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ORIGINAL ARTICLE

Effect of Salinity Stress on Growth, Water Content, and Guggulsterone Production in Callus Cultures of *Commiphora wightii* (Arnott.) Bhandari

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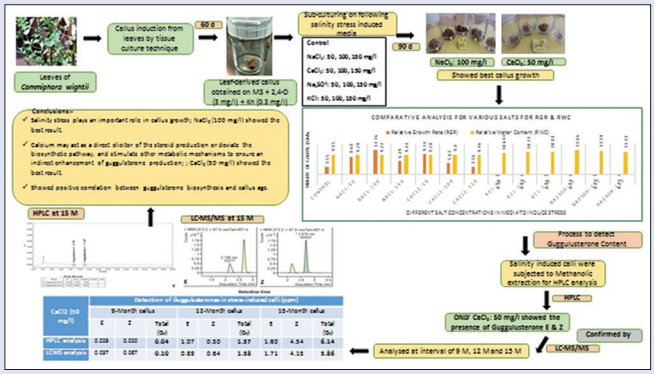
ABSTRACT

Objectives: A traditional herb *Commiphora wightii* (Arnott.) is well known for the management of different ailments, including hypolipidemic and hypocholesterolemic disorders. Overexploitation has resulted in enlisting it under endangered species list; thus, *in vitro* studies are desirable to circumvent further depletion of the wild population. The objective of our study is to analyze the effect of salinity stress on growth and guggulsterone content in callus cultures. **Materials and Methods:** The establishment of leaf-derived calli of *C. wightii* and the effect of salinity stress such as calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride, and ammonium sulfate at different concentrations and time periods on callus growth and steroid biosynthesis were studied. High-performance liquid chromatography and liquid chromatography-mass spectrometry/mass spectrometry-based spectrometric systems were carried to identify the guggulsterones. **Results:** The 50 mg/L $CaCl_2$-induced callus was found to have the highest content of guggulsterones after 15 months of incubation as compared to 9 and 12 months and indicated a positive correlation between callus age and guggulsterone biosynthesis. **Conclusion:** Salinity stress played an important role in callus growth of *C. wightii*, while calcium-induced calli showed *in vitro* synthesis of guggulsterones, and this can be further explored for the synthesis of guggulsterones *in vitro* on large commercial scale using bioreactor, and thus helps to conserve the endangered species in its natural habitat.

Key words: Callus proliferation, *Commiphora wightii*, salinity stress, salt tolerance, tissue culture

SUMMARY

- The leaf-derived, 50 mg/L calcium chloride ($CaCl_2$)-induced callus of *Commiphora wightii* was found to have highest content of guggulsterone steroid in 15 months as compared to 12 and 9, indicating a strong correlation between steroid content and callus age. Further, 100 mg/L sodium chloride and 50 mg/L $CaCl_2$-induced calli were found to have maximum relative growth rate and relative water content response. This study result suggests that salinity stress played an important role in callus growth while calcium may act as a direct elicitor of the steroid production.



Abbreviations used: ANOVA: Analysis of Variance, DW: Dry Weight, FW: Fresh Weight, FWi: Initial Weight, FWf: Final Weigh, HPLC: High Performance Liquid Chromatography, Kn: Kinetin, LC-MS: Liquid Chromatography and Mass Spectrometry, MS: Murashinge and Skoog, RGR: Relative Growth Rate, RWC: Relative Water Content, CaCl2: Calcium Chloride, HCl: Hydrochloric acid, NaCl: Sodium Chloride, KCl: Potassium Chloride, Na2SO4: Ammonium Sulphate, NaOH: Sodium Hydroxide, 2,4-D: 2,4-Dichlorophenoxyacetic acid

