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Micropropagation of a rare medicinal Dasamoola plant and analysis of its phytochemical constituents

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Abstract

O. indicum Vent., a valuable medicinal tree with pharmacologically important plant parts has fallen victim to overexploitation, thereby being pushed to the vulnerable and endangered lists in different Indian states. This necessitates an urgent need for its conservation through micropropagation and restoring its biodiversity. Tissue culture techniques can also be used for developing an alternative source for whole plant/parts or secondary metabolites. The callusing responses of *O. indicum* Vent. in the present study have increased the scope of its re-establishment through micropropagation, in auxin-cytokinin containing Murashige and Skoog medium, with maximum biomass attained in media fortified with the growth regulator formulation 6-benzyladenine at 3 gm/litre and 1-naphthalene acetic acid at 1gm/litre. Phytoconstituents were revealed through preliminary screening, HPTLC and UPLC-Q-TOF-MS analysis.

Key words: *O. indicum* Vent., micropropagation, phytoconstituents

Introduction

Plants are rich sources of bioactive secondary metabolites that resist them against predators, pathogens and physical stress. A vast variety of them provide health benefits to humans. Different classes of secondary metabolites – phenolic compounds (Azmi *et al.*, 2006, Apostolou *et al.*, 2013), flavonoids (Ren *et al.*, 2003, Chahar *et al.*, 2011), terpenoids (Huang *et al.*, 2012), alkaloids (Wall and Wani, 1996), tannins (Marzouk *et al.*, 2007), saponins (Man *et al.*, 2010) and steroids (Bradford and Awad, 2007) - in plants have been proved to be potent anti cancer agents. But, according to an article published in collaboration by the IUCN and TRAFFIC (the wildlife trade monitoring network), the population of many important species of medicinal plants have declined in India due to their overexploitation for meeting the demands of native and foreign drug markets

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(e-Article, 2008). Non sustainable collection methods, indiscriminate felling of trees and opportunistic marketing have led to the exhaustion of many plant resources (Schippmann *et al.*, 2006).

O. indicum Vent., which is also documented as a valuable medicinal tree species possessing pharmacologically important plant parts - leaves, roots, stem, fruits and seeds (Deka *et al.*, 2013) - has also fallen victim to overexploitation and subsequent population depletion (Yasodha *et al.*, 2004). This valuable tree got pushed to the vulnerable list in Karnataka and Andhra Pradesh and endangered in Kerala, Maharashtra, M.P. and Chhattisgarh and is feared to become endangered soon in other states too, as studied by the Foundation for Revitalisation of Local Health Traditions (FRLHT), Bangalore, India (Ravikumar and Ved, 2000). Natural propagation of the tree is limited by the low viability and high rate of abortion (30.56 to 47.62 %) of its seeds (Gunaga *et al.*, 2012). This necessitates an urgent need for its conservation and regeneration through micropropagation (Najar and Agnihotri, 2012). Moreover, *O. indicum* Vent. is also used as an ingredient of *Dasamoola*, the Ayurvedic formulation comprising roots of ten plants. The plants have been subjected to severe uprooting, with no concomitant replanting. Identifying the crisis, attempts of micropropagating some of these plants (*Gmelina arborea* and *Aegle marmelos*) were begun initially, – as reviewed by Yasodha *et al.* (2004) - followed by a few studies on *O. indicum* Vent. also (Dalal and Rai, 2004, Gokhale and Bansal, 2008, Gokhale and Bansal, 2009). Along with the benefit of regenerating medicinal plants facing biodiversity threats, tissue culture techniques can also be used for developing an alternative source for whole plant/parts or secondary metabolites (DiCosmo and Misawa, 1995, Mulabagal and Tsay, 2004).

During *in vitro* propagation, selection of the culture medium and the presence of growth regulators are critical in callusing, shoot and root development. The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported by Skirvin *et al.* (1990). The commonly used synthetic auxins are indole acetic acid (IAA), 1-naphthalene acetic acid (NAA) and indole butyric acid (IBA). 6-benzyladenine (BA) and kinetin are the commonly used synthetic cytokinins. Standardisation of the medium with the suitable growth regulator is a must before micropropagation, since the plant growth responses vary with the species and environmental conditions. The preliminary part of this work involved the micropropagation of *O. indicum* Vent. using media fortified with plant growth regulators and the initiation of callus cultures.

