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Research Article

Induction of *in vitro* root tubers in *Holostemma annulare* (Roxb.) K. Schum. for the production of bioactive metabolites

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Abstract *Holostemma annulare* (Family Asclepiadaceae) is an invaluable vulnerable medicinal plant; the root tubers are used in Ayurveda medicine and by folk healers to treat various ailments. In this study, Schenk and Hildebrandt medium fortified with the cytokinins 6-benzyl adenine, kinetin, and auxins, including indole 3-butyric acid, indole 3-acetic acid, α-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid, were checked for their efficiency on root tuber induction from different explants. Adventitious root tubers were more successfully induced from *in vitro* leaf segments and shoots when cultured in Schenk and Hildebrandt medium supplemented with 0.5 mg/l of α -naphthaleneacetic acid. In addition, preliminary phytochemical analysis of *in vitro* root tubers and identification of different secondary metabolites were conducted. Thin layer chromatography and high performance thin layer chromatography analysis of the crude methanolic extracts of the *in vitro* root tuber identified the presence of lupeol, a bioactive triterpene. Adventitious root tuber induction offers a novel method for the *in vitro* production of bioactive metabolites that can be scaled up by bioreactors, thus ensuring the conservation and sustainable utilization of *H. annulare*. The study warrants further scale-up production and pharmacological investigation that can be extended for pharmaceutical needs.

Keywords Adventitious *in vitro* root tubers, *Holostemma annulare,* high performance thin layer chromatography (HPTLC), lupeol, Schenk and Hildebrandt (SH) medium

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Introduction

Holostemma annulare (Roxb.) K. Schum. (Syn. *Holostemma ada-kodien* Schult) is a lactiferous medicinal herb with conspicuous flowers and tuberous roots, locally known as "*Adapathiyan*" belonging to Family Asclepiadaceae is reported to be endangered in Kerala (Gowthami et al. 2021) and it is chiefly distributed in the tropical rain forests such as India, West peninsula, Sri Lanka and China (Sivarajan and Balachandran 1994). It is used in traditional system of medicine for maintaining youthful vigour and potentiality (Sudhakaran 2017). Traditionally the plant is used as an alternative, astringent to the bowels; cures ulcers, diseases of the blood, worms (Irimpan et al. 2011), itching, leucoderma; useful in gonorrhoea as tonic and stomachic, aphrodisiac agent (Kirtikar and Basu 2005). The root tubers of this plant are medicinally important due to the presence of terpenoid sugars and aminoacids and are useful in ophthalmopathy, orichitis, cough, fever, burning sensation, stomachalgia and also as expectorant, tonic, stimulant, aphrodisiac and galactagogue (Geetha et al. 2009; Irimpan et al. 2011; Warrier et al. 1995). Roots are used as ingredient for the preparation of the drug '*Jivanti*' (Kolammal 1979; Raviraja Shetty 2019). According to Khare (2004), Charaka and Bhavaprakasha described *Jivanti* as Shakashreshtha (the best among leafy vegetables), an agent for spermatogenesis and galactagogue by Sushruta, and according to Dhanvantari Nighantu and Kaiyadeva Nighantu it is known to promote vision. The drug is considered to have the capability of maintaining youthful vigour and strength. The leaves, flowers and fruits are eaten as vegetable and the root is used in many ayurvedic formulations such as 'Jeevanthyadi ghritham', 'Aswaghandhadi ghritham', 'Jeevanthyadi yamakam', 'Balaristam', 'Anuthailam', 'Punarnavabaladi kashayam', 'Chandanadi thailam' (Vedavathy 2004).

The tuberous roots of *Holostemma* have huge demand in South Indian pharmacies (Gowthami et al. 2021) and

more than 150 tonnes of root tubers are required every year for ayurvedic preparations in these pharmacies (Raviraja Shetty et al. 2019; Sunil et al. 2016). The destructive and ruthless utilization of root tubers, the plant population is gradually reducing, which leads to the species being listed as vulnerable medicinal plant in FRLHT red list (Pushparajan and Surendran 2014). The species is conventionally propagated through seeds, stem cuttings and root cuttings and it is hampered due to several reasons. During summer season the plant is dormant and young shoots which develop during pre-monsoon and monsoon seasons are eaten by herbivores and insects due to the presence of the sweet latex (Tuppad et al. 2017). It flowers once in a year and the fruit set is poor due to incompatibility. Though there are limited reports on micropropagation through direct and indirect organogenesis (Decruse and Seeni 2002; Decruse et al. 2004; Martin 2002; Balkrishnan et al. 2018), somatic embryogenesis (Martin 2003), hairy root induction (Karmarkar et al. 2001), cryopreservation (Decruse et al. 1999) and *in vitro* medium-term conservation (Keshavachandran et al. 2005; Tuppad et al. 2017) of *Holostemma* species, still there is lacuna on *in vitro* tuberous root induction in this taxa. So the present study focused on the development of a faster and efficient method of *in vitro* propagation and root tuber induction followed by phytochemical determination of the bioactive secondary metabolite 'lupeol' using HPTLC all of which in turn can contribute to the *in vitro* conservation and sustainable utilization efforts in this medicinal wealth.

