

Enhanced production of lupeol through elicitation in *in vitro* shoot cultures of snake grass (*Clinacanthus nutans*)

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Abstract

Clinacanthus nutans (Acanthaceae), generally known as ‘snake grass’, has diverse uses in customary system of herbal medicine. The species is endowed with various bioactive compounds exhibiting extensive pharmacological properties. The present investigation focused on elicitor-intervened *in vitro* shoot biomass cultivation and scale-up production of the anti-cancerous compound ‘lupeol’, one of the foremost constituents in this species. For the augmented production of lupeol, the shoot cultures were elicited with various concentrations of yeast extract (YE), chitosan and methyl jasmonate (MeJA). Maximum shoot biomass yield and production of lupeol was detected in MS medium supplemented with 1.0 mg l⁻¹ BA and 400 mg l⁻¹ YE. The petroleum ether extracts of selected samples upon TLC analysis proved R_f values corresponding to lupeol. HPTLC analysis revealed that the sample treated with YE displayed relatively higher amount (975.50 ng) of lupeol than the *in vivo* plant (713.69 ng). Hence the *in vitro* shoot culture system with elicitor (YE) treatment propose an appropriate method for the elevated synthesis of lupeol which can be scaled up *via* bio-reactor technology in doing so profiting the pharmaceutical appliances.

Keywords: chitosan; *Clinacanthus nutans*; elicitors; HPTLC; lupeol; methyl jasmonate; yeast extract

Introduction

Clinacanthus nutans (Burm. f.) Lindau belonging to family Acanthaceae commonly known as “Sabah snake grass” is indigenous to the tropical provinces of Southeast Asia, particularly Thailand, southern China and some temperate region (Haida and Hakiman, 2019). The taxa have immense pharmaceutical properties and has been utilized traditionally as anti-venom, anti-inflammatory, analgesic, anti-diabetic, anti-rheumatic, antiviral and antioxidant. Fresh leaves have been suggested as an effective remedy in treating venomous snake and insect bites, burns, allergic reactions and skin rashes. Leaves are chewed for internal consumption and

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crushed leaves are applied on the area of snake bite (Thongharb and Tejasen, 1977; Tejasen and Thongthaap, 1978). The dried leaves are used to treat fever, diarrhoea and dysuria (Sriwanthana *et al.*, 1996; Shim *et al.*, 2013); while the infusion or decoction of dried leaves and stem is recommended for hepatitis infection (Teshima *et al.*, 1998). The leaf extracts also employed for the cure of varicella-zoster virus (VZV) gashes (Tuntiwachwuttikul *et al.*, 2004). Besides, this species has gained considerable attention amongst the Malaysian populace for the treatment cancer (P'ng *et al.*, 2013). In Malaysia, the fresh leaves are boiled in water and consumed as herbal tea; several manufacturers are there selling the herbal tea product of snake grass tea. A greater part of the bioactive compounds reported from *C. nutans* includes triterpenes, C-glycosyl flavones and sulfur containing glucosides. Occurrence of six known C-glycosyl flavones namely, schaftoside, vitexin, isovitexin, orientin, isoorientin and isomollupentin-7-O- β -D-glucoside have also been described in the dried leaves and stem segments of this species (Teshima *et al.*, 1997). Preceding studies disclosed the presence of lupeol and β -sitosterol in the stem (Wanikiat *et al.*, 2008), betulin, lupeol and β -sitosterol in dried up rhizomes (Dampawan *et al.*, 1977).

