed with *D. prazeri* at 2.5 years to 3.0 years of its growth.

Chromatogram obtained on biochemical analysis of *in vitro* regenerated plants. (A) The HPLC profile of 5µg of standard with methanol as isocratic solvent system; (B) Diosgenin peak obtained with *in vitro* regenerated *D. prazeri*
Fig. 3. 11 Comparative overlay of Diosgenin (Standard) and the Sample (Extract from tubers of *in vitro* propagated plants).

**Table 3.1** The various *D.prazeri* parts used for Diosgenin assay and the content (HPLC)

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Plant material</th>
<th>Diosgenin content on HPLC analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>In vitro</em> tubers</td>
<td>2.85±0.1</td>
</tr>
<tr>
<td>2</td>
<td>Wild plant tubers</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>3</td>
<td><em>In vitro</em> leaves</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>4</td>
<td>Wild plant Leaves</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>5</td>
<td><em>In vitro</em> roots</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>6</td>
<td>Wild plant roots</td>
<td>0.40±0.02</td>
</tr>
</tbody>
</table>

*The data is on an average value ± standard deviation of 5 replicates*
Table 3.2  Diosgenin estimation data on HPLC analysis of tuber extract from different growth phases of Dioscorea from in vitro propagated plants

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Ret.Time</th>
<th>Channel</th>
<th>Area</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant extract from 1.0 y old plant</td>
<td>13.96</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>3824403</td>
<td>293997</td>
</tr>
<tr>
<td></td>
<td>13.96</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>3824403</td>
<td>293995</td>
</tr>
<tr>
<td></td>
<td>16.06</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>4405292</td>
<td>333151</td>
</tr>
<tr>
<td></td>
<td>16.06</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>4405292</td>
<td>333150</td>
</tr>
<tr>
<td>Plant extract from 1.6 y old plant</td>
<td>16.03</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>4405283</td>
<td>333153</td>
</tr>
<tr>
<td></td>
<td>20.62</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>5673669</td>
<td>377406</td>
</tr>
<tr>
<td></td>
<td>20.62</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>5673671</td>
<td>377408</td>
</tr>
<tr>
<td>Plant extract from 2.0 y old plant</td>
<td>20.62</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>5673668</td>
<td>377405</td>
</tr>
<tr>
<td></td>
<td>24.73</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>6828457</td>
<td>444522</td>
</tr>
<tr>
<td></td>
<td>24.73</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>6828456</td>
<td>444521</td>
</tr>
<tr>
<td>Plant extract from 3.0 y old plant</td>
<td>24.73</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>6828459</td>
<td>444525</td>
</tr>
</tbody>
</table>

Table 3.3  Diosgenin estimation data on HPLC analysis of in vitro propagated plants

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Ret.Time</th>
<th>Channel</th>
<th>Area</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro grown 1</td>
<td>13.96</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>3824403</td>
<td>293997</td>
</tr>
<tr>
<td>In vitro grown 2</td>
<td>13.96</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>384401</td>
<td>293995</td>
</tr>
<tr>
<td>In vitro grown 3</td>
<td>13.96</td>
<td>6.9</td>
<td>Ch2 205 nm</td>
<td>3824399</td>
<td>293994</td>
</tr>
</tbody>
</table>

Table 3.4  Data on Chromatographic analysis of D. prazeri plant extract

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Rt</th>
<th>Channel</th>
<th>Area</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro grown 1</td>
<td>28.861</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8770718</td>
<td>665978</td>
</tr>
<tr>
<td>In vitro grown 2</td>
<td>28.861</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8770717</td>
<td>665976</td>
</tr>
<tr>
<td>In vitro grown 3</td>
<td>28.861</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8770716</td>
<td>665975</td>
</tr>
<tr>
<td>Donor plant 1</td>
<td>28.065</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8528347</td>
<td>664038</td>
</tr>
<tr>
<td>Donor plant 2</td>
<td>28.065</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8528349</td>
<td>664037</td>
</tr>
<tr>
<td>Donor plant 3</td>
<td>28.065</td>
<td>7.0</td>
<td>Ch2 205 nm</td>
<td>8528346</td>
<td>664038</td>
</tr>
</tbody>
</table>
3.3.7 Diosgenin assay from *D. prazeri* plant extract - TLC

Petroleum ether extracts showed yellow bands on treatment with anisaldehyde as a detection reagent. The bands were observed on heating the TLC plate on hot plate for around 2-3 minutes. The chromatogram of the plant materials extracted with chloroform showed lighter band than Petroleum ether but appeared better with iodine vapours. The compound of interest was observed in comparison with the standard.

The Rf value of the *D. prazeri* tuber extracts in comparison with standard (Diosgenin) (SIGMA) has shown that the extract contained Diosgenin. The Rf values of the extract showed clear bands against the band of the standard (Diosgenin with chloroform and the petroleum ether as solvents). Chloroform (Table 3.5a) was found to be a good solvent for Diosgenin than Petroleum ether (Table 3.5b). The TLC was used as a pre requisite study for the confirmation of the presence of Diosgenin in *Dioscorea prazeri* tubers.