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INTRODUCTION

Commiphora wightii, commonly called Guggulu, belongs to the *Burseraceae* family. It is an endangered, slow-growing halophytic dwarf tree with immense therapeutic properties, found in the arid and rocky tracts of Rajasthan and Gujarat (India). *C. wightii* is a commercially valuable plant primarily because of its tree exudates which comprise true gums, resins, gum resins, oleo-resins, and mucilage. The bioactivities of guggulsterones extracted exudates from Guggulu such as lipid-lowering,[1] antimicrobial,[2,3] anti-inflammatory,[4] hypolipidemic, and hypocholesterolemic activities[5-10] are reported which are possibly used in the treatment of a varied range of ailments. Further, Guggulu is reported as a reliever from epilepsy, ulcer, obesity, and rheumatoid arthritis.[11-14] Slow growth, poor seed set, faulty gum tapping method, indiscriminate wild collection, and inadequate replenishment strategies

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have led to overexploitation and have resulted it enlisted in the critically endangered status.[15] Therefore, an alternate *in vitro* guggulsterone biosynthesis protocol and conservation strategy is important for the sustainable utilization of Guggulu. Due to the significance of guggulsterones from *C. wightii*, several *in vitro* studies have been reported using morphactin,[16] modified media,[17,18] growth retardants,[19] plant gums,[20] and calcium deprivation.[21] Scientists and biotechnologists considered plant cell, tissue, and organ cultures as an alternate to produce secondary metabolites[22] from callus/cell suspension cultures as the chemical synthesis of secondary metabolites was achieved through field cultivation in plants where natural growth was difficult.[23]

Several callus culture studies have reported the production of steroids such as saponins,[24-28] saikosaponins,[29] diosgenin,[30-33] cardenolides,[34-40] phytosterols,[41-44] sterols and phenolic compounds,[45] diosgenin and 20E, 20E-3-acetate,[46-50] polypodine B,[51,52] paclitaxel,[53,54] taxoids,[55] ecdysterone,[56] and withanoids.[57,58]

The plants remodel their plasticity to abiotic and biotic stresses[59] and enable accumulation of secondary metabolites as a defense response and activated by elicitors.[60,61] Elicitation is a strategy widely used in *in vitro* cultures to enhance the production of secondary metabolites by abiotic elicitors such as physical agents (i.e., osmotic pressure, heat, cold, and ultraviolet light) and chemical agents (i.e., heavy metals, ethylene, fungicides, salts, and antibiotics). Elicitation also helps control gene expression in response to the stimulus,[62] induce enzyme synthesis, and thus stimulate the synthesis of various secondary metabolites, such as steroids, phenylpropanoid, polypeptides, and flavonoids.[63] Under stress, two mechanisms are activated, i.e., enzymatic and non-enzymatic.[64] The former mechanism is governed by the enzymes, superoxide dismutase, catalase (CAT), and peroxidase, while the latter is formed by antioxidant molecules within the cell[65] such as phenolic compounds, flavonoids, steroids, and various secondary metabolites.[66] In plants, salinity stress may create both ionic and osmotic stress and result in an increase or decrease of particular secondary metabolites.

The establishment of an *in vitro* callus induction and guggulsterone biosynthesis technique for *C. wightii* will highly facilitate the production of the steroid to various industries and accolade conservation strategies. Since *C. wightii* is listed under rare and endangered category, the current natural population will be insufficient to achieve the raw material demand. Hence, the establishment of a reliable *in vitro* synthesis of guggulsterone is vital to acquire the steroid demand as raw materials for product development. The effect of plant growth regulators on callus growth was previously studied;[66] however, the effect of salinity stress on callus growth in *C. wightii* has not yet been researched. Therefore, this investigation was aimed to study the influence of salinity stress on callus growth and to establish an efficient process of *in vitro* guggulsterone biosynthesis. This study will help as a mode of expediting the guggulsterone production on a bioreactor-based large scale[67] and help to conserve the germplasm of this endangered species in its natural habitat.

MATERIALS AND METHODS

Plant material and surface sterilization

The young leaves of *C. wightii* were collected from the field. Leaves were kept under running water for 15 min to eliminate the foreign particles/microbes. The leaves were then surface sterilized by first dipping in a soap solution (10 mL Labolene® + 5 ml cetrimide [Savlon] + few drops polyethylene glycol sorbitan monolaurate [Tween-20]) for 15 min, after which the leaves were disinfected by soaking in 1.5% (w/v) methyl N-(1H-benzimidazol-2-yl) carbamate (Bavistin) for 20 min. The leaves were rinsed thoroughly with sterile distilled water at least 2–3 times to remove all traces of Bavistin. Finally, the leaves were surface sterilized by 0.15% (w/v) mercuric chloride for 8 min and washed three times with sterile water.