Apart from few phytochemical studies, the literature related to *in vitro* multiplication of this species is scanty and the plant is often vegetatively propagated by stem cuttings (Chen *et al.*, 2015). However, the demand for quality raw materials to the pharmaceutical industry as well as herbal tea manufacturers is very high and conventional multiplication could not meet the widening gap between demand for quality raw material and supply. Consequently, development of a rapid propagation scheme through biotechnological intervention is an attractive proposition to bridge the demand-supply gap. Furthermore, the popularity of *C. nutans* has encouraged several investigators to execute *in vitro* culture technique, a more rapid propagation means than stem cuttings in order to increase the consistent supply of true to type clones (Chen *et al.*, 2015) whereas merely callus induction from explants was reported in some (Ng, 2013; Gunasekaran, 2014), while Bong *et al.* (2021) developed callus and cell suspension cultures in this species. Also, Haida *et al.* (2020) reported *in vitro* responses of plant growth factors on growth, yield, phenolics content and antioxidant activities of *C. nutans*. Conversely, perusal of literature reveals that there are no attempts carried on high frequency elicitor mediated *in vitro* biomass cultivation for the enhanced production of bioactive compounds followed by bioassay of *in vitro* biomass, which forms the basis of the present research; wherein the established *in vitro* shoot cultures of *C. nutans* were subjected to treatment with different concentrations of elicitors *viz.*, yeast extract, chitosan and methyl jasmonate in order to examine the production of lupeol using chromatographic techniques.

Materials and Methods

Plant material for in vitro studies

Healthy shoots collected from the natural habitat of Thiruvananthapuram district were planted and reared in green house of the Botany Department of University College, Thiruvananthapuram, Kerala, India. The plant species were identified and the voucher specimens were deposited in the herbarium. Explants were taken from the green house grown plants to develop the *in vitro* system in the present study.

In vitro shoot culture establishment

Top cuttings of the shoots with 4-5 nodes collected from mature plant were washed in running water and sliced to appropriate size. Subsequently, the explants were cleansed in 0.5% (v/v) labolene for 10 min and then in running tap water for 30-45 minutes along with 4-5 times rinses in distilled water, before taken to the laminar air flow hood. During surface sterilization inside the airflow chamber, the explants were treated with 10% (v/v) sodium hypochlorite solution for 20 min. Following rinsing 3-4 times in sterile distilled water, the explants were trimmed to appropriate sizes (0.8-1.0 cm) and inoculated vertically into Murashige and Skoog

(MS) medium (Murashige and Skoog, 1962) fortified with 0.5 mg^l⁻¹ BA. The shoots/shoot buds induced from nodal cultures were excised out for fostering auxiliary biomass harvesting in medium complemented with 1.0 mg^l⁻¹ BA. Shoot biomass cultures maintained in MS basal medium were used in elicitation experiments.

Effect of elicitors in shoot multiplication

In vitro shoots were transferred to SM (medium for Shoot Multiplication), i.e., MS medium augmented with 1.0 mg^l⁻¹ of BA (6-benzyl adenine) permuted with different concentrations of elicitors *viz.*, 50, 100, 150 and 200 mg^l⁻¹ chitosan, 100, 200, 300 and 400 mg^l⁻¹ yeast extract (YE) and 50, 100, 150 and 200 µM methyl jasmonate (MeJA) keeping SM as control. All the inoculated culture tubes were incubated in a culture room (26±2 °C) under 12-hour photoperiod at a photon flux intensity of 50-60 µEm⁻²s⁻¹ PFD provided by cool, white, fluorescent tubes (Philips, India) under 50-60% RH. The cultures were set aside for a period of 6 weeks for supplementary chromatographic analysis.

Experimental design, data collection and statistical analysis

The whole experiment was performed in randomized block design. Each treatment consisted of ten replicates and was repeated thrice. The cultures were consistently examined and the remarks on number of shoots, shoot length (cm) and number of nodes per shoot were recorded after 4 weeks. Results were presented as mean value ± standard error. Data were subjected to ANOVA and the means were compared by Duncan's multiple range test ($p \leq 0.05$) using the computer software SPSS (PC + version 10; SPSS Inc., Chicago, USA 1999).

Chemical analysis for the elucidation of lupeol

Extract preparation and Thin Layer Chromatography (TLC)

Four samples (control and treated samples) i.e., *in vivo* plant (absolute control), *in vitro* plant (control), *in vitro* plant treated with yeast extract and chitosan were dried up and powdered using mortar and pestle and afterward extracted with petroleum ether. These extracts were used for loading on TLC plates. Authentic standard of lupeol purchased from Sigma Aldrich was used for confirmation. The solvent system used was petroleum ether: methanol (9:1) and the spots developed were visualized under ultra-violet chamber (Kemi) (365 nm).

