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Research Article

ISOLATION IDENTIFICATION AND QUANTIFICATION OF STEROIDS FROM *B.MONOSPERMA*

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ABSTRACT

Butea monosperma is an important medicinal plant known for its vast potential. It is a valuable plant which is commonly used in traditional system of medicine for relieving pain and inflammation, as well as in a number of metabolic disorders such as diabetes and obesity. In the present study, steroids from *B. monosperma* was identified and quantified *in vivo* and *in vitro*. Steroids were identified using chromatographic and spectral studies. Diosgenin was identified by IR and GC-MS. It was observed that in GC-MS profiling of steroids total 33 compounds were found, out of which maximum amount was of Linoleic acid ethyl ester having maximum area of around 22.72 %. It is the first report on steroids from the experimental plant.

INTRODUCTION

Plant tissue culture has brought a revolutionary break through because of its several advantages in micro propagation and production of secondary metabolites. Plant tissue culture has facilitated in producing and raising the yield of commercially important biosynthetic compounds. Plant tissue culture techniques have become especially important in the agricultural community over the past ten years. The therapeutic efficacies of many indigenous plants for several disorders have been described by practitioners of traditional herbal medicines (Natarajan *et al*., 2003).

Butea monosperma is commonly known as Flame of forest, belonging to the family Fabaceae. It is locally called as palas, palash, mutthuga, bijasneha, dhak, khakara, chichra, Bastard Teak, Bengal Kino, Nourouc and is common throughout India, Burma and Ceylon except in very acrid parts. The seeds of *B. monosperma* administered as crude powder at doses of 1, 2 and 3 g/kg to sheep naturally infected with mixed species of gastrointestinal nematodes exhibited a dose and a time-dependent anthelmintic effect. An extract from the flowers of *B. monosperma*, a plant drug used in India for the treatment of liver disorders, showed significant activity in different models of liver damage (Wagner *et al*., 1986).

All plants synthesize chemical compounds as part of their normal metabolic activities. These phytochemicals are divided into two groups- (1) primary metabolites such as sugars and fats, which are found in all plants; and (2) secondary metabolites such as alkaloid and steroids which are found in a smaller range of plants, serving a more specific function (Meskin and Mark, 2002). It is these secondary metabolites and pigments that can have therapeutic actions in human race and which can be refined to produce drugs e.g. Quinine from the Cinchona, Morphine and Codeine from the Poppy, and Digoxin from the Foxglove (Meskin and Mark, 2002).

Various secondary metabolites produced by some medicinal plants are involved in plant defense responses and facilitate plant adaptation to their environment by enhancing their general fitness and well-being. These compounds are also sources of pharmaceuticals, pesticides, flavoring agents, fragrances, and food additives. Different plants produce diverse products and their production is often related to a particular developmental stage, and is profoundly affected by seasonal variations. Cell cultures are attractive alternatives to whole plants for production of high value secondary metabolites due to consistency in quality and quantity of the desired product (Rao and Ravishankar, 2002; Sarin, 2005).

Steroids are derivatives of cyclopentenoperhydrophenanthrene and include sterols, steroidal sapogenin, steroidal glycosides, and cardiac glycosides and hormones, corticosteroids and oral contraceptives (Coppen, 1979). Among various steroids, diosgenin is one of the promising compounds which have

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attracted lot of researchers as it is found in a few higher plant species and interest in its medicinal properties has increased (Liu *et al*, 2005). It has been reported that diosgenin can be absorbed through the gut and plays an important role in the control of cholesterol metabolism (Roman *et al*, 1995). Diosgenin is generally used as starting material for partial synthesis of oral contraceptives, sex hormones, and other steroids (Zenk, 1978). The partial synthesis of steroids from plant-based precursors has been a boon because of the increasing demand for corticosteroids, contraceptives, sex hormones, and anabolic steroids since about 1960 (Hall & Walker, 1991).

