Isolation and characterization of diosgenin from in vitro cultured tissues of *Helicteres isora* L

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Received: 26 June 2013 / Revised: 3 September 2013 / Accepted: 6 September 2013 / Published online: 22 November 2013
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Abstract The objective of the present study was to isolate, characterize, quantify and compare the accumulation of bioactive secondary metabolite—diosgenin from in vitro cultured cells of *Helicteres isora* and plant parts. The levels of this secondary compound were examined by using various biochemical techniques. The result showed that maximum diosgenin was obtained from in vitro cultured cells as compared to the plant parts. The fallout of this study is important, since levels of diosgenin detected in the in vitro cultured cells were more than in the plant parts. In vitro cultured cells accumulate comparatively higher amount of diosgenin making *Helicteres isora* a potentially new and indigenous source of diosgenin.

Keywords Bioactive metabolite · Diosgenin · *Helicteres isora* · In vitro · Quantify

Introduction

Medicinal plants are gaining great interest in pharmaceutical industries for the production of high valued secondary compounds (Rout et al. 2000; Das and Rout 2002). Plant cell and tissue culture assures controlled production of myriad secondary metabolites on demand (Hussain et al. 2012). The production of useful secondary metabolites from plant tissue culture has created a new methodology for their commercialization (Bolta et al. 2000).

Diosgenin is an steroidal sapogenin belonging to the triterpene group and is found in a few higher plant species, and interest in its medicinal properties has been increased

recently (Liu et al. 2005). It is among the ten most important sources of steroids and is also the most often prescribed medicine of plant origin. Diosgenin is a precursor for the chemical synthesis of steroidal drugs, oral contraceptives and is tremendously important to the pharmaceutical industry (Wagle et al. 2007; Li and Ni 2011 and Kalailingam et al. 2012). Diosgenin plays an important role in control of cholesterol metabolism, variation in the lipooxygenase activity of human erythroleukaemia cells and responsible for morphological and biochemical variations in megakaryocyte cells (Sharareh 2011). It is reported (Cheng et al. 2003) that methyl protodioscin - the potent agent with anti—tumor properties has been synthesized from diosgenin. The partial synthesis of steroids from plant based precursors is a boon because of the increasing demand for corticosteroids, contraceptives, sex hormones and anabolic steroids since 1960 (Hall and Walker 1991). Recent studies have found that diosgenin has been used in traditional medicine as an antihypercholesterolemia, antihypertriglyceridemia antihyperglycemic agent (Chen et al. 2011) To date, diosgenin and related steroidal sapogenins were commercially obtained from the tubers of various *Dioscorea* species; however it is crucial to discover new and alternative sources of these compounds due to decreasing plant resources as well as increasing demand (Savikin et al. 1998).

Helicteres isora Linn (Sterculiaceae) is a recently investigated as an important source of Diosgenin (Barik et al. 1998). *Helicteres isora*, commonly known as East Indian screw tree or red isora or spiral bush is an arborescent sub deciduous shrub or small tree. It contains a range of phytochemicals viz. alkaloids, flavonoids, triterpenoids, saponins and sugars (Badgujar and Jain 2009; Deshpande and Bhalsing 2011). Almost all the parts of the plant are used in Ayurveda. In traditional medicine, the root juice and bark of *Helicteres isora* is claimed to be useful in snake bite, diabetes, asthma, blood disorder, cough, colic, diarrhoea, dysentery, stomach affections, intestinal infections, emphysema, and also as a

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urinary astringent (Shriram et al. 2008). The fruits are astringent, acrid, refrigerant, demulcent, constipating, and stomachic possessing anti HIV activity (Otake et al. 1995). The main aim of the study is to isolate diosgenin form in vitro cultured tissues of *Helicteres isora* and characterize it by using various biochemical techniques.

Materials and methods

Plant material and explant collection

The plantlets of *Helicteres isora* were directly obtained from Dr. Punjabrao Deshmukh Agricultural University, Akola, India. They were authenticated by a taxonomist from Botany department of Moolji Jaitha College, Jalgaon

Culture media and culture conditions

The explants were surface sterilized initially in 70 % (v/v) ethanol for 30 s, subsequently in 0.1 % (w/v) mercuric chloride solution for 60 seconds. Finally the explants were washed thoroughly with autoclaved distilled water for several times to remove the traces of HgCl_2 . These sterilized explants were cut into suitable size (5×5 mm) and cultured on MS medium (Murashige and Skoog 1962) in culture tubes fortified with different concentrations and combinations of auxins and cytokinins. Throughout the experiment MS medium with 3 % (w/v) sucrose and gelled with 0.8 % (w/v) agar was used. Its pH was adjusted to 5.8 with 1 N NaOH or 1 N HCL prior to autoclaving. Each hormonal combination was tried in three replicates of 10 test tubes. All the cultures during callus induction were maintained in a sterilized culture room at $27 \pm 2^\circ\text{C}$ under a 16 h photoperiod. White light was provided by fluorescent tubes at a photon rate of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity (Deshpande et al. 2010).