Materials and Methods

Plant material

Holostemma annulare (Roxb.) K. Schum. (Syn. *Holostemma ada-kodien* Schult) collected from the native habitat of Palode in Kulathooppuzha forest range of Southern Western Ghats and maintained in the green house of Department of Botany, University college, Thiruvananthapuram, Kerala, India were used as the source of explants for the present study. The plant specimens were identified by taxonomic experts and deposited in the indexed herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India (Voucher Number TBGT 84555).

Shoot culture establishment

Explants like nodes and shoot tips collected from disease-free plants of *H. annulare* were used for establishing *in vitro* culture system. The explants were washed thoroughly with running tap water for 20 minutes to remove all easily removable particles. They were then soaked in 20% (v/v) labolene for 10 minutes, washed by agitation for 15-20 minutes and kept under running tap water for 30 minutes with constant gentle shaking. This was followed by successive washing five to six times with distilled water. Subsequent surface sterilization procedures were done inside the laminar air flow chamber. The explants were immersed in 10% (v/v) Sodium hypochlorite (NaOCl) for 10-15 minutes and then rinsed in sterilized distilled water. After washing, the explants were again surface sterilized in 0.1% (w/v) mercuric chloride for 6-10 minutes, then thoroughly washed with sterile distilled water 5-6 times. The segmented explants were inoculated to SH medium (Schenk and Hildebrandt 1972) fortified with 0.25, 0.5, 1.0, 2.0 mg/l BA either individually or 0.25, 0.5, 1.0, 2.0 mg/l BA in combination with 0.5 mg/l Kinetin or 0.1, 0.5, 1.0, 1.5 mg/l NAA. For increasing the multiplication rate, the initiated shoots were transferred to SH medium augmented with various plant growth regulators like 0.5, 1.0 and 2.0 mg/l BA along with 0.5, 1.0, 1.5, 2.0 mg/l Kinetin or (1.0, 2.0 mg/l BA with 0.5, 1.0 mg/l IBA or NAA.

In vitro adventitious root tuber induction

The *in vitro*-raised shoots (4-5 cm) were transferred to SH medium augmented with different concentrations and combinations of auxins like NAA/ IBA/ IAA/ 2,4-D (0.05, 0.1, 0.5, 1.0, 1.5, 2.0 mg/l) and the leaf explants were transferred to SH medium supplemented with 0.05, 0.1, 0.5, 1.0, 2.0, 3.0 mg/l NAA and 0.05, 0.1, 0.5 mg/l IBA for initiating adventitious root tubers.

Adventitious roots induced were subcultured to liquid SH medium containing the growth regulator that evoked better induction of *in vitro* roots and were kept under constant agitation on a gyratory shaker (Scigenics) at 80 rpm for enhancing adventitious branching and to establish root suspension culture.

Phytochemical analysis of *in vitro* root tubers

For detecting the presence of bioactive metabolites in the *in vitro* root tubers harvested from root suspension culture, preliminary phytochemical analyses were carried out using standardized procedures and compared with root tubers collected from *in vivo* source plants.

Chromatographic separation of bioactive component *viz.* lupeol was done by TLC and HPTLC.

TLC (Thin Layer Chromatography)

About 2.25 g *in vitro* and *in vivo* samples were weighed and dried under room temperature. They were grinded in 3 ml methanol using mortar and pestle and the extract was spotted onto the baseline of TLC plate. The solvent system used was petroleum ether : methanol in different ratios (5:5, 7:3, 9:1). The chromatogram was developed till the solvent front migrated to ¾th of the silica gel plate (TLC silica gel $60F_{254}$, Merck). The spots developed were visualized under ultra-violet chamber (Kemi) (365 nm). Authentic reference standard of lupeol (Sigma) was used for confirming the presence of this bioactive triterpene.

HPTLC (High Performance Thin Layer Chromatography)

HPTLC analysis of methanolic extracts of root samples (used for TLC separation) were carried out in HPTLC system (CAMAG, Switzerland), CAMAG twin-trough plate development chamber, using silica gel plates (60F254 Manufacturer E. MERCK KGaA). Sample application was carried out on CAMAG Linomat 5 instrument (CAMAG TLC Scanner 3, CAMAG Reprostar 3 photodocument system and WinCATS Software 4.03). The submitted samples are applied to a Silica gel 60 F254 TLC plate (E. Merck, Germany) (10 cm × 10 cm) using Linomat V sample applicator as 8 mm wide bands along with the standard lupeol (1 mg lupeol dissolved in 1 ml chloroform). It is then developed up to 80 mm in a twin trough glass chamber using the Mobile Phase: 16:4, hexane : ethyl acetate. The plate is then derivatised using anisaldehyde sulphuric acid reagent, heated at 110°C for 10 min. The plate is then scanned densitometrically at 580 nm using TLC Scanner 3 equipped with WinCats software. The derivatised plate is then photo documented using CAMAG reprostar 3.

Statistical analysis

Each *in vitro* culture experiment consisted of ten replicates with one explant per culture vessel and all the experiments were repeated three times. The data were recorded after 4 weeks and statistically analyzed by Analysis of Variance (ANOVA). The means were compared by Duncan's multiple range test ($p < 0.05$) using the computer software SPSS/ PC + version 4.0 (SPSS Inc., Chicago, USA).