MATERIALS AND METHODS

Tissue culture studies for callus cultures

In the present study following plant parts were used as explants:-

Seeds- Seeds and in vitro seedlings used for culture work were inoculated to MS medium without hormone and MS medium supplemented with various concentrations and combinations of growth hormones.

Nodal segments- Both nodal, internodal segments were taken from young juvenile tree.

Culture Medium

MS medium was used for all tissue culture studies.

Stock preparation

Stock solutions of organic and inorganic nutrients were prepared in sterile distilled water, stored in a refrigerator at 40^0C. Main growth regulators used were auxins viz 2, 4-Dichloro-phenoxy Acetic Acid (2,4 –D), Indole Acetic Acid (IAA) , Naphthalene Acetic Acid (NAA), Indole Butyric Acid (IBA) and Cytokinins viz Kinetin (Kn) , Benzyl Amino Purine (BAP). The auxins were dissolved in small quantity of absolute alcohol and cytokinins in 1N HCl. All stocks were prepared using sterile double distilled water. Double glass-distilled water was used only up to 2 days of preparation and 200 mL of each stock solution was made.

Media preparation

Medium was prepared by dissolving required amount of stock solutions. Sucrose (w/v 30 gL-1) was dissolved, filtered, mixed in stock solutions measured for the preparation of media and was made to final volume. The required growth hormones were added to the medium and for solidifying the medium 0.8% of bacteriological grade agar was used. The pH of the medium was adjusted to 5.8 with the help of 1N NaOH and 1N HCl. In each flask (100mL Borosil) approximately 30 mL and in a test tube 20 mL medium was dispensed. Slants were made to provide the larger surface area for inoculation. All the culture vessels which contained medium were plugged with non - absorbent cotton and mouth of flask/ test tube was wrapped with aluminum foil or paper and these vessels were autoclaved at 15 lbs pressure for 15-20 min.

Surface Sterilization and inoculation

Seeds were surface sterilized with mercuric chloride ($HgCl_2$) solution (0.1%; w/v) for 2 min and subsequently rinsed thrice with sterile distilled water where as nodal segments were treated with antibiotic (Ciprofloxacin, 250mgL-1) prior to inoculation in order to remove any kind of microbial interactions. Surface sterilization and inoculation of seeds and nodal segments were done in a Laminar flow hood fitted with ultraviolet light. Before starting the inoculation work, slab of Laminar flow was cleaned with rectified spirit and culture vessels containing autoclaved media, petri dishes, and spirit lamps, cotton and other things required were kept on the slab of transfer chamber. A day before inoculation of work, transfer chamber was fumigated with fumes obtained by heating formic acid and potassium permanganate (KMnO4). The forceps, scalpels, needles; scissors were kept in a glass tube column containing rectified spirit. UV light was used for an hour to sterilize the chamber. Seeds as well as nodal segments were inoculated in the flasks containing culture medium aseptically. Cultured flasks were incubated in culture chamber. The temperature of chamber was maintained at 25± 1^0 C using air conditioner and light intensity (1200 lux) was provided from fluorescent tubes (40 watt) and incandescent bulbs (40 watts). A photoperiod of 16h light was provided. The cultures were observed and examined every week and final morphogenetic data were recorded.

Steroidal Sapogenin

Extraction

Each of the dried plant parts of *B. monosperma* (Flowers, leaves and seeds) and callus were powdered weighed and defatted separately in soxhlet apparatus in petroleum ether for 24 h on a water bath. Each mixture was hydrolyzed with 15% ethanolic HCl (1g/5 mL: w/v) for 4 h by refluxing on water bath (Tomita *et al,* 1973). Each hydrolysate was filtered and filtrate extracted thrice with ethyl acetate. The ethyl acetate fractions of each sample was pooled and washed to neutrality by repeated washings with distill water, dried *in vacuo*, reconstituted in chloroform, filtered, dried again and weighed. Each test sample was replicated thrice. Thin glass plates coated with silica gel (250mμ thick) were dried at room temperature, thereafter kept at 100^0C for 30 min to activate. The freshly prepared activated plates were used for qualitative as well as quantitative analysis.