Preparation of in vitro cell culture extract

The isolation of crude cell culture extract of leaf derived callus tissue of *Helicteres isora* was performed as per the protocol of Nikam et al. 2009. Leaf derived in vitro cultured tissues (callus tissue) of different age of *Helicteres isora* was sun dried and grained into a coarse powder (Fig. 1). The dried leaf derived callus tissue (2 g) was hydrolyzed by heating under reflux with 2 N H_2SO_4 (15 ml) in 70 % isopropanol and boiled under reflux for 8 h. The solution was filtered and 15 ml water was added. The aqueous phase of the solution extracted four times with hexane solvent. The resulting hexane extracts were pulled together and further neutralized with 1–2 N NaOH (10 ml). Finally the hexane fractions were concentrated and evaporated on a rotary evaporator (Buchi, R – 124, Switzerland) to obtain a solid crude residue. The resulting solid crude

Fig. 1 Leaf derived callus tissue of *Helicteres isora*. (100 % callusing frequency was routinely achieved on MS basal medium fortified with $10 \mu\text{M}$ 2, 4 – D)

extract of callus tissue of *Helicteres isora* was reconstituted in 10 ml of acetonitrile, which was further used for characterization using various biochemical techniques each time using standard diosgenin for comparison (Peiqin et al. 2012).

Results and discussion

Characterization of diosgenin

Thin layer chromatography (TLC)

Crude sapogenin extract of callus tissue of *Helicteres isora* was chromatographed on pre-coated silica gel plates (60 F254, 20×10 cm, Merck, Germany) with solvent mixtures of chloroform: acetic acid: methanol: water (6.4:3.2:1.2:0.8) and analyzed by thin layer chromatography (TLC). The spots were visualized by spraying the plates with a mixture of ethanol, sulphuric acid, glacial acetic acid and anisaldehyde reagent (135:5:1:3.7) and heating them in an oven for 3–5 min at 90°C (Bhalsing et al. 2000)

The R_f value of extracted residue was in agreement corresponding to standard diosgenin, (R_f 0.56) which confirmed the presence of diosgenin. This observation was similar with previous findings in *Dioscorea alata* Var purpurea by Shah and Lele 2012.

High performance liquid chromatography

Chromatographic experiments were conducted on YL9100 HPLC system (South Korea). HPLC instrument comprising variable wavelength programmable UV—visible UV 730D detector. This instrument was controlled by the use of

Fig. 2 HPLC chromatogram of standard diosgenin (a) and HPLC chromatogram of in vitro cell culture extract of *Helicteres isora* (b)

Autochro - 3000 software installed with the equipment. Samples were injected by using a rheodyne injector fitted with a 20 μ l fixed loop. Standard and sample solutions were filtered through 0.10 μ m syringe filter before use. The mobile phase was acetonitrile: water (90:10, v/v). The separation was achieved by using column with 4.6 $\times$ 250 mm, particle size 5 μ m grace C₁₈ column.

Diosgenin was quantified in in vitro culture extract by gradient flow at 273 nm. Flow rate and injection volume were 0.7 ml/min and 20 μ l respectively. The chromatographic peaks of the analytes were confirmed by comparing the retention times and UV spectra with those of the reference standards. All chromatographic operations were carried out at room temperature (ICH guidelines 2003; Dalene and Elizabeth 2010; Mohd et al. 2012)

Owing to high reliability of High Performance Liquid Chromatography, a simple rapid HPLC method was developed for quantification of diosgenin for the first time in *Helicteres isora* as mobile phase consisted of acetonitrile: water (90:10, v/v) and flow rate of 0.7 ml/minute and elution was monitored at 273 nm with total run time of 10.00 min. The retention time of diosgenin cell culture extract was found to be 4.1 min which was similar to standard diosgenin (Fig. 2a and b).

Due to its high feasibility, HPLC method can be used for routine analysis of diosgenin in the said medicinal plant. Similar to our studies, quantitative analysis of diosgenin by HPLC has been reported in *Dioscorea deltoida* and *Dioscorea zingiberensis* by Mohd et al. 2012 and Yang et al. 2013 respectively.

Fourier transform infrared spectroscopy (FTIR)

Diosgenin C₂₇H₄₂O₃, the structure of which is determined by the presence of –OH, (C=C), methyl vibration, methylene, spiroketal ring, 25 R configuration and Sp10 H. The FTIR spectral analysis revealed a broad peak at 3,533 cm^{-1} indicating the presence of a stretching vibration of hydroxyl group (–OH) and the absorption bands at 1,632 cm^{-1} indicated the presence of (C=C) group. The peaks at 1,449 and 1,409 cm^{-1} revealed the presence of methylene vibration and methylene respectively. The absorption band at 922 cm^{-1} indicate the presence of spiroketal ring and 25 R configuration, while a absorption peak at 1,421 cm^{-1} indicated the presence of (Sp10 H). These data are comparable to the spectral data for diosgenin reported earlier in *Costus igneus* (Kalailingam et al. 2012) (Fig. 3).