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Since plant extracts are mixtures of different types of compounds, the separation and analysis of compounds is to be attained precisely, which involves chromatographic procedures like TLC, HPLC, HPTLC, column chromatography etc, as well as non-chromatographic procedures such as phytochemical screening assays and immunoassays, following solvent extraction (Sasidharan *et al.*, 2011). The study also involved the phytochemical evaluation of the callus extract through preliminary screening, HPTLC and UPLC-Q-TOF-MS analysis.

Materials and methods

Chemicals

Murashige and Skoog medium for micropropagation was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India; and the plant growth regulators - BA, IAA, IBA and NAA for fortification were purchased from Sigma-Aldrich Inc., St Louis, USA.

Micropropagation of *O. indicum* Vent.

Callus induction

All micropropagation studies of *O. indicum* Vent. were conducted in MS medium. The dried pods of *O. indicum* Vent. were collected from the Ayurvedic Garden maintained in the Amala Cancer Hospital campus, Thrissur, Kerala, India. Seeds were inoculated into basal MS media and maintained under at a temperature of 25 ± 2^0 C, in dark for a period of 2 weeks and thereafter for 4 weeks under a photoperiod (16-hour light/8 hour dark) with light intensity 1000-1500 lux. Nodal explants of length ranging from 10-15 mm were used for establishment of tissue culture. They were inoculated into MS media supplemented with combinations of the cytokinin, 6-benzyl aminopurine (BA) at a concentration of 1 mg/L and auxins, 1-naphthalene acetic acid (NAA- 0.5 mg/L) or indole acetic acid (IAA- 0.5 mg/L). The culture tubes were incubated in dark at 25 ± 2^oC for four weeks. From the calluses initiated, (~) 500 mg (fresh weight) inoculates were cut out and subcultured into MS media supplemented with combinations of cytokinin (BA- 2 and 3 mg/L) and auxins (NAA- 0.5 and 1 mg/L or IAA- 0.5 and 1 mg/L). The increase in biomass in a time span of three weeks was assessed with cultures maintained under a photoperiod (16-hour light/8 hour dark) with light intensity 1000-1500 lux.

Extract preparation

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The tissues harvested from micropropagation were lyophilized and ground into fine powder. This was extracted using 70% methanol by periodic (every 4 hours) stirring and warming at 45-50^{o}C. The extracts were filtered and evaporated to get a dry residue, until all traces of solvent was removed. The percentage yield of extract obtained from callus (OCM) was 5.17.

Preliminary phytochemical screening

The OCM extract was tested for the presence of alkaloids, phenolic compounds, tannins, flavonoids, phytosterols, steroids, saponins and carbohydrates according to the standard procedures (Harborne, 1984, Sofowora, 1993, Trease and Evans, 2002).

HPTLC profiling

The OCM extract was subjected to qualitative high performance thin layer chromatography (HPTLC) (Sharma *et al.*, 2014) on pre-coated TLC plates of silica gel 60 F_{254} (Merck, India). The solvent system used was toluene: ethyl acetate: formic acid (5:5:0.5). The developed plates were scanned at 366 and 254 nm with CAMAG TLC Scanner 3. Derivatization of the plate was performed by spraying with anisaldehide – sulphuric acid reagent and heating for 10 min in a hot air oven at 115ºC. The data were analyzed using CAMAG WinCat software. The number of bands formed was determined and the R_f values were recorded.