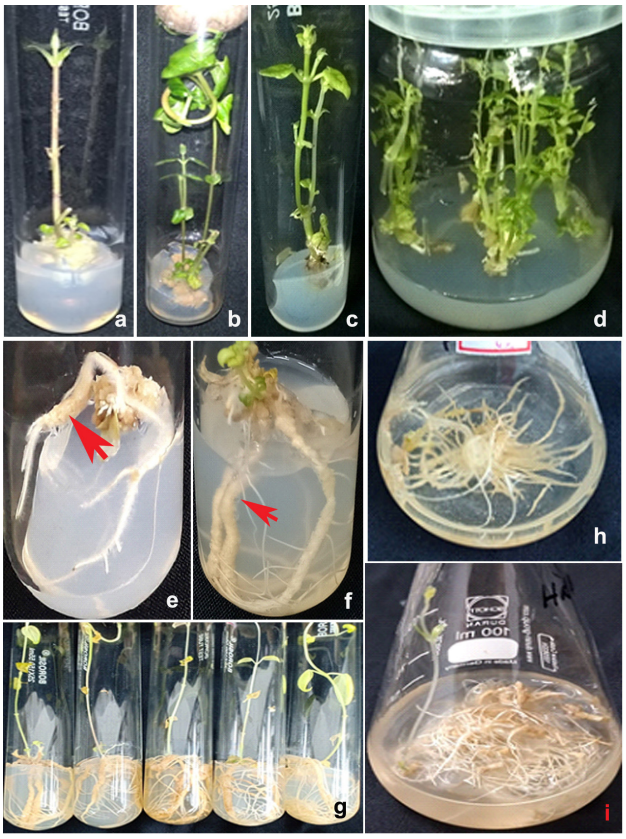

Fig. 1 *In vitro* multiplication and tuberous root induction in *Holostemma annulare*. (a) Shoot initiation in Murashige and Skoog (MS) medium supplemented with 0.25 mg/l 6-benzyl adenine (BA). (b) Shoot multiplication in ½ MS medium supplemented with 0.25 mg/l (BA). (c) Shoot initiation in Schenk and Hildebrandt (SH) medium supplemented with 0.5 mg/l BA. (d) Shoot multiplication in SH medium containing 2.0 mg/l BA in combination with 0.5 mg/l kinetin and 1.0 mg/l indole 3-butyric acid. (e) and (f) Tuberous root induction from leaf and shoots, respectively, in SH medium fortified with 0.5 mg/l α-naphthaleneacetic acid. (g) Tuberous roots after 32 days of culture. (h) Root biomass in root suspension culture. (i) Shoot initiation from the *in vitro* tuberous roots in suspension culture

Results and Discussion

Shoot culture establishment

When the effect of different medium formulations such as MS, ½ MS and SH media were tested for shoot culture initiation in *H. annulare.* The major demerit noticed in MS medium is the occurrence of basal callus formation from below the inoculated explants (Fig. 1a) and its intensity increased as the concentration of cytokinins especially BA increased in the medium. The shoots initiated in ½ MS medium also exhibited basal callusing in lesser intensity and the shoots were pale and much elongated (Fig. 1b). Among the different nutrient formulations, SH medium

Table 1 Effect of plant growth regulators in Schenk and Hildebrandt medium on shoot culture initiation in *Holostemma annulare*

BA	Kinetin	NAA	Number of shoots		Length of shoots (cm)		Number of nodes per shoots	
			Node	Shoot tip	Node	Shoot tip	Node	Shoot tip
0.25	-	-	1.88 ± 0.01^{b}	1.83 ± 0.44^{b}	11.91 ± 0.01^{b}	10.21 ± 0.21^{b}	5.51 ± 0.31^{b}	5.83 ± 0.10^{b}
0.5	-	-	1.95 ± 0.12^{a}	1.92 ± 0.21^{a}	10.82 ± 0.02^{c}	10.01 ± 0.43^{b}	6.31 ± 0.31^{a}	6.16 ± 0.05^{a}
1.0	-	-	1.45 ± 0.37^{d}	1.56 ± 0.42^{e}	7.38 ± 0.01^{f}	8.21 ± 0.31^{d}	5.91 ± 0.43^{b}	5.71 ± 0.03^{b}
2.0	-	-	1.34 ± 0.36^{e}	1.36 ± 0.16^{f}	6.21 ± 0.01^{g}	6.41 ± 0.36^{f}	5.12 ± 0.22^{b}	5.13 ± 0.11^{b}
0.25	0.5	-	1.67 ± 0.43^{c}	1.66 ± 0.21^{d}	10.52 ± 0.02^{c}	9.22 ± 0.41^{c}	3.92 ± 0.32^{e}	2.63 ± 0.22^{e}
0.5	0.5	-	1.24 ± 0.32^{f}	1.77 ± 0.43^{c}	8.23 ± 0.03^{e}	8.03 ± 0.26^{d}	3.22 ± 0.23^{e}	3.34 ± 0.04^{d}
1.0	0.5	-	1.13 ± 0.04^{g}	1.15 ± 0.09^{g}	6.24 ± 0.04^{g}	6.14 ± 0.13^{f}	3.22 ± 0.22^{e}	3.23 ± 0.13^{d}
2.0	0.5	-	1.04 ± 0.08^{h}	1.05 ± 0.05^{h}	4.03 ± 0.02^{h}	3.92 ± 0.02^{g}	2.82 ± 0.41^{f}	2.74 ± 0.33^{e}
0.25	-	0.1	1.97 ± 0.21^{a}	1.95 ± 0.03^{a}	13.72 ± 0.02^{a}	12.83 ± 0.13^{a}	6.52 ± 0.28^{a}	6.43 ± 0.42^{a}
0.5	-	0.5	1.85 ± 0.42^{b}	1.73 ± 0.07^{c}	9.52 ± 0.02^{d}	9.33 ± 0.23^{c}	4.34 ± 0.34^{d}	4.23 ± 0.17^{c}
1.0	-	1.0	1.64 ± 0.11^{c}	1.54 ± 0.20^{e}	8.43 ± 0.03^{e}	8.23 ± 0.25^{d}	4.12 ± 0.13^{d}	4.33 ± 0.21^{c}
2.0	-	1.5	1.22 ± 0.32^{f}	1.34 ± 0.72^{f}	7.93 ± 0.03^{f}	7.53 ± 0.28^{e}	3.22 ± 0.15^{e}	3.32 ± 0.32^{d}