Media and culture condition

The leave explants were cultured in Murashige and Skoog,[68] MS basal medium containing 30 g/L sucrose and 100 mg/L myo-inositol supplemented with plant growth hormones 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.3 mg/L kinetin (Kn). The pH of the medium was adjusted to 5.8–5.9 using 1 N NaOH or HCl before adding 3 g/L Clarigel® (HiMedia, India). Semi-solid MS media prepared were poured in culture bottles, secured with polypropylene caps, and autoclaved at 121°C for 20 min at 15 psi.

Establishment of callus cultures

The surface sterilized leaves were excised (measuring 1 cm × 1 cm) aseptically with a sterile sharp scalpel blade to reduce injury and place onto sterilized media. The cultures were maintained under 16 h/8 h (light/dark) photoperiod at 23°C ± 2°C with a light intensity of 30 µmol/m/s using cool white fluorescent tubes; and the callus was regularly sub-cultured at 21-day intervals by transferring 0.5 g callus to a fresh medium and was maintained for 1 year. The callus cultures were maintained for 1 year 4 months on the mentioned media; and their growth and steroid formation were measured after an interval of every 3 months. The minimum amount of callus required for analysis was obtained at 9 months, followed by 12 and 15 months.

Elicitation experiment of guggulsterones with salt stress

The culture bottles containing 25 mL of standardized MS medium, added with three iso-osmotic concentrations (50, 100, and 150 mg/L) of sodium chloride (NaCl), calcium chloride ($CaCl_2$), potassium chloride (KCl), and ammonium sulfate (Na_2SO_4), were inoculated with 0.5 g callus, which were fragmented as described in the previous section. Samples were taken at an interval of 9, 12, and 15 months, and the callus growth, water content, and guggulsterone content were monitored. The experiment was carried out in quintuplicate.

Harvesting for measuring responses

For culture growth and water content, the calluses were harvested and weighted on the 90th day of inoculation in all experiments, and the growth and water content were measured. Relative growth rate (RGR) was calculated using the formula (FWf – FWi)/FWi, where FWf is the final weight and FWi is the initial weight (here 500 mg) of the calli,[69,70] while relative water content (RWC) was determined using the formula = (FW – DW)/DW, where FW and DW represent fresh weight and dry weight (DW), respectively.[70] DW was determined by drying the callus overnight at 60°C in an oven. For guggulsterone content, the calluses were harvested at intervals of 9, 12, 15 months from the day of inoculation from NaCl- and $CaCl_2$-induced media (as KCl- and Na_2SO_4-induced media failed to proliferate), and the guggulsterone content was measured.

Sample preparation

The extraction of guggulsterone from *C. wightii in vitro* callus was carried out following the methodology of Mathur *et al.*, 2007.[17] The 0.2 g of dried callus was finely crushed using pestle mortar and extracted overnight with 25 ml methanol. The methanol layer was evaporated, and the residue was extracted thrice with 2 ml of ethyl acetate. Finally, the ethyl acetate was evaporated to dryness in the sample concentrator. The crude extracts were stored at 4°C until analysis. For high-performance liquid chromatography (HPLC) and liquid chromatography

mass spectrometry (LCMS), the callus extract was dissolved in methanol (HPLC-grade) to yield a final concentration of 200 mg dried tissue per 25 ml. Samples were filtered through syringe filters (0.45 µm, 4 mm nylon filter, Axiva Sichem Pvt. Ltd., India) to remove suspended particles and transferred to autosampler vials and used for HPLC and LCMS analysis.