Qualitative Analysis

The crude steroidal sapogenin extract of each sample was examined on TLC, along with the reference steroidal sapogenin (diosgenin). The plates were developed in a solvent system of chloroform, hexane and acetone (23:5:2), air dried and sprayed with 50% sulphuric acid (Bennett & Heftmann, 1962) and anisaldehyde reagent (composed of 0.5 mL of anisaldehyde, 1mL of conc. sulphuric acid and 50mL of acetic acid) separately and heated to 100^0C until the characteristics colors developed. The fluorescence response as well as permanent black zones was recorded. The times required for the initial appearance of a colour reaction, the initial colour in day light and after heating for 10 minutes and the colour in UV light (360nm) were recorded. A combination of other solvent systems such as benzene and ethyl acetate (85:15; Heble *et al,* 1968) and acetone and benzene (1:2; Khanna & Jain, 1973) were also used but solvent system of chloroform, hexane and acetone (23:5:2) was comparatively better than other solvent system. Three replicates were run and Rf values were calculated.

Quantitative Analysis

Preparative thin layer chromatography (PTLC)

PTLC was used to isolate diosgenin from crude steroidal sapogenin extract on silica gel G plates by using solvent mixtures of chloroform, hexane and acetone (23:5:2). The spots were marked on TLC by spraying with anisaldeyde reagent, to one of the columns on each plate and spots corresponding to the standard diosgenin were marked and scrapped separately from the unsprayed plates/column. The PTLC was repeated until about 20mg of the substance was obtained. Co –TLC of crystallized isolated substance along with reference marker (standard diosgenin) was carried out to test the purity of isolated compounds. Such chromatograms were also visualized by spraying a solution of antimony trichloride in conc. HCl (Kadkade *et al,* 1976). After PTLC the diosgenin was crystallized from methanol-acetone (Kaul & Staba, 1968) and examined for mp, mmp and infra –red spectral studies.

Spectrophotometry for Diosgenin- The spectrophotometric method of Sanchez *et al,* (1972) was followed to estimate quantitatively the levels of diosgenin in each sample. It removed the preparation of regression curve of the standard diosgenin from it's stock solution (1mg/mL) prepared in chloroform, from which different concentrations (20µg to 200µg) were made and applied on silica gel G plates with fine applicators separatively. The plates were then developed along with a parallel run of blank in an organic solvent of chloroform, hexane and acetone (23:5:2), which were later on dried and exposed to iodine vapours. The resultant dark yellowish spots as also the spots corresponding in the blank were marked and the plate were heated to 100^0C for 15 minutes to remove iodine. Each of the marked spot was scrapped along with the adsorbent, transferred to separate test tube and eluted with 5 mL of methanol. The mixture was then centrifuged, the supernatant transferred to separate test tube and evaporated to dryness. To the dried residues, 4mL of 80% methanolic sulphuric acid was added and left at room temperature for about 2 hours by intermittent shaking. Optical density of the reaction mixture was read at 405nm against a blank solution (80% methanolic sulphuric acid). Three replicates of each concentration were taken and average optical density was calculated. A regression curve between various concentrations and their respective optical density was computed which followed the Beer's law.

Each of the crude extract of plant and tissue samples were dissolved in 5 mL of chloroform and applied (0.1mL) on silica gel G plates along with standard diosgenin as marker, developed in solvent mixture of chloroform, hexane and acetone (23:5:2) which were later on dried and exposed to iodine vapours. The resultant dark yellowish spots and corresponding spot of the standard authentic sample were marked on each plate, scrapped, eluted with methanol, dried and then treated with 80% methanolic sulphuric acid as above. The concentration of diosgenin in each case samples were worked out referring to their optical densities in the standard curve and the results were calculated on dry weight basis. Three replicates of each sample were taken and their mean values calculated.