BA - 6-benzyl adenine; NAA - α-naphthaleneacetic acid

Data represents the mean ± SE of ten replicates, repeated thrice. Mean values followed by the same superscript letter do not differ significantly based on the ANOVA and Duncan's multiple range test ($p \leq 0.05$).

responded better. Here, the explants inoculated in SH medium fortified with different concentrations of BA and Kinetin induced bud breaking after one week. SH medium supplemented with cytokinin combinations (0.25 mg^{-1} BA and 0.5 mg/l Kinetin) produced 3.51 ± 0.01 shoots with a mean length of 10.52 ± 0.21 cm having 3.92 ± 0.32 nodes per shoots (Fig. 1c) (Table 1). Healthy plantlets with expanded green leaves and very little basal callusing were noticed in this combination. Leaf abscission was reduced and minimal basal callusing was also observed in low concentrations of these plant hormones. Similarly maximum shoot induction and elongated shoots without basal callusing was noticed from the shoot tip explants of *Woodfordia fruticosa* (Asclepiadaceae) in SH medium augmented with BA and Kinetin (Krishnan and Seeni 1994). The synergic effect of BA in combination with an auxin has been demonstrated very earlier in Asclepidacean medicinal plants like *Hemidesmus indicus* (Sreekumar et al. 2000), *Ceropegia noorjahaniae* (Kedage et al. 2017) and *Gymnema sylvestre* (Veerashree and Kumar 2018). According to Balakrishnan et al. (2018) shoots were initiated from nodal explants in *H. ada-kodien* the when MS basal medium was supplemented with 0.1 mg/lBA and 1.0 mg/l Kinetin. Better shoot initiation and multiplication devoid of basal callusing in SH medium augmented with combination of BA and NAA is the first reported data in *H. annulare*, whereas the previous studies have used MS medium for shoot culture establishment in *Holostemma* spp. and basal callogenesis was evident in these reports which might have hindered the extended shooting response, due to the limited nutrient uptake occurred because of the callus formation from the cut margins of the explants.

Shoot multiplication

The initiated shoots subcultured to SH medium supplemented with 2.0 mg/l BA in combination with 0.5 mg/l Kinetin and 1.0 mg/l IBA evoked maximum 7.16 ± 0.15 shoots with a mean length of 6.53 ± 0.03 cm and 4.53 ± 0.05 nodes per shoot (Fig. 1d) (Table 2). Likewise, Gayathri et al. (2009) reported multiple shoot induction in *Woodfordia fruticosa* using shoot tips in SH medium fortified with 0.5 mg/l BA and 0.5 mg/l NAA. A reduction in the number of shoots and shoot length was observed in increased concentrations of BA, Kinetin and IBA.

In vitro adventitious tuberous root induction

Better root tuber induction devoid of callusing was noticed in SH medium containing 0.5 mg/l NAA (Fig. 1e-g) that produced 6.14 ± 0.32 cream coloured long roots having 4.40 ± 0.10 cm length and 0.62 ± 0.02 cm diameter from *in vitro* shoots (Table 3). Also *in vitro* shoots displayed better root induction than leaf segments (Table 3). With 0.5 mg/l IBA, approximately 5.17 ± 0.41 tuberous roots along with normal roots of 4.2 ± 0.10 cm length were produced. When SH medium supplemented with 0.05 mg/l IBA was tested, root induction was also noticed and the

Table 2 Effect of plant growth regulators in Schenk and Hildebrandt medium on shoot multiplication in *Holostemma annulare*