UPLC-Q-TOF-MS analysis

The Ultra high pressure liquid chromatographic (UPLC) analysis of the extract was performed using methanol and 0.1% aqueous formic acid as the mobile phase through a BEH C18 column (with specifications, 50 mm × 2.1 mm × 1.7 µm, purchased from Waters, USA) using gradient elution (0-5 min, 5% acetonitrile; 5-7 min, 95% methanol; 8-9 min 5% methanol) at a flow rate of 0.3 ml/min. Detection was achieved at a wavelength of 210 - 400 nm. The MS and MS/MS data was retrieved from Xevo G2 (Waters, USA) Quadrapole – Time-of-Flight (Q-TOF) system. Mass spectrometric operations were carried out and the results were compared to previous MS/MS fragmentation reports to identify the compounds. Some of the active constituents were thus identified.

Results

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Callus induction

Nodal explants of *O. indicum* Vent. showed best callusing ability in MS media fortified with the growth regulator formulation BA3 NAA1 with an increase in biomass of 1.8 ± 0.48 g, as given in table 1. Stages of callus initiation are represented in figure 1.

Figure 1. Stages of callus initiation from nodal explants of *O. indicum* Vent. in MS media fortified with BA3 and IBA1. a) Callus initiation from nodal explant; b) subcultured callus; c) fully grown callus

a) b) c)

Table 1. Biomass (g) of callus generated from *O. indicum* Vent. nodal sections in MS media fortified with the growth regulators- BA, NAA, IAA and IBA in specified combinations. *Values expressed are mean ± SD for 72 culture tubes.

BA (mg/L)	**NAA (mg/L)**	**IAA (mg/L)**	**IBA (mg/L)**	**Increase in biomass(g) per inoculate**
2	0.5	-	-	0.79±0.13*
2	1	-	-	0.91±0.16
3	0.5	-	-	0.85±0.29
3	1	-	-	1.61±0.67
2	-	0.5	-	0.39±0.35
2	-	1	-	0.46±0.13
3	-	0.5	-	0.40±0.68
3	-	1	-	0.87±0.11

2	-	-	0.5	0.89 ± 0.32
2	-	-	1	0.89 ± 0.45
3	-	-	0.5	1.74 ± 0.51
3	**-**	**-**	**1**	**1.8 ± 0.48**

Preliminary phytochemical screening

The presence of phytoconstituents in the callus (OCM) extract is given in table 2. It tested positive, with moderate presence of phenols, flavonoids, alkaloids; carbohydrates, tannins and phytosterols also, though detected in low amounts. No terpenoids, saponins or sterols were detected.

Table 2. Phytoconstituents detected in *O. indicum* Vent. wild root bark (OIM) and callus (OCM) extracts

Phytoconstituent	**Detection (+/-) (presence /absence) in OCM**
Phenolics	++
Flavonoids	++
Alkaloids	+
Saponins	_
Terpenoids	_
Tannins	+
Carbohydrates	+
Phytosterols	+
Steroids	_

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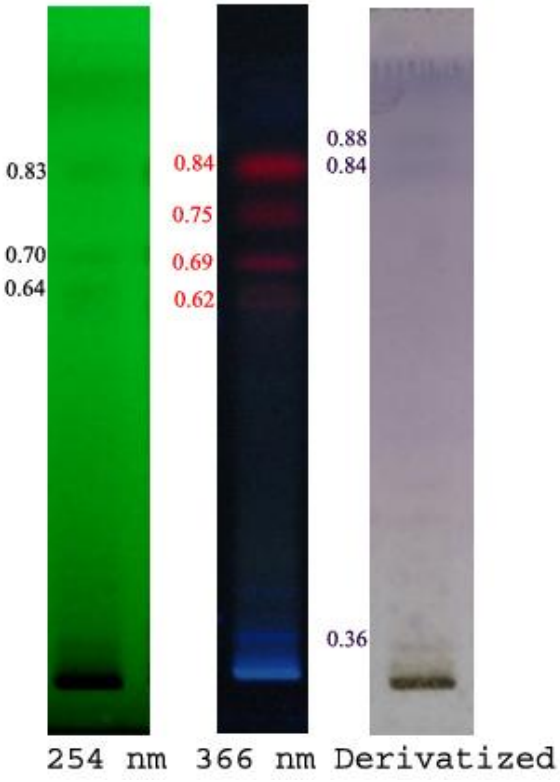
(The presence of phytoconstituents in high, moderate and low amounts is indicated as '+', '++' and '+++' respectively. '_' indicates absence of a phytoconstituent).