HPTLC (High Performance Thin Layer Chromatography)

HPTLC method was used for the quantification of lupeol in dried powdered samples of *C. nutans* plantlets (both *in vivo* and *in vitro*), that was used for TLC. The samples were analyzed using HPTLC system where they were spotted in the form of bands of width 7 mm and 10 mm apart with a Camag microlitre syringe on precoated silica gel aluminium Plate 60F254 having 10 cm × 10 cm dimensions (E. Merck, Darmstadt, Germany) using a Camag Linomat IV sample applicator (Muttentz, Switzerland). The mobile phase consisted of toluene, ethyl acetate, glacial acetic acid in the volume ratio of (6:1.5:0.1). Linear ascending development was carried out in 10 × 10 cm twin trough glass chamber (Camag, Muttentz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 80 mm. Subsequent to the development; applied with methanolic sulphuric acid and plate was dried at 110 °C for 5 minutes. Densitometric scanning was performed on Camag TLC scanner III in the reflectance absorbance mode at 580 nm and was operated by CATS software (Camag). The amount of lupeol present in the sample was determined in nano gram units as per the system generated values using the standard curve.

Results and Discussion

The axenic shoots of *C. nutans* derived from established *in vitro* shoot cultures while treated with different elicitors exhibited varied responses with regard to the concentrations and type of elicitors being tested.

Effect of elicitors in shoot multiplication

An elicitor can stimulate any kind of plant defense, upholding secondary metabolism to protect the cell and the whole plant and once it is introduced in minute concentrations to a living cell system, it initiates or boost up the biosynthesis of specific compounds. Therefore, elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amount of elicitors (Radman *et al.*, 2003). Growth, regeneration and moreover the production of bioactive molecules of *in vitro* cultured plants can be enhanced by elicitation. Biotic and abiotic elicitors equally stimulate product accretion in whole plants, plant organs and in plant cell cultures as a consequence of their defensive, protective or offensive reactions. The quantity of these substances required for elevated product accumulation varies with species and genotype as well. The addition of elicitors for the enhanced production of secondary metabolites is well documented in many medicinal plants (Namdeo, 2007; Naik and Al-Khayri, 2016; Shakya, 2017). Protocol optimization by way of accomplishment of elicitors for the bioproduction of secondary metabolites is highly promising in medicinal herbs for their sustainable utilization. However, no such attempts have been made yet in *C. nutans* concerning this aspect and in this study the effect of three elicitors *viz.*, chitosan, YE and methyl jasmonate were examined to check their role in the synthesis of bioactive metabolites.

Effect of Chitosan on shoot morphogenesis

The explant exhibited varied response with regard to the treatment with different concentrations of chitosan. The *in vitro* shoot explants displayed a linear decrease in the mean number of shoots and mean number of axillary shoots with the increase in concentration of chitosan (Figure 1a). Maximum 4.4 ± 0.16 shoots only were produced per explant in MS medium supplemented with 1.0 mg l^{-1} BA + 50 mg l^{-1} chitosan within 6 weeks against the control, which evoked the production of 5-6 shoots. This was analogous to the findings in *Lilium longiflorum*, wherein the number of shoots decreased with the increase of chitosan concentration and the combinations of chitosan and BA were less effective in promoting shoot multiplication when compared to chitosan or BA alone (Kanchanapoom, 2012). A mean number of 3.3 ± 0.79 axillary shoots obtained at 50 mg l^{-1} concentration of chitosan was dropped down to 1.3 ± 0.15 when the concentration was increased to 200 mg l^{-1} (Table 1).

Table 1. Effect of chitosan on shoot multiplication of *C. nutans*

Concentration of chitosan (mg l^{-1})	Number of shoots	Shoot length (cm)	Number of axillary shoots	Number of nodes per shoot	Remarks
0	5.8 ± 0.20^a	3.31 ± 0.31^c	3.3 ± 0.79^a	5.8 ± 0.29	Aerial roots present
50	4.4 ± 0.16^b	3.26 ± 0.03^c	$3.2 \pm 0.25^{a\#}$	2.3 ± 0.15^a	Presence of basal roots
100	3.3 ± 0.26^c	3.72 ± 0.04^c	2.2 ± 0.25^b	2.4 ± 0.16^a	Basal roots absent; only aerial roots
150	2.5 ± 0.22^d	4.54 ± 0.04^b	1.7 ± 0.15^c	2.5 ± 0.17^a	Basal roots absent; only aerial roots
200	2.2 ± 0.20^d	5.26 ± 0.04^a	1.3 ± 0.15^c	2.5 ± 0.17^a	Basal roots (1-2) present. Aerial roots around the basal region

Values represents mean \pm SE of 10 replicates repeated thrice. Mean values followed by the same letter in a column do not differ significantly based on ANOVA and Duncan's multiple range test at $p \leq 0.05$.