BA	Kinetin	IBA	NAA	Number of shoots	Length of shoots (cm)	Number of nodes per shoots
1.0	0.5			2.13 ± 0.15^{f}	2.21 ± 0.31^{e}	2.06 ± 0.05^{c}
2.0	0.5	-	-	2.23 ± 0.25^{f}	2.11 ± 0.20^{e}	1.62 ± 0.41^{d}
2.0	1.0	-	-	3.14 ± 0.10^{e}	2.52 ± 0.24^{e}	2.23 ± 0.10^{c}
1.0	-	0.5		4.23 ± 0.20^{d}	3.21 ± 0.01^{d}	2.63 ± 0.25^{c}
2.0	-	0.5	-	4.06 ± 0.20^{d}	2.42 ± 0.02^{e}	3.13 ± 0.11^{b}
2.0	-	1.0	-	3.36 ± 0.35^{e}	2.82 ± 0.02^{e}	3.43 ± 0.20^{b}
1.0	-	-	0.5	4.26 ± 0.20^{d}	1.23 ± 0.03^{f}	2.41 ± 0.36^{c}
2.0	-	-	0.5	3.33 ± 0.28^{e}	1.81 ± 0.10^{f}	2.44 ± 0.05^{c}
2.0	-	-	1.0	3.1 ± 0.100^{e}	2.07 ± 0.06^{e}	2.32 ± 0.10^{c}
1.0	0.5	0.5	-	4.06 ± 0.05^{d}	5.23 ± 0.03^{b}	3.43 ± 0.32^{b}
2.0	0.5	0.5	-	6.13 ± 0.15^{b}	6.33 ± 0.25^{a}	4.46 ± 0.28^{a}
2.0	0.5	1.0	-	7.16 ± 0.15^{a}	6.53 ± 0.03^{a}	4.53 ± 0.05^{a}
1.0	0.5	-	0.5	4.06 ± 0.05^{d}	4.23 ± 0.15^{c}	3.12 ± 0.10^{b}
2.0	0.5	-	1.0	5.16 ± 0.15^{c}	5.33 ± 0.13^{b}	3.16 ± 0.20^{b}
2.0	1.0	-	1.0	3.13 ± 0.12^{e}	4.19 ± 0.06^{c}	2.33 ± 0.30^{c}

BA - 6-benzyl adenine; IBA - indole 3-butyric acid; NAA - α-naphthaleneacetic acid
Data represents the mean ± SE of ten replicates, repeated thrice. Mean values followed by the same superscript letter do not differ significantly based on the ANOVA and Duncan's multiple range test ($p \leq 0.05$).

Table 3 Effect of plant growth regulators Schenk and Hildebrandt medium on tuberous root production in *Holostemma annulare in vitro* shoots

NAA	IBA	IAA	2,4-D	Number of tuberous roots	Length of tuberous roots (cm)	Diameter of tuberous roots	Color of tuberous roots
0.05	-	-	-	2.12 ± 0.12^{e}	2.29 ± 0.18^{c}	0.13 ± 0.03^{f}	Cream
0.1	-	-	-	3.70 ± 0.15^{d}	3.28 ± 0.19^{b}	0.34 ± 0.04^{d}	Cream
0.5	-	-	-	6.14 ± 0.32^{a}	4.40 ± 0.10^{a}	0.62 ± 0.12^{a}	Cream
1.0	-	-	-	4.52 ± 0.24^{c}	3.50 ± 0.34^{b}	0.53 ± 0.13^{b}	Cream
1.5	-	-	-	3.52 ± 0.35^{d}	3.31 ± 0.22^{b}	0.42 ± 0.08^{c}	Cream
2.0	-	-	-	3.23 ± 0.23^{d}	3.00 ± 0.26^{b}	0.22 ± 0.12^{e}	White
-	0.05	-	-	1.21 ± 0.12^{f}	0.66 ± 0.15^{e}	0.55 ± 0.17^{b}	White
-	0.1	-	-	1.03 ± 0.13^{f}	1.57 ± 0.29^{d}	0.15 ± 0.34^{f}	White
-	0.5	-	-	5.17 ± 0.41^{b}	4.20 ± 0.10^{a}	0.54 ± 0.21^{b}	Cream
-	1.0	-	-	4.36 ± 0.43^{c}	3.42 ± 0.43^{b}	0.33 ± 0.13^{d}	Cream
-	1.5	-	-	4.21 ± 0.21^{c}	3.31 ± 0.22^{b}	0.42 ± 0.12^{c}	Cream
-	2.0	-	-	3.33 ± 0.22^{d}	2.83 ± 0.44^{c}	0.33 ± 0.25^{d}	Cream
-	-	0.05	-	1.34 ± 0.33^{f}	1.02 ± 0.32^{d}	0.53 ± 0.21^{b}	White
-	-	0.1	-	1.44 ± 0.22^{f}	1.14 ± 0.11^{d}	0.44 ± 0.11^{c}	White
-	-	0.5	-	1.45 ± 0.41^{f}	1.17 ± 0.21^{d}	0.34 ± 0.13^{d}	White
-	-	1.0	-	1.64 ± 0.32^{f}	1.15 ± 0.30^{d}	0.24 ± 0.22^{e}	White
-	-	1.5	-	1.75 ± 0.43^{f}	1.06 ± 0.32^{d}	0.21 ± 0.41^{e}	White
-	-	-	0.05	1.13 ± 0.32^{f}	0.55 ± 0.43^{e}	0.10 ± 0.30^{f}	White
-	-	-	0.1	1.15 ± 0.14^{f}	0.56 ± 0.24^{e}	0.15 ± 0.05^{f}	White
-	-	-	0.5	1.16 ± 0.32^{f}	0.44 ± 0.33^{e}	0.18 ± 0.21^{f}	White
-	-	-	1.0	1.15 ± 0.17^{f}	0.43 ± 0.36^{e}	0.15 ± 0.11^{f}	White
-	-	-	1.5	1.14 ± 0.42^{f}	0.32 ± 0.21^{e}	0.12 ± 0.01^{f}	White
0.1	0.1	-	-	2.12 ± 0.37^{e}	1.44 ± 0.25^{d}	0.43 ± 0.41^{c}	White
0.5	0.1	-	-	3.36 ± 0.23^{d}	1.90 ± 0.04^{d}	0.32 ± 0.32^{d}	White
1	0.1	-	-	3.75 ± 0.26^{d}	1.80 ± 0.06^{d}	0.41 ± 0.31^{c}	Cream
0.5	0.5	-	-	4.14 ± 0.19^{c}	2.36 ± 0.21^{c}	0.13 ± 0.23^{f}	Cream
0.5	1	-	-	3.46 ± 0.27^{f}	2.21 ± 0.17^{c}	0.10 ± 0.24^{f}	Cream
1	0.5	-	-	4.27 ± 0.43^{c}	2.53 ± 0.15^{c}	0.12 ± 0.32^{f}	Cream
1	1	-	-	3.66 ± 0.39^{d}	1.93 ± 0.13^{d}	0.33 ± 0.21^{d}	Cream
2	0.5	-	-	2.16 ± 0.32^{e}	1.42 ± 0.16^{d}	0.24 ± 0.23^{e}	Cream
2	1.0	-	-	1.44 ± 0.13^{f}	1.36 ± 0.14^{d}	0.42 ± 0.12^{c}	Cream