RESULTS

Tissue culture

Effect of auxins (2,4-D and NAA) in combination with cytokinin (BAP/Kn) on callus induction

It was observed that BAP (2.5 mg/L) in combination with NAA (0.5 mg/L) was best for callus induction and establishment (Fig 1 and 2). The callus so produced was compact and blackish in color. It grew profusely and possessed high capacity of growth. This callus when sub cultured show vigorous growth. Kn did not proved to be beneficial for producing callus**.**

Fig 1 Initiation of callus from nodal segments of *B. monosperma* in BAP+NAA (2.0+0.5 mg/L)

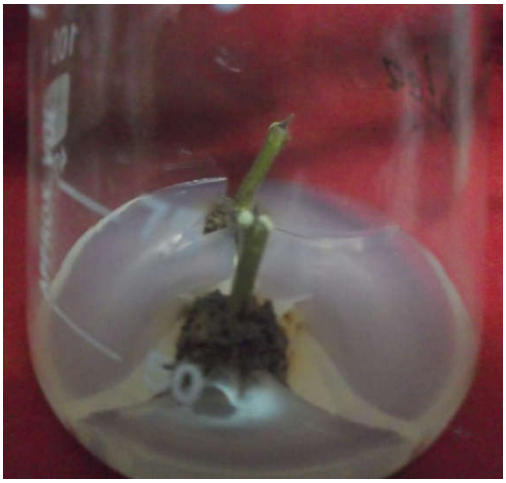

Fig. 2 Blackish and compact callus induction from nodal segments of *B. monosperma* in BAP+NAA (2.5+0.5 mg/L)

Steroids

Qualitative

Thin layer chromatography of isolated sapogenin gave single flouroscent spot under UV lamp when developed in solvent system of Chloroform: Hexane: Acetone (23:5:2). A greenish yellow coloured spot was observed after spraying of these developed plates with anisaldehyde reagent and with 50% sulphuric acid. Spot having R_f 0.58 coincided with that of standard diosgenin. Isolated fraction was purified and subjected to crystallization when subjected to determination of melting points, corresponded with that of respective diosgenin standard (diosgenin-203-205^{0}C). The characteristic peaks of IR

spectra of isolated diosgenin also superimposible with IR spectra of reference compound (Table 1 and Fig. 3.)

Table 1 Chromatographic Behavior and Physico-chemical Characteristics of Isolated Diosgenin

Isolated Compound	In UV	R_f Value		Color After Spray		Melting Point (°C)
		S_1	S_2	R_1	R_2	
Diosgenin	BR-BL	0.58	0.57	GN	GN	203-205

BR- Brownish Red
BL- Brownish light yellow
GN- Greenish yellow
S1- solvent having Chloroform: Hexane : acetone in ratio of 23:5:2
S2- Solvent having Benzene and ethyl acetate in ratio of 85:15
R1- color after spray with 50% Sulphuric acid
R2- color after spray with anisaldehyde reagent (composed of 0.5 mL of anisaldehyde , 1mL of conc. sulphuric acid and 50mL of acetic acid)

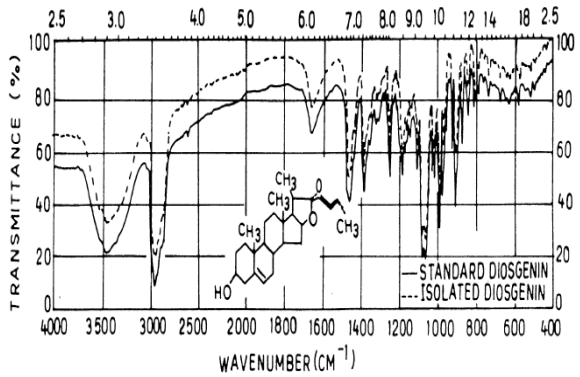

Fig 3 Infrared spectra of standard and isolated Diosgenin

Quantitative

The data revealed that the total diosgenin content was found to be maximum in seeds (2.78 mg/gdw) followed by 6 week old callus cultures (2.27mg/gdw) and minimum in 2 week old callus culture (0.82) and minimum (0.97 mg/gdw) in leaves **(Fig. 4 and 5)**