The length of regenerated shoots increased as the chitosan milieu increased (Figure 1a); the mean shoot length of 5.26 ± 0.04 cm was recorded at 200 mg l^{-1} chitosan, while it was 3.26 ± 0.03 cm at 50 mg l^{-1} concentration. Maximum number of 2.5 ± 0.16 nodes per shoots was observed at 200 mg l^{-1} chitosan. Thus, higher concentrations of chitosan in the growth medium supported shoot growth in *C. nutans* as a consequence of which the shoots remained elongated with proliferation in the mean number of nodes per shoot. In strawberry also, the highest number of shoots, diameter of shoots, number of leaves and dry weight of plant mass were obtained in 1.0 or 2.0 mg l^{-1} BA without chitosan; but the highest length of shoot was observed in combination of 1.0 or 2.0 mg l^{-1} BA along with 30 mg l^{-1} chitosan (Jalili Marandi *et al.*, 2011). Basal rooting (4-5 cm length) was noticed in 50 mg l^{-1} chitosan (Table 1) and these roots were having root hairs with powdery appearance. In 100 and 150 mg l^{-1} chitosan, the shoots were devoid of basal roots and they exhibited merely aerial lateral roots. However, in 200 mg l^{-1} chitosan, basal roots (1-2) were present and the aerial lateral roots were seen around the sub-aerial parts (Figure 1a).



Figure 1. Morphogenic responses of the *in vitro* shoots of *C. nutans* in different concentrations of elicitors. (a) chitosan, (b) yeast extract

Effect of yeast extract (YE) on shoot morphogenesis

Yeast extract, a source of amino acids and vitamins in particular inositol and thiamine is used as an additive in plant tissue culture media (George, 2007). In *C. nutans*, the treatment with YE exhibited good range of influence on the explants than control. The *in vitro* shoot explants differed in the mean number of shoots in different concentrations of YE (100, 200, 300, 400 mg l⁻¹) (Figure 1b). Maximum 8.5±0.22 shoots were produced in MS medium incremented with 1.0 mg l⁻¹ BA (SM) + 400 mg l⁻¹ YE which was 7.0±0.21 at 100 mg l⁻¹ YE (Table 2). Though statistically insignificant, the mean number of shoots obtained with 300 mg l⁻¹ YE was found to be 7.2±0.20, while at 200 mg l⁻¹ YE 7.7±0.15 shoots were proliferated. The mean shoot length enhanced linearly with increase in YE concentrations (Figure 1b). Maximum 6.38±0.03 cm long shoots were obtained at 400 mg l⁻¹ whilst the shoot length was 5.54±0.03 cm at 100 mg l⁻¹ YE (Table 2). The mean number of nodes per shoot remained constant at 100 and 200 mg l⁻¹, i.e., 2.7±0.15. Axillary shoots were produced only at 200 and 300 mg l⁻¹ YE and it was missing in 100 and 400 mg l⁻¹ concentrations. Presence of basal roots was invariably observed in all the concentrations of YE tested in the present study. Among the four concentrations of YE examined, 400 mg l⁻¹ was exceedingly favourable which increased the shoot proliferation, shoot length and number of nodes per shoot in *C. nutans*. In agreement with this, the promotive effect of YE on shoot multiplication was reported earlier in *Lavandula latifolia* (Gras and Calvo, 1996), *Eryngium foetidum*, *Stevia rebaudiana*, etc. (Sridhar and Aswath, 2014).

Effect of methyl jasmonate (MeJA) on shoot morphogenesis

The treatment with MeJA at different concentrations (50, 100, 150 and 200 µM) induced strong stress on the explants so that all of them turned glassy and died within 2-3 days after inoculation. The explants exhibited abscission and become pale coloured (data not shown). The reason for this may be the lethal effect occurred due to the high concentration of MeJA selected for the experimentation. So other minute concentrations need to be experimented to check the efficiency of the same.