NAA - α-naphthaleneacetic acid; IBA - indole 3-butyric acid; IAA - indole 3-acetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid
Data represents the mean ± SE of ten replicates, repeated thrice. Mean values followed by the same superscript letter do not differ significantly based on the ANOVA and Duncan's multiple range test ($p \leq 0.05$).

Table 4 Effect of plant growth regulators in Schenk and Hildebrandt medium on tuberous root production in *Holostemma annulare* leaf explants

NAA	IBA	Mean number of tuberous roots	Length of tuberous roots (cm)	Diameter of tubers	Color of tuberous roots
0.05	-	2.04 ± 0.10^{c}	2.26 ± 0.01^{a}	1.24 ± 0.02^{b}	Cream
0.1	-	2.76 ± 0.15^{b}	1.18 ± 0.01^{c}	1.05 ± 0.01^{c}	Cream
0.5	-	3.21 ± 0.26^{a}	2.00 ± 0.10^{b}	1.45 ± 0.03^{a}	Cream
1.0	-	-	-	-	-
2.0	-	-	-	-	-
3.0	-	1.23 ± 0.05^{d}	0.52 ± 0.10^{d}	0.16 ± 0.05^{d}	White
-	0.05	-	-	-	-
-	0.1	-	-	-	-
-	0.5	1.02 ± 0.08^{e}	0.23 ± 0.15^{e}	0.12 ± 0.07^{e}	White

NAA - α-naphthaleneacetic acid; IBA - indole 3-butyric acid
Data represents the mean ± SE of ten replicates, repeated thrice. Mean values followed by the same superscript letter do not differ significantly based on the ANOVA and Duncan's multiple range test ($p \leq 0.05$).

roots were thin, long and white in colour. In this experiment, rooting response was low in SH medium augmented with 0.1 mg/l IBA. Contrastingly, best rooting response was observed in shoot tip explants of *Woodfordia fruticosa* in SH medium fortified with the 0.2 mg/l IBA (Gayathri et al. 2009). Also, Sudha et al. (1998) and Martin (2002) previously reported the success of IBA in promoting efficient root induction using MS medium in *Holostemma annulare* and in *H. adakodien.* Moreover, IBA favored rooting in some other Asclepiadaceae medicinal plants such as *Ceropegia candelabrum* (Beena et al. 2003), *Hemidesmus indicus* (Sreekumar et al. 2000) and *Gymnena sylvestre* (Komalavalli and Rao 2000).

Leaf explants of *H. annulare* when transferred to SH medium supplemented with 0.05 and 0.5 mg/l NAA, it produced 3.20 ± 0.26 tuberous roots with a mean length of 2.00 ± 0.10 cm (Table 4). Here also root tuber formation was very low in IBA compared to NAA supplemented medium while using the leaf segments as explants. Rhizogenesis with callus formation was noticed in SH medium fortified with 3.0 mg/l NAA and the roots were white coloured in appearance.