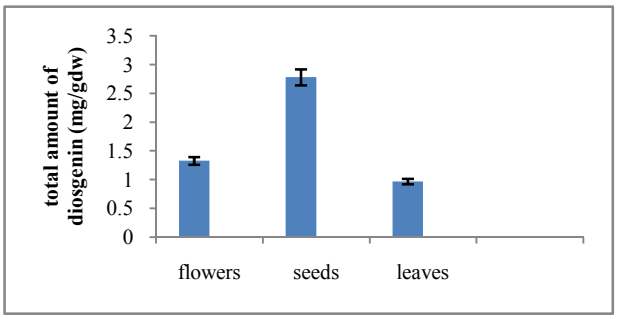

Fig 4 Yield of Diosgenin isolated (mg/gdw) from various plant parts of B. monosperma

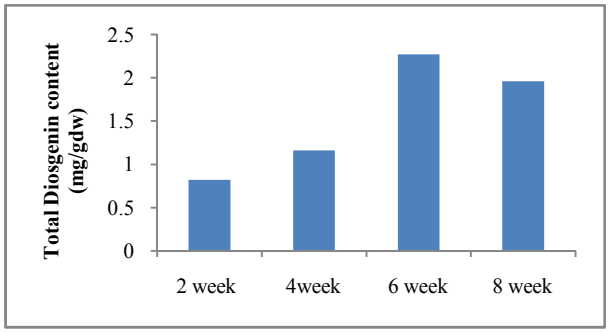

Fig 5 Yield of Diosgenin isolated (mg/gdw) from various stages of callus cultures of B. monosperma

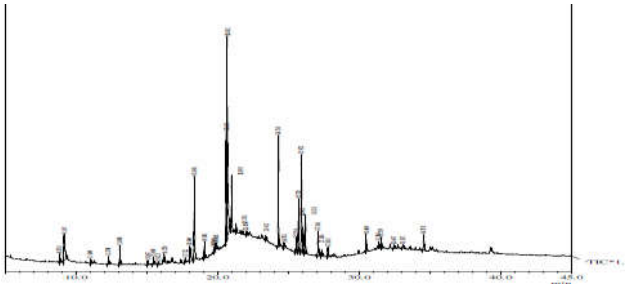

Fig 6 GC-MS analysis of compounds in steroidal extract of *B. monosperma*

Table 2 Various compounds identified by GC –MS in steroidal extract of *B. monosperma*

RT	Compound Name	Area	Area %
8.870	1-Tridecene	125591	0.54
9.165	3-Buten-2-one, 4-hydroxy-4-phenyl-	1398181	5.96
11.049	Phenol, 2,4-BIS(1,1-Dimethylethyl)-	61807	0.26
12.309	Phosphonic Acid, Dioctadecyl Ester	124861	0.53
13.089	2,4-Dimethyl-6-phenylpyridine	330972	1.41
15.067	Benzene, 1,1'-(1,2-Cyclobutanediyl)BIS-, CIS-	78146	0.33
15.469	Phosphonic Acid, Dioctadecyl Ester	132600	0.56
15.821	2-Ethylhexyl salicylate	38607	0.16
16.250	FARNESYL ACETATE 3	81079	0.35
17.722	Cyclododecene, (Z)-	93379	0.40
18.044	Dibutyl phthalate	161396	0.69
18.368	Hexadecanoic Acid, Ethyl Ester	1801733	7.68
19.081	Hexadecanoic acid, trimethylsilyl ester	309090	1.32
19.754	6,11-HEXADECADIEN-1-O	53865	0.23
19.817	9-Octadecenoic acid (Z)-, methyl ester	54249	0.23
19.885	2-Buten-1-one, 3-amino-1-phenyl-4,4,4-trifluoro	126386	0.54
20.682	Linoleic acid ethyl ester	5333270	22.72
20.995	Ethyl Oleate	1088620	4.64
21.302	Oelsaeure, Trimethylsilylester	165942	0.71
22.050	Ethyl 9-Hexadecanoate	62180	0.26
23.412	Ethyl Nonadecanoate	84159	0.36
24.310	Benzonitrile, m-phenethyl-	2076274	8.85
24.702	Oxalic acid, decyl 3,5-difluorophenyl ester	98330	0.42
25.554	Bis(2-ethylhexyl) phthalate	345981	1.47
25.720	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans	1188041	5.06
27.106	,4-Dibenzoyl-2,4-Diamino-Nitrobenzol	688488	2.93
27.366	2,4-Dibenzoyl-2,4-Diamino-Nitrobenzol	141042	0.60
27.801	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	257908	1.10
30.499	Squalene	366920	1.56
31.386	Hentriacontane	94569	0.40
31.579	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	229362	0.98
32.467	Docosahexaenoic Acid, 1,2,3-Propanetriyl Est	74828	0.32
34.570	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	461805	1.97