Fig. 3 FTIR spectra of in vitro cell culture extract of *Helicteres isora*

Estimation of diosgenin

A stock solution of standard diosgenin ($1,000 \mu\text{g mL}^{-1}$) was prepared in acetonitrile. To each tube containing different concentration of diosgenin standard ($100\text{--}1,000 \mu\text{g mL}^{-1}$), 2 ml of acetonitrile and 1 ml of 70–72 % perchloric acid was added. Then the tubes were incubated at room temperature till the stable yellow chromogen developed. The resultant yellow colour chromogen was scanned in the UV region within 300 to 700 nm. In both cases, well defined identical peaks were found at 430 nm for standard diosgenin as well as for the crude sapogenin extract of *Helicteres isora* respectively (Fig. 4). The resultant yellow colour chromogen was measured by spectrophotometer (Shimadzu, UV – Visible 1601, Japan) at 430 nm against blank and a standard calibration curve was made and used for further studies (Peiqin et al. 2012). Diosgenin and its chromogen have been earlier detected in *Trigonella foenum – graecum*. (Sharareh 2011) and in *Costus igneus* (Kalailingam et al. 2012).

In vitro cell culture of medicinal plants with enriched bioactive principles and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds (Ramawat et al. 2009). Eight week old callus tissues of *Helicteres isora* restrain utmost amount of diosgenin (2.3 %) while, two, four and six week old callus tissues accumulate least amount of diosgenin (0.316, 0.412 & 0.560 %) respectively as compared with the in vivo parts namely stem, (0.166 %) leaf (0.394 %) and fruits (0.510 %) (Figs. 5 and 6). In our study, diosgenin content was observed to increase continuously within 2 – 8 weeks, as in vitro cultured tissue grows, the callus growth follows a typical logarithmic pattern involving slow initial cell division, induction period requiring auxin, a rapid cell division phase involving active DNA and RNA synthesis and protein followed by a gradual termination of cell division along with differentiation of callus mass (Hartmann et al. 1999).

Fig. 4 Absorption spectra of cell culture extract of *Helicteres isora*

Fig. 5 Diosgenin content in in vitro culture tissues of *Helicteres isora*

Similar to our studies, diosgenin quantification has been reported earlier in *Dioscorea deltoidea* by Mohd et al. 2012. The callus tissues derived from the nodal segments of *Dioscorea deltoidea* accumulate more amount of diosgenin (0.08 %) as compared to plant (0.017 %) (Mohd et al. 2012). Diosgenin quantification has been studied in fresh tubers of different *Dioscorea* species like *D. bulbifera*, *D. hispida*, *D. tomentosa*, *D. oppositifolia*, *D. esculenta*, *D. alata* (Kambaska et al. 2010). The biochemical analysis amongst different species revealed that diosgenin content was adequately highest in *D. bulbifera* (1.38 %) followed by *D. hispida*, (0.82 %) while the lowest quantity was found in *D. alata* (0.095 %). However, diosgenin content varies from species to species (Kambaska et al. 2010).

Fig. 6 Diosgenin content in in vivo parts of *Helicteres isora* *. *(In vivo parts of *Helicteres isora* were treated similar to callus tissue as per the protocols of Nikam et al. 2009; Peiqin et al. 2012)

This indicates that in vitro cultured tissues of *Helicteres isora* act as good alternative for diosgenin production. Thus plant cell cultures act as an attractive alternative source to whole plant for the production of high-valued secondary metabolites (Bhalsing et al. 2000)

Conclusion

The present study was aimed towards development of simple, faster and cost effective bio analytical methods to detect and estimate the marker compound (Diosgenin) from the selected plant using TLC, HPLC, FTIR and UV – visible spectroscopy for routine analysis. The present study is significant from the point of view of the fact that the diosgenin detected in the callus tissue of *Helicteres isora* was extracted and quantified for the first time. The content of diosgenin was also found to be higher in the in vitro leaf derived callus tissue (2.3 %) as compared to the plant parts. This study may be useful for the estimation and characterization of diosgenin from other species. Further studies looking for increase in high yield in diosgenin content, by studying the effect of precursor, additive and elicitor in the callus can be checked. Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics.

Acknowledgments Authors gratefully acknowledge financial assistance from UGC; New Delhi through grant no: 37- 280/2009 (SR) to SRB. The financial assistance towards instrumentation facility at School of Life Sciences from UGC, New Delhi under SAP-DRS program and DST, New Delhi under FIST program is duly acknowledged.

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