Adventitious roots induced when subcultured to liquid SH medium containing 0.5 mg/l NAA that induced better formation of *in vitro* roots and kept under constant agitation on a gyratory shaker at 80 rpm have established root suspension culture and enhanced the lateral branching (Fig. 1h). Initiation of new shoots from the *in vitro* roots was also noticed in the root suspension culture system (Fig. 1i) which offers a three in one system for *in vitro* propagation, conservation and sustainable utilization and secondary metabolite production in the targeted species.

Phytochemical analysis of *in vitro* root tubers

In the present study the phytochemical analysis of methanolic extracts of *in vivo* and *in vitro* root tubers of *H. annulare* revealed that the samples possess alkaloids, flavonoids, triterpenoids, tannin, phenol and carbohydrates (Table 5). Supporting this previous reports regarding the phytochemical screening of aerial plant part extracts of *Holostemma* reports that the possesses flavanoids, tannins, saponins, anthocyanins, steroids, alkaloids and phenols (Sunil et al. 2016). Similarly phytochemical screening of the leaf aqueous extract of the plant showed positive test for alkaloids, tannins, phlobatannins, flavonoids, terpenoids, glycosides and phenol. Also, Irimpan et al. (2011) demonstrated that the preliminary phytochemical screening of stem and leaf samples of the herb detected flavonoids, hydrolysable tannins, phenols, saponins, sterols and terpenoids; while According to Kumar et al. (2014) *Holostemma* leaves exhibited the presence of flavonoids, tannins, saponins, anthocyanin's and phenols in the methanolic extract and hydro-alcoholic extracts. Substantiating these findings, Archana (2017) carried out the preliminary phytochemical screening of leaves and concluded that *H. adakodien* is a promising source of potential antioxidants which can be used for various diseases. All these reports strongly support our present research that the *in vitro* tubers of *H. annulare* can be exploited as a good alternative source for drug preparations and it also warrants an in depth study in this aspect.

Table 5 Phytochemical analysis of methanolic root tuber extracts of *Holostemma annulare*

Phytochemicals	Test	Inference of phytoconstituents	*In vivo* root tubers	*In vitro* root tubers
Alkaloids	Wagner's test	Formation of yellow or brown precipitate	+++	++
	Dragendorff's test	Formation of a reddish-brown precipitate	++	++
Flavonoids	Alkaline test	A yellow color observed at the ammonia layer	+	++
	Shinoda test	A pink, scarlet, crimson red, or occasionally green to blue color appeared after a few minutes	+++	+
Triterpenoids	Salkowski test	A red-brown color formed at the interface	++	+
Tannins	$FeCl_3$ test	Formation of bluish black color	+	+
Phenols	-	Formation of greenish-black color	+	+
Anthraquinones	-	Appearance of red color	-	-
Carbohydrates	Molisch's test	A reddish-violet or purple ring at the junction of the two liquids	++	+
Saponins	Foam test	Stable foam	−	−
Protein	Biuret test	Formation of purple or violet color	+	+
Phytosterol	Liebermann-Burchard test	A brown ring formation at the junction	++	−

('+' for presence, '−' for absence)

TLC

Lupeol is an immense bioactive triterpenoid present in different medicinal plants having anti-inflammatory, anti-microbial, anti-protozoal, anti-tumor, anti-proliferative, anti-invasive, and cholesterol lowering properties. Chromatographic separation of methanolic extracts of *in vitro* and *in vivo* root tuber samples was carried out in the present study in order to detect the presence of the bioactive terpenoid 'lupeol'. The solvent system petroleum ether: methanol (9:1) was ideal for separation of triterpenoids. Intensified spots having R_f value 0.63 corresponding to lupeol were developed when visualised under ultra-violet chamber (365 nm). The separation of lupeol using the same solvent system *viz*. petroleum ether: methanol (9:1) was earlier reported in *Wrightia tinctoria* (Jain and Bari 2010) thereby substantiating the finding presented here. Lupeol was detected in the benzene extract of root samples of *Holostemma* (Manikpuri et al. 2010).

HPTLC

HPTLC is a valuable quality assessment tool for the evaluation of botanical samples efficiently and offers high degree of selectivity, sensitivity and rapidity along with single-step sample preparation. Also it is a reliable method for the quantification of nanograms level of samples. This technique was explored by Sunil et al. (2017) who reported the antioxidant activity of a bioassay-guided fractionation and its active components/compounds in *Holostemma adakodien* where the compounds were isolated by HPTLC and their structures were established by mass spectrometry (MS), Nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FT-IR) spectroscopic analyses. In the present study *in vitro* root tubers induced from SH medium fortified with 0.5 mg^{-1} NAA and *in vivo* root tubers were subjected to High Performance Liquid Chromatography for the estimation of lupeol. HPTLC analysis of methanolic extracts of both samples revealed the presence of lupeol up on derivatization of TLC plate (Fig. 2, 3). The band having R_f value 0.53 corresponds to lupeol as per the HPTLC scan report. The amount of lupeol content in *in vivo* root tuber was 0.30 $\mu g\mu l^{-1}$ where as in *in vitro* root tubers it was 3.33 $\mu g\mu l^{-1}$. Similarly Pathak et al. (2017) reported quantification of lupeol in wild and *in vitro* samples of *Hemidemus indicus* and stated maximum lupeol content (0.187 mgg^{-1}) was estimated in shoots regenerated in BA and Kinetin fortified medium than wild shoots (0.185 mgg^{-1}). Earlier Misra et al. (2005) reported that plant growth regulators influence the synthesis of lupeol in *in vitro* shoots of *Hemidemus indicus*. Also Sakakibara et al. (2006) noticed that medium fortified with cytokinins have high lupeol content due to the fact that they are known to express or up-regulate genes involved in various secondary metabolite biosynthetic pathways by repressing certain micronutrient transporters. According to