Table 2. Effect of yeast extract (YE) on shoot multiplication of *C. nutans*

Concentration of YE (mg l ⁻¹)	Number of shoots	Shoot length (cm)	Axillary shoots	Number of nodes per shoot	Basal roots
0	5.8±0.20 ^c	3.31±0.31 ^c	Present (3.33±0.79)	5.8±0.29	Aerial roots present as usual
100	7.0±0.21 ^b	5.54±0.03 ^b	Nil	2.7±0.15 ^a	1-2 basal roots of 4-5 cm length
200	7.2±0.15 ^b	6.07±0.02 ^a	Present (1.33±0.79)	2.7±0.15 ^a	1-2 slender basal root of 1 cm length
300	7.7±0.20 ^b	6.19±0.03 ^a	Present (2.00±1.00)	2.8±0.13 ^a	1-2 very slender basal roots of 5.6 cm length
400	8.5±0.22 ^a	6.38±0.03 ^a	Nil	2.9±0.10 ^a	3 long basal roots with root hairs, 8.5 cm length

Values represents mean±SE of 10 replicates repeated thrice. Mean values followed by the same letter in a column do not differ significantly based on ANOVA and Duncan's multiple range test at p≤ 0.05.

Comparative analysis of elicitor-mediated shoot multiplication in C. nutans

Among the three types of elicitors analyzed, the explants responded to only two of them i.e., YE and chitosan. The values obtained for different parameters at various chitosan concentrations as well as YE showed significant differences from that of the control. The mean number of shoots rose with increase in concentration of YE, but decreased upon chitosan treatments (Figures 2a and 2b). The mean shoot length increased

concurrently as the amount of chitosan and YE increased. The results of this study demonstrated that YE is complementing the vigorous growth of *C. nutans* compared to chitosan.

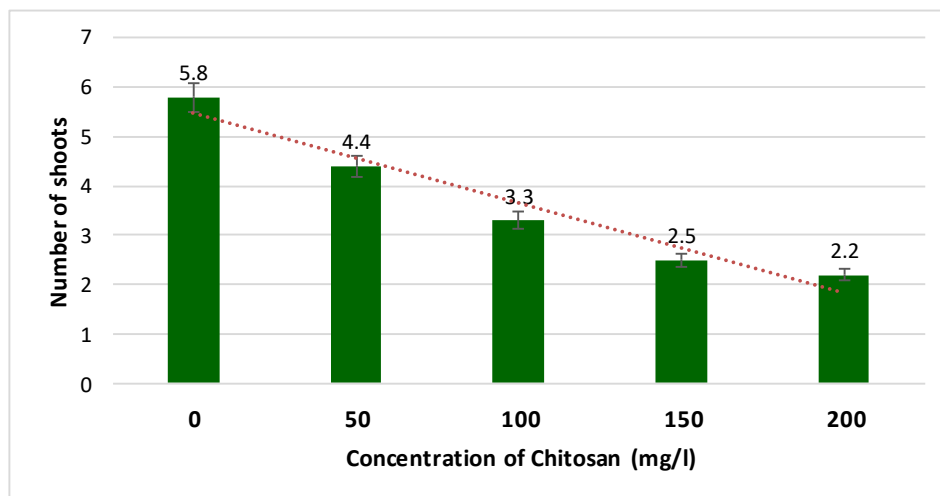


Figure 2a. Shoot multiplication in *in vitro* shoots of *C. nutans* in different concentrations of chitosan