It was observed that in GC-MS profiling of steroids total 33 compounds were found, out of which maximum amount was of Linoleic acid ethyl ester having maximum area of around 22.72 %.

DISCUSSION

Plant tissue culture technique is now a well-established technology to improve the quality and quantity of useful plants. Like many other technologies, it has gone through different stages of evolution; scientific curiosity, research tool, novel applications and mass exploitation. Initially, plant tissue culture was exploited as a research tool and focused on attempts to

culture and study the development of small, isolated segments of plant tissues or isolated cells. Around the mid twentieth century, the notion that plants could be regenerated or multiplied from either callus or organ culture was widely accepted and practical application in the plant propagation industry ensued. The technique was heralded as the universal mass clonal plant propagation system for the future and the term 'micropropagation' was introduced to describe more accurately the processes. In the present study callus cultures of *B. monosperma* were raised from nodal segments of this plant. The observations of present investigation are in agreement with Thorpe and Patel (1984) that tissue or organs used as source of explants can also be determinant for the success of plant tissue culture (Akbas *et al.*, 2008). It has been observed that juvenile and actively dividing plant responded effectively *in vitro* condition due to vigorous vegetative development stage and absence of reproductive structure formation. Even in juvenile stage, tissue and organ regeneration is reported to be more with the younger and actively dividing tissues (Endress, 1994).

Medicinal plants are believed to be important source of bioactive compounds with potential therapeutic effects (Eisner, 1990). The secondary metabolites of plants are source of various phytochemicals that could be directly used as intermediates for the production of new drugs. Traditional medicines still plays an important role in the modern primary healthcare system of the developing countries. The natural products are believed to be more acceptable to the human body, as compared to modern synthetic drugs used to cure many diseases. Thus the most important factor needed is to derive the maximum benefit from the traditional system of medicine for providing adequate healthcare service to rural people (Ghani, 1990).

Sapogenins are C_{27} steroids, widely distributed in a number of families. Steroidal sapogenins, the pharmaceutically important natural products have been reported from a number of plants (Mangla & Kamal, 1989). Diosgenin, useful in contraception is industrially important, which is either microbially or chemically converted to medicinally useful steroids commercially extracted from underground portion of various *Dioscorea* species (Staba, 1977). Nino *et al* (2007) reported the percentages of diosgenin obtained from the seventy four accessions of *D. polygonoides* on range from 0.02 to 2.64%, which was significant since there are several reports where the diosgenin contents was very low. In the present investigation one sapogenin namely diosgenin confirmed in plant. Parts and callus cultures of *B. monosperma*. The data revealed that the total diosgenin content was found to be maximum in 6 weeks old callus cultures (2.27mg/gdw). Among various plant parts maximum content as observed in seeds (2.78 mg/gdw) and minimum (0.97 mg/gdw) in leaves

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