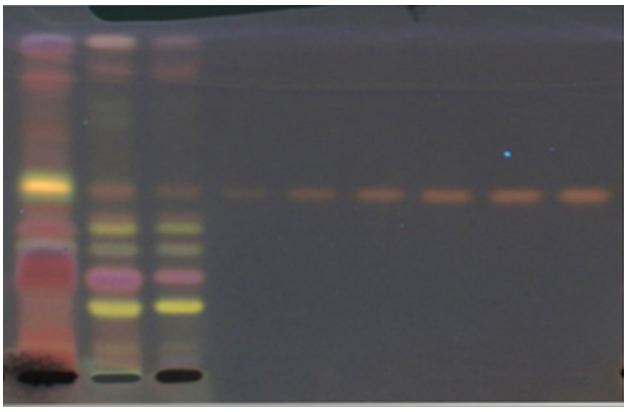

Fig. 2 HPTLC chromatogram of root samples of *Holostemma annulare* (Derivatized plate under UV 366 nm) [Track 1: *In vivo* root sample (Control), Track 2 & 3: *In vitro* root samples, Track 4-9: Different concentrations of the standard, lupeol]

Lee et al. (2011), under *in vitro* conditions, plant growth regulators influence the secondary metabolite production. Hence the high lupeol content recorded with *in vitro* root tubers may be due to the stimulating role of PGRs especially cytokinins on secondary metabolite production. Hence the *in vitro* culture of this *H. annulare* might offer an alternative method for the production of important pharmaceuticals (Lupeol) which would reduce the collection pressure of this vulnerable plant. The presence of lupeol in *in vitro* root tubers of *Holostemma annulare* as depicted here also offers a novel way to exploit the *in vitro* system for the production and scaling up of bioactive secondary metabolites using bioreactors for phyto-pharmaceutical applications.

Conclusion

The present study standardized a novel consistent system of *in vitro* propagation and the induction of *in vitro* root tubers of *H. annulare*. The presence of lupeol in *in vitro* root tubers offers the way to exploit the *in vitro* system for the production and scaling up of bioactive secondary metabolites for phyto-pharma needs. This system can further be extended for the rapid propagation, conservation and sustainable utilization efforts for this genetic stock.

Contribution

PTS and ASH conceptualized the study, SPS executed the research work, PTS and ASH analysed the data and written the manuscript.

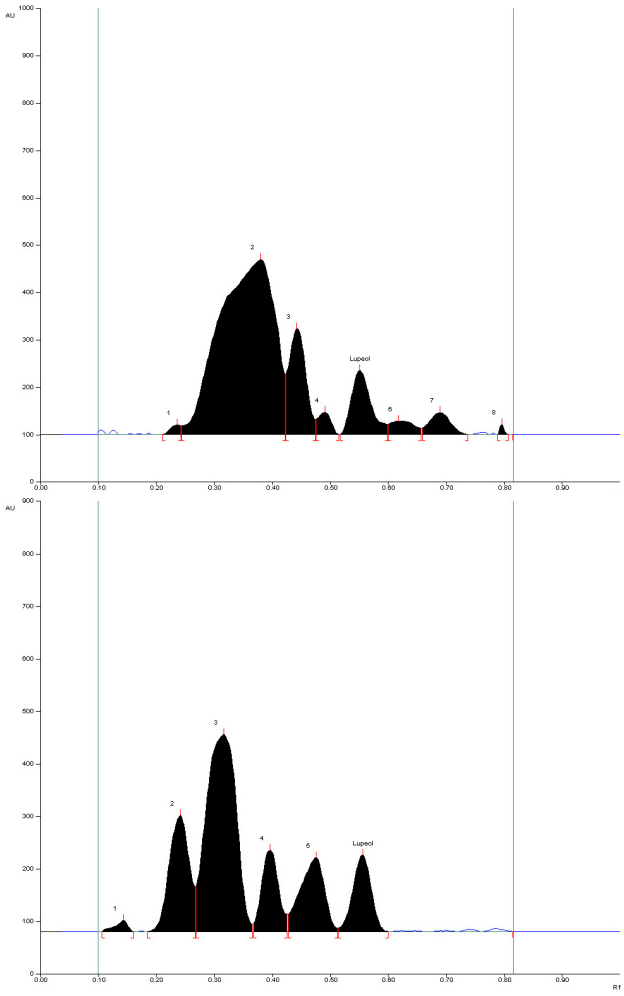

Fig. 3 HPTLC results: Peak display of *in vivo* (top) and *in vitro* (bottom) root samples of *Holostemma annulare*

Conflict of interest

Authors have no conflict of interest

Announcement for Submission

Authors declare that this manuscript has not been submitted fully or partially anywhere.

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