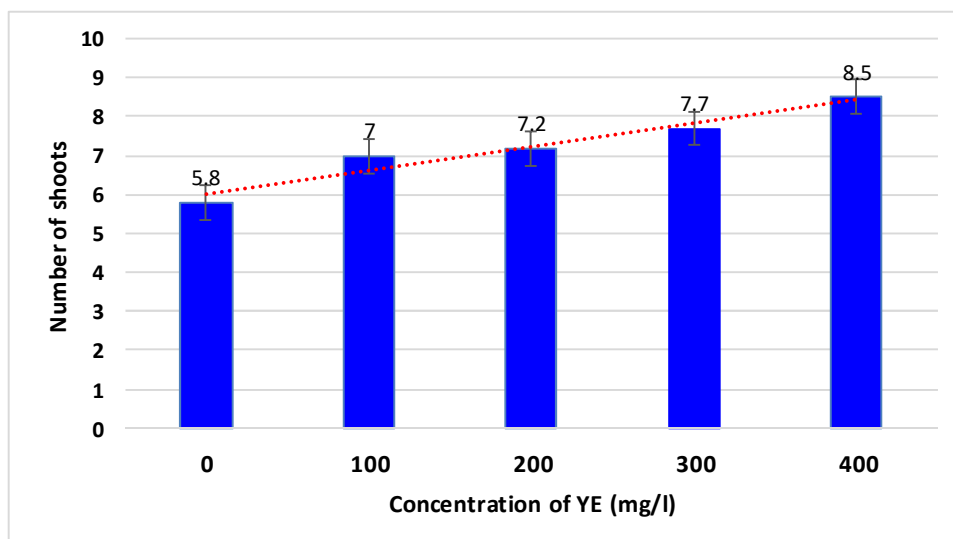


Figure 2b. Shoot multiplication in *in vitro* shoots of *C. nutans* in different concentrations of yeast extract

Chemical analysis for qualitative and quantitative estimate of lupeol

Thin layer chromatography

Thin layer chromatographic separation of samples extracted in petroleum ether indicated intensified bands in all the samples (Figure 3) and the R_f values of the chemical constituent corresponded to lupeol was determined in them (Table 3). This is the first and foremost report regarding the presence of lupeol in the *in vitro* shoot cultures of *C. nutans*. The partitioning 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 our study.

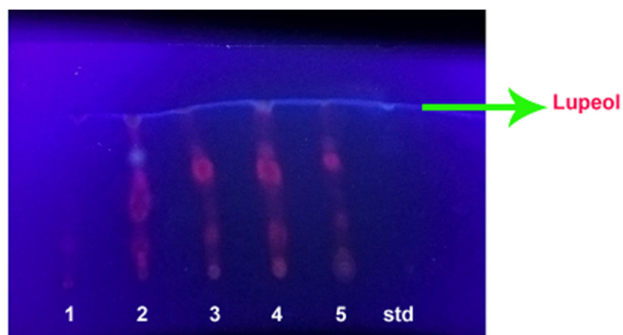


Figure 3. TLC chromatogram of selected samples of *C. nutans*. Track 1&2: *In vivo* plant (absolute control), Track 3: *In vitro* plant (control), Track 4: *In vitro* YE-treated plant, Track 5: *In vitro* chitosan-treated plant, Track 6: Standard (Lupeol)

Table 3. R_f values of the bands corresponding to lupeol in selected samples of *C. nutans* in TLC

Sl. No.	Samples	R _f Values
1	<i>In vivo</i> plant (absolute control)	0.60
2	<i>In vitro</i> (control) plant	0.60
3	<i>In vitro</i> yeast extract-treated plant	0.61
4	<i>In vitro</i> chitosan-treated plant	0.62
5	Standard (Lupeol)	0.62

High performance thin layer chromatography (HPTLC)

HPTLC, a sophisticated and automated separation technique of TLC is a valuable quality measurement tool for the assessment of botanical materials proficiently, thereby offering high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. It is a consistent method for the quantification of nanogram level of sample components. In the present study, HPTLC chromatogram of samples in petroleum ether extract revealed the presence of lupeol (Figures 4a-d) and the band having R_f value 0.62 corresponds to lupeol as per the HPTLC scan report.