HPTLC analysis

As given in Figure 2, at 254 nm, 3 bands were detected in the lane (with R_f - 0.64, 0.70 and 0.83). Plates scanned at 366 nm revealed 4 red bands (with R_f - 0.62, 0.69, 0.75 and 0.89). Derivatized plate revealed 3 violet bands from the extract (with R_f - 0.36, 0.84 and 0.88).

Figure 2. HPTLC profile of *O. indicum* Vent. callus (OCM) extract on pre-coated silica gel 60 F_{254} TLC plates with toluene: ethyl acetate: formic acid (5:5:0.5) as solvent. The plates scanned at 366 and 254 nm as well as the derivatized plate sprayed with anisaldehyde – sulphuric acid reagent is shown in the figure. (R_f values of bands are shown).



UPLC-Q-TOF-MS analysis

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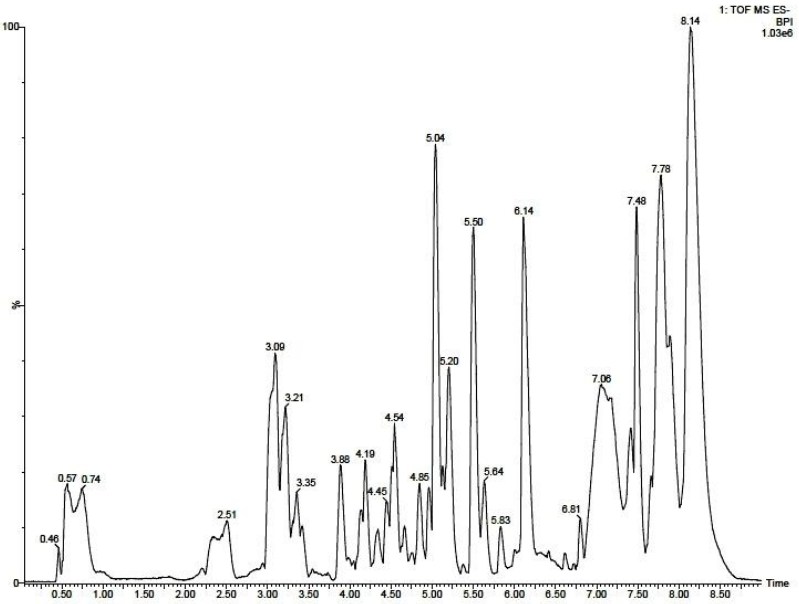
Figure 3. shows the total ion chromatogram of the OCM extract subjected to *UPLC-Q-TOF-MS analysis*.



The compounds provisionally identified based on previous fragmentation data from literature are enlisted in table 3.

Sl. No:	**RT (Min)**	***m/z* and relative abundance %**	**Molecular weight (kDa)**	**Molecular formula**	**Name of the compound**
1	3.09	507.1807	508.12169	$C_{23}H_{24}O_{13}$	Syringetin-3-O-glucoside
2	3.88	163.0421	164.04734	$C_9H_8O_3$	2-Coumaric acid
3	4.85	445.0854	446.08491	$C_{21}H_{18}O_{11}$	Baicalein-7-O-glucuronide (Baicalin)
4	5.179	315.0562	316.05830	$C_{16}H_{12}O_7$	5,7-Dihydroxy-2-(4-hydroxyphenoxy)-6-methoxychromone (Capillarisine)
5	5.64	269.0502 (100%)	270.05282	$C_{15}H_{10}O_5$	5,6,7-trihydroxyflavone (Baicalein)

6	6.14	283.0658 (60%)	284.068	$C_{16}H_{12}O_5$	5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one (Biochanin A)
7	7.49	357.2137	358.214409	$C_{21}H_{20}O_{11}$	Luteolin-6-C-glucoside (Iso orientin)