**Table 3.5a** The Rf value obtained on treatment of *D. prazeri* plant extract with Petroleum ether and standard on TLC analysis

<table>
<thead>
<tr>
<th>SI No</th>
<th>Distance traveled by solvent (cm)</th>
<th>Distance traveled by solute (cm)</th>
<th>Rf Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Standard</td>
<td>7.9</td>
<td>4.05</td>
<td>0.5126</td>
</tr>
<tr>
<td>2. DpTub Ex1 (Chloroform)</td>
<td>8.0</td>
<td>4</td>
<td>0.5000</td>
</tr>
<tr>
<td>3. Dp tsb Ex2 (Petroleum ether i)</td>
<td>8.05</td>
<td>4</td>
<td>0.4968</td>
</tr>
<tr>
<td>4. Dp tub Ex 3(Petroleum ether ii)</td>
<td>8.0</td>
<td>4.5</td>
<td>0.5625</td>
</tr>
</tbody>
</table>

TLC plate coated with solid adsorbent of Silica gel 60 F 254 10 cm x 5 cm size. Isolate of Dp Tub Ex: *Dioscorea prazeri* tuber extract.

**Table 3.5b** The Rf value obtained on treatment of *D. prazeri* plant extract with Petroleum ether on TLC analysis.

<table>
<thead>
<tr>
<th>SI No</th>
<th>Extract</th>
<th>Distance traveled by solute (cm)</th>
<th>Rf Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dp tub Ex1(Petroleum ether)</td>
<td>4.1</td>
<td>0.5061</td>
</tr>
<tr>
<td>2</td>
<td>Dp tub Ex1(Petroleum ether i)</td>
<td>4.1</td>
<td>0.5061</td>
</tr>
<tr>
<td>3</td>
<td>Standard (Petroleum ether ii)</td>
<td>3.9</td>
<td>0.4814</td>
</tr>
</tbody>
</table>
TLC plate was coated with solid adsorbent of Silica gel 60 F 254 10 cm x 5 cm size. Distance traveled by solvent in all the cases was found to be 8.1 cm.

**Fig. 3.12** The pictorial representation of whole process of Diosgenin extraction and characterization from *D. prazeri*. 
3.4 Discussion
Diosgenin, a plant steroid (5-spirostan- 3-ol) has been used for various steroidal drugs since they have isolated in 1930s (Yang, 1981; Afrose S., 2010; Akinpelu D., 2008). Steroidal drugs are considered to be some of the costliest and most important medicines used throughout the world today. With recent reports of Diosgenin’s function in inducing differentiation of erythroleukemia through changing lipoxygenase activities (Beneytout et al., 1995) and inducing apoptosis and cell cycle arrest in the human oestrosascoma 1547 cell line (Moalic et al., 2001), the value of Diosgenin has further increased. The most recent finding concerning Diosgenin was found to inhibit migration and invasion of human prostate cancer PC-3 Cells by reducing matrix metalloproteinases expression. These findings reveal new therapeutic potential for Diosgenin in anti-metastatic therapy (Pin-Shern Chen, 2011). So the enhancement and its characterization hold high significance from D. prazeri, an indigenous source.

The TLC was conducted for the detection of compound Diosgenin with reference to standard as instigation for the enhancement of secondary metabolite in D. prazeri. Diosgenin (Standard) and the tuber extracts of D. prazeri was dissolved in chloroform and petroleum ether and detected in TLC using petroleum ether as a solvent system as it showed the value as 0.49 as Rf value as mentioned (Wang., 2011; Benjamin., 1984) The iodine vapor and anisaldehyde were used as detection reagents that showed the presence of Diosgenin.

The percentages of Diosgenin obtained from the seventy four accessions on HPLC analysis, of D. polygonoides on this work are in the range from 0.02 to 2.64%, which is significant since there are several literature data where the Diosgenin contents found to be low as exemplify as with D. polygonoides (0.2%); (Coursey et.al., 1981) Dioscorea althaeoides (0.2-2.3 %); Dioscorea prazeri (1.92%); Dioscorea villosa (1.3%); 2 years old Dioscorea zingiberenzis (0.18-0.55%), several Dioscorea species (0.04-0.93%), among others (Nino, 2007). Furthermore, this heterogeneity found on the steroidal sapogenin contents as mentioned might depend on factors such as the genotype, the physiological state, the climatic conditions as well as the geographic localization of plants as stated (Dinan et.al., 2001). These findings correlate with the determination of the steroidal sapogenin contents by HPLC, where significant differences were found.
depending on the origin and part of the plant used for extraction (Oleszek et al., 2002; Ganzera et al., 2001). Hence it was essential to standardize a protocol to obtain higher concentration of Diosgenin from *D. prazeri* and was achieved with higher yield in this study. This study on *D. prazeri* was carried out with reference to certain important factors influence the characterization of the compound like the choice of plant material, conditions of the growth, and geographical locations and it was crucial for the further research conducted on enhancement of Diosgenin. These optimized conditions will be used for the biochemical assay on Diosgenin of transgenic plants.

* Dioscorea prazeri * was one among the main natural source of Diosgenin manufacturing (Behera, 2010), but now it is listed in rare species due to exploitation. The high therapeutic characteristics of the compound, Diosgenin can be utilized by the preservation of the plant germplasm, micropropagation and enhancement of the compound in natural source. The conditions for estimation of Diosgenin were standardised using *D. prazeri* plant and obtained high yield. This can be utilized for extraction and characterization of the active compound. The plant re-established to the natural environment through this study and the high content of Diosgenin can be utilized for medicinal and commercial application.
3.5 References III