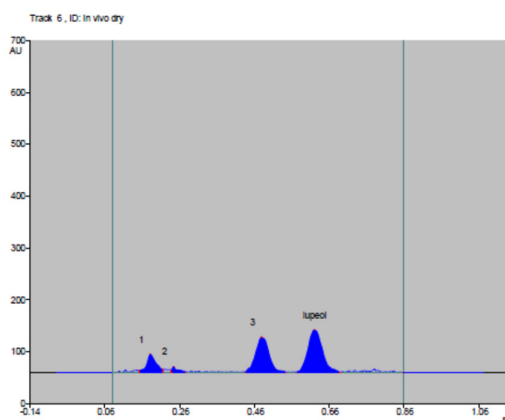


Figure 4a. HPTLC chromatogram of *in vivo* plant (absolute control)

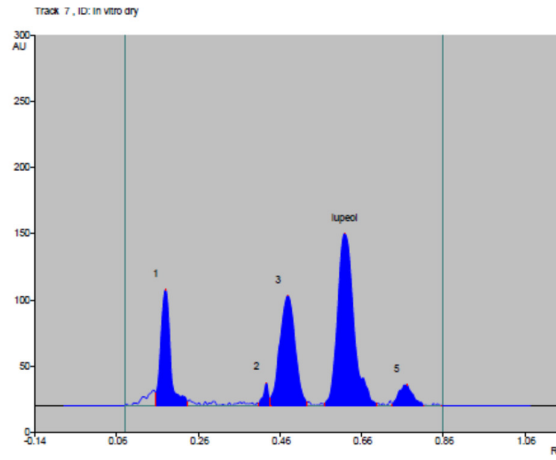


Figure 4b. HPTLC chromatogram of *in vitro* plant (control)

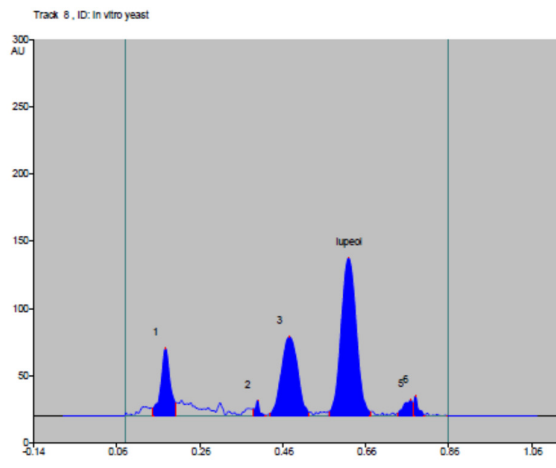


Figure 4c. HPTLC chromatogram of *in vitro* yeast extract-treated plant

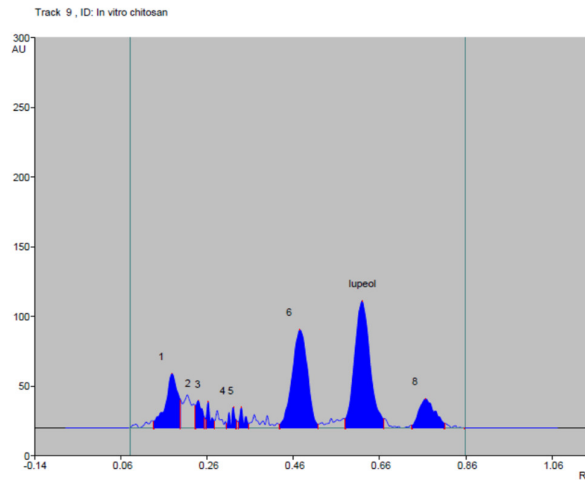


Figure 4d. HPTLC chromatogram of *in vitro* chitosan-treated plant

Quantification of lupeol

The amount of lupeol in the selected samples was determined as per the standard procedures (Figure 5). In the *in vivo* plants of *C. nutans* (absolute control), lupeol was detected with an R_f value of 0.62. It also showed other three unknown compounds with R_f values of 0.18, 0.24 and 0.48 and the amount of lupeol found in this sample was 713.69 ng (Figure 5). While, in *in vitro* plants (control, without elicitation) lupeol was detected at R_f value 0.62. This sample showed five compounds including lupeol and the other four compounds seen were having R_f values 0.18, 0.43, 0.48 and 0.77; the amount of lupeol in the same was quantified as 1067.0 ng. In elicited samples, *in vitro* shoots of *C. nutans* treated with YE also showed lupeol at R_f value 0.62; with 975.50 ng lupeol in it. In addition to this, five other unknown compounds were detected at R_f values at 0.18, 0.40, 0.48, 0.77 and 0.78. Lupeol in *in vitro* shoots of *C. nutans* treated with chitosan was 761.26 ng (Figure 5) which was also at an R_f value of 0.62. In addition, there were seven compounds with R_f values 0.18, 0.24, 0.26, 0.32, 0.34, 0.47 and 0.77.