High-performance liquid chromatography analysis

The extracted callus was analyzed with the Waters HPLC system equipped with a separation module (Model e2695), PDA detector (Model 2998), controlled with "Empower-3" software (Waters Corporation, Milford, MA, USA) and reverse-phase column (LUNA; 250 mm × 4.6 mm C_{185} µm). The column oven temperature was programmed at 25°C and the column flow rate was 1 mL/min. The mobile phase consisted of 65% (v/v) acetonitrile (A) and 35% (v/v) water (B).[71] The isocratic elution program was used and monitored at 242 nm. Standards of guggulsterone E (T17D025; Natural Remedies) and guggulsterone Z (T17D026; Natural Remedies) 2 mg/L were used as references to identify the presence of guggulsterones. The experiment was carried out in triplicates.

Liquid chromatography-mass spectrometry/mass spectrometry analysis

The extracted callus were analyzed with an Agilent 1260 Liquid Chromatography coupled with Agilent 6460 MS (LC-MS/MS) triple quad with Jetstream instrument equipped with column (2.7 µm × 2.1 EC-C_{18} column [100 mm; Porashell 120]) and the flow rate was 0.3 mL/min. The mobile phase consisted of 35% (v/v) water (A) and 65% (v/v) acetonitrile (B). The isocratic elution program was used. Under positive ESI mode, pure isomers of guggulsterones produced abundant protonated molecule $(M + H)^+$ at 313.2. Dominant fragments ions at *m/z* 97, 109.2, and 135.2 were derived from guggulsterone [Supplementary Table 1]. Similar results were inferred by Bhatta *et al.*, 2010.[72] Experiments were conducted in triplets.

Standards of guggulsterone E (T17D025; Natural Remedies) and guggulsterone Z (T17D026; Natural Remedies) 2 mg/L were used as references to identify the presence of guggulsterones in HPLC and LC-MS/MS analysis. The experiment was carried out in triplicates.

Statistical analysis

The data were expressed as mean ± standard deviation (SD), and $P < 0.05$ was considered to be statistically significant. The mean and SD were calculated by MS Excel tools. The effect of salinity stress on growth and water content of callus was assessed by one-way ANOVA, while the correlation between (a) RGR and RWC and (b) callus age and guggulsterone content was measured by Pearson's R correlation coefficient. Supplementary Table 2 shows the mean ± SD of the quintuplicate.

RESULTS

Callus induction and growth

Friable callus was developed on MS medium fortified with 3 mg/L 2,4-D and 0.3 mg/L Kn (our unpublished previous work). The callus was initiated from young leaves explants after 4 weeks of inoculation; however, profuse and measurable growth was obtained after 90 days. The non-embryogenic callus was white to cream in color but subsequently turned darker with time, which may be due to the presence of phenolic substances within the cells.

Effect of salinity stress on callus growth

To optimize the concentration of salinity stress on the growth of callus, water content, and biosynthesis of guggulsterone *in vitro*, an experiment was designed to grow *C. wightii* callus cultures in three iso-osmotic concentrations (50, 100, and 150 mg/L) of NaCl, $CaCl_2$, KCl, and Na_2SO_4 [Supplementary Table 2 and Figures 1a-e, 2a-d, 3a-c, and 4a-c]. Results showed that MS medium with 100 mg/L NaCl resulted in the highest growth of callus (11.76 ± 0.18 g) [Figure 1e] and, when supplemented with 50 mg/L $CaCl_2$, exhibited second-highest callus growth (9.74 ± 0.17 g); [Figure 2d]. Callus without stress weighed 3.55 ± 0.06 g [Figure 1a]. The elicitation using KCl and Na_2SO_4 showed inhibition of calli growth. Our results showed that the study for rate of callus growth was significant at $P < 0.05$ [Supplementary Table 3]. The growth of NaCl-induced calli showed a bell-shaped curve from low to high salinity concentration (50 mg/L <100 mg/L >150 mg/L = 8.68 ± 0.03 g <11.76 ± 0.18 g >6.39 ± 0.12 g, respectively). In contrast to the observation in NaCl-induced calli, $CaCl_2$-induced calli showed decreased growth of callus from low to high salinity concentration (50 mg/L >100 mg/L >150 mg/L = 9.74 ± 0.17 g >5.61 ± 0.36 g >3.56 ± 0.27 g, respectively). From Supplementary Table 2, it is evident that the growth of the callus was suppressed at all concentrations of KCl and Na_2SO_4; the latter is more detrimental.