Discussion

In the context of depleting medicinal plant biodiversity, the scope of *in vitro* regeneration is accelerating pace, thereby ensuring continuous supply of resources for producing plant based medicines (Tripathi and Tripathi, 2003); and the results of the present study suggest possibilities of re-establishing the biodiversity of *O. indicum* Vent. and ensuring its continuous supply. The standardization of culture conditions is a crucial step in tissue culture because the nutritional requirements, optimal growth conditions and responses of plants to growth regulators vary among species. In the present study, nodal explants from *O. indicum* Vent. showed callusing responses in the provided culture conditions. The nodal explants developed green friable calluses later turning compact, when kept in BA (cytokinin)-NAA (auxin) medium. Dark condition favoured callus initiation and growth. MS medium supplemented with BA (3 mg/L) and NAA (0.5 mg/L) was found to be superior for callus growth, compared to the other growth regulator combinations. Auxins, cytokinins and their interactions influence cell growth, cell division, tissue differentiation and organogenesis in plants (Su *et al.*, 2011). Cytokinins stimulate cell division, release lateral bud dormancy and promote adventitious lateral bud formation. Auxins initiate cell division, promote root development and organisation of meristems to calluses (Aloni *et al.*, 2006, Müller and Leyser, 2011). But, when used in combination, the requisite for each plant varies. Here, with auxin concentrations kept unchanged (1 mg/L or 0.5 mg/L), increasing cytokinin concentration (3 mg/L) promoted higher growth rate of callus than 2 mg/L formulation. Similarly, keeping cytokinin concentrations constant (2 or 3 mg/L), it was found that 1 mg/L of the auxin NAA favoured better growth rate than 0.5 mg/L application. Hence it is conclusive that both auxins and cytokinins positively influence callus development and growth in *O. indicum* Vent. in tissue culture systems.

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The validation of phytochemical properties of tissue culture derived plant/plant parts is as important as their regeneration. The bioactive properties are attributed mainly to the presence of secondary metabolites in plants, whose production shows qualitative and quantitative variations in accordance with the growth stages and ambient conditions. Bioactive compound classes such as flavonoids and phenols, though detected in OCM, gave feeble positive tests, during preliminary phytochemical screening. The number of prominent bands revealed through HPTLC also indicated the presence of metabolites, irrespective of its undifferentiated stage. UPLC-Q-TOF-MS fingerprint reconfirmed the presence of already reported flavonoids such as baicalein and biochanin A (Zaveri *et al.*, 2008), along with flavonoid compounds such as baicalin, Luteolin-6-C-glucoside (Iso orientin) Syringetin-3-O-glucoside2-Coumaric acid5,7-Dihydroxy-2-(4-hydroxyphenoxy)-6-methoxychromone (Capillarisine). The compounds which revealed their presence are attributed with anti proliferative properties- biochanin A (Kole *et al.*, 2011), baicalin (Peng *et al.*, 2015) and baicalein (Roy *et al.*, 2007) against cancer cell lines. The anti oxidant properties of baicalein (Shieh *et al.*, 2000, Kang *et al.*, 2012) and biochanin A (Zhang *et al.*, 2011a) have been established previously, and is also discussed in earlier chapters. Baicalin, the glucoronide of baicalein is also antioxidant in nature (Peng-fei *et al.*, 2013). Most of the compounds identified in the UPLC-Q-TOF-MS analysis of the extract were flavonoids, which are already appreciated for their anti inflammatory effects (Kim *et al.*, 2004, Serafini *et al.*, 2010).

Development of callus cultures has been identified as an alternative source for biologically important secondary metabolites (Rao and Ravishankar, 2002, Ali *et al.*, 2013), and therefore, can evade situations of sacrificing whole plants. In the context of the rarity of the plant and its demand in the Ayurveda pharmaceutical market, the positive callusing response of *O. indicum* Vent. is recommendable of it to be used as a substitute for medicinal preparations. For this, as a pre-requisite, a comparative evaluation of the phyto-pharmaceutical properties of the callus with the wild plant part is to be done; which will validate the use of the micropropagated plant part as an alternative.

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