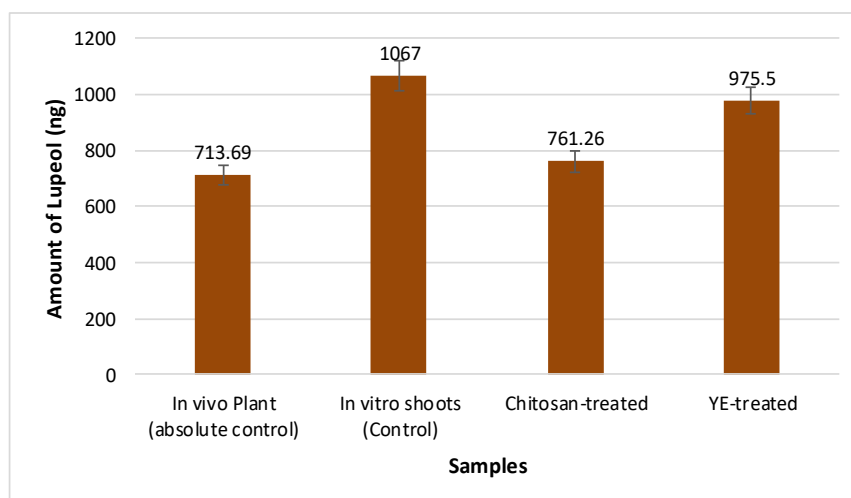


Figure 5. Quantification of lupeol in the selected samples of *C. nutans*

All the samples analysed here contained lupeol and showed characteristic R_f value of 0.62. Most of the unknown compounds present in different samples showed same R_f values such as 0.18, 0.24, 0.47, 0.48 and 0.77. Among the treated samples, elicitation with YE resulted in comparatively higher amount (975.50 ng) of lupeol. However, the values corresponding to lupeol content was moderately higher in the control (1067 ng) than YE elicitation (975.50 ng). This may be because in the control i.e., the shoot cultures raised in BA-supplemented medium, where the plant growth regulator, cytokinin (BA) itself may be acting as the elicitor thereby eliciting the production of lupeol. Similarly, in *Hypericum perforatum*, modified MS medium, with 50% reduction in ammonium nitrate and potassium nitrate with supplements of BA (0.44 μ M) and indole 3-butyric acid (0.049 μ M), have resulted in the increased production of hypericins (Liu *et al.*, 2007). In the present study, the *in vitro* shoots (both control and treated samples) exhibited higher production of lupeol than the absolute control i.e., the *in vivo* plant (713.69 ng). On the other hand, YE-treated plants exhibited higher shoot multiplication rate than control. Hence the *in vitro* shoot culture system with elicitor treatment (especially YE treatment) offers an enhanced shoot biomass production as well as elevated synthesis of lupeol, compared to the *in vivo* plants (absolute control).

Conclusions

Shoot culture system established in MS medium augmented with 1.0 mg l⁻¹ BA was found to be essential for *in vitro* shoot biomass cultivation of *C. nutans*. The present investigation has also demonstrated elicitation (especially YE treatment) in *in vitro* system as an efficient method to tackle the obstacle of low lupeol content in *in vivo* plant (absolute control; 713.69 ng) and it offers the enhanced shoot biomass production as well as elevated synthesis of lupeol in culture condition. Maximum elicitation was recorded in YE with a 1.4-fold increase in lupeol and 1.5 fold and biomass production respectively. The present investigation demonstrated the feasibility of using elicitation as a method for enhancing the accumulation of lupeol and shoot biomass in *C. nutans*. The study also suggests further experiments for optimization of culture parameters in suitable bioreactor system for large scale shoot biomass production followed by elucidation of compound (lupeol) to cater the demands in phyto-pharma and herbal tea industries.

Authors' Contributions

TSP has designed the experiments, SLC and VTAV conducted it and wrote the manuscript, ASH helped in the analysis of the results and checking the draft and PPP provided overall supervision of the work. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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