Effect of salinity stress on the water content in callus

Contrasting cellular responses, it is to note that the total water content in callus followed a reverse trend to callus growth. Results indicated that MS medium fortified with 50 mg/L Na_2SO_4 showed the highest water content [11.66 ± 0.52 g; Figure 4a], while 100 mg/L NaCl the lowest (9.22 ± 0.01 g) [Figure 1e] and followed by 50 mg/L $CaCl_2$ [9.28 ± 0.09 g; Figure 2d]. Our study was significant at $P < 0.05$ [Supplementary Table 4], and also, Pearson's correlation coefficient R resulted in a strong negative correlation (−0.861) and supported the fact that the growth of callus is indirectly related to its water content.

Effect of salinity stress on guggulsterones production

Column chemistry, solvent type, solvent strength (volume fraction of organic solvent[s] in the mobile phase and pH of the buffer solution), detection wavelength, and flow rate were diverse to regulate the

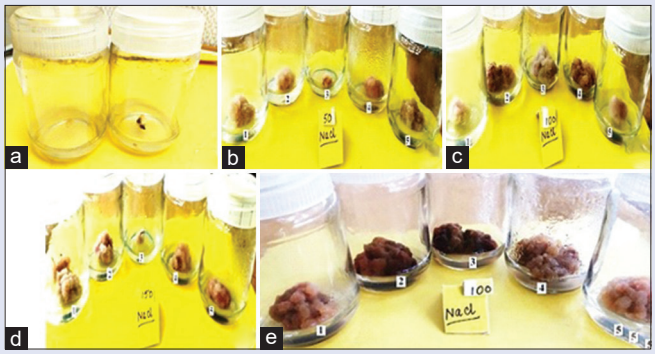

Figure 1: Callus proliferated from the tender leaves of *Commiphora wightii* cultured on MS basal with 3 mg/L 2,4-dichlorophenoxyacetic acid + 0.3 mg/L kinetin within 30 days. (a) Untreated (control), (b) sodium chloride (50 mg/L), (c) sodium chloride (100 mg/L), (d) sodium chloride (150 mg/L), and (e) sodium chloride (100 ml/L) within 90 days

chromatographic conditions giving the best separation. The mobile phase conditions were adjusted to avoid interference from solvent and other compounds. For HPLC analysis, primarily, many mobile phases were tried in attempts to obtain the best separation and resolution between guggulsterones E and Z; however, the optimized chromatographic conditions for an adequate resolution were obtained by using the mobile phase consisting of isocratic elution of acetonitrile (65% [v/v]) and water (35% [v/v]). Under this system, the chromatogram of guggulsterones E and Z from *C. wightii* extract is shown in Figure 5a-c. The retention times for guggulsterones E and Z were 9.046, 8.76 and 8.75 min and 11.50, 11.077 and 11.08 min at 9, 12, and 15 months, respectively. Our results indicated that MS medium fortified with 50 mg/L $CaCl_2$ showed the presence of guggulsterones by HPLC analysis, when measured (in ppm) at an interval of 9, 12, and 15 months of incubation, and the highest was recorded at 15 months [Supplementary Table 5]. Furthermore, an LC-MS experiment was performed to confirm the study Figure 6a-c.

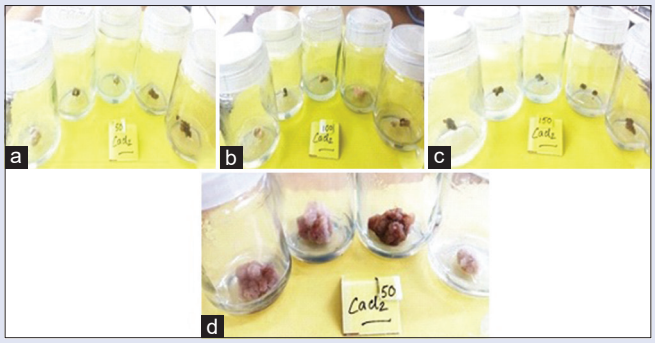

Figure 2: Callus proliferated from the tender leaves of *Commiphora wightii* cultured on MS basal with 3 mg/L 2,4-dichlorophenoxyacetic acid + 0.3 mg/L kinetin within 30 days. (a) Calcium chloride (50 mg/L), (b) calcium chloride (100 mg/L), (c) calcium chloride (150 mg/L), and (d) calcium chloride (50 mg/L) within 90 days

The increase of basal $CaCl_2$ concentration by 18% promoted the accumulation of guggulsterone (E and Z) when kept for 15 months. The results suggest that 50 mg/L $CaCl_2$ is an effective stress inducer and higher calcium is required for guggulsterone production in *in vitro* callus cultures of *C. wightii*. Our data suggest a strong positive correlation (Pearson's *R* coefficient is 0.962) between the age of the callus and the guggulsterone content.

DISCUSSION

The occurrence of phenolic substances within the cells, amassed in the cytoplasm, undergoes oxidation and polymerization and oxidized products appear brown in the callus.[73] Our result corroborates with the findings that the osmotic stress tolerance in intact plants is not necessarily matched by tolerance exhibited by callus,[74] as *C. wightii* in natural habitat preferred arid climate and sandy loam soil with alkaline pH (7.5–9); however, at *in vitro* level, the pH optimum for growth of the callus was observed to be subacidic (5.8–5.9). Our study also suggested that salinity stress affects the growth of the callus; similar results were reported in Cicer arietinum[75] and suggested that NaCl-stressed media increase callus weight.

It is observed that when the salt concentration is increased beyond a threshold level, the growth of the calli progressively decreases and ultimately affects the size of most plant species;[76] NaCl-induced calli showed declined growth of callus at a higher concentration in rice species,[77] like indica rice cultivars Pusa Basmati 1 and Basmati 370;[78] and also in soybean;[79] while in *Populus euphratica*, calli reported maximum growth at 50 mM NaCl and reduced at 150 and 250 mM.[80] Similar results were recorded in the present study as, with increase of salt concentration, RGR reduced [Supplementary Table 5] for NaCl and $CaCl_2$ salts. The reduced weight with increased salinity could be attributed to either due to ion toxicity or low exterior osmotic potential or reduced P, K, and Fe and increased Na, Mn, Cu, and Zn uptake,[81] and thus, increased NaCl in the culture media may indicate enhanced sodium uptake and translocation.[82] Thus, our results corroborated

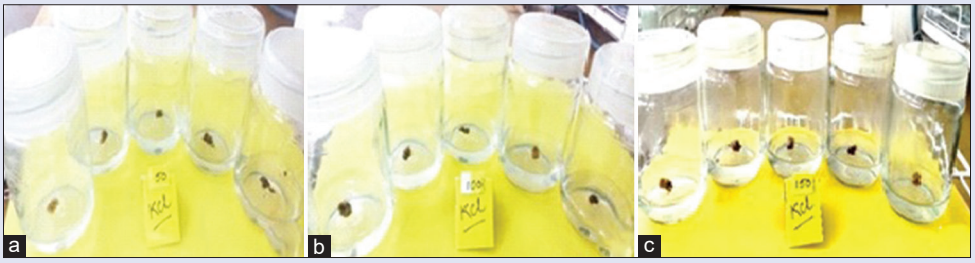

Figure 3: Callus proliferated from the tender leaves of *Commiphora wightii* cultured on MS basal with 3 mg/L 2,4-dichlorophenoxyacetic acid + 0.3 mg/L kinetin within 90 days. (a) Potassium chloride (50 mg/L), (b) potassium chloride (100 mg/L), and (c) potassium chloride (150 mg/L)

Figure 4: Callus proliferated from the tender leaves of *Commiphora wightii* cultured on MS basal with 3 mg/L 2,4-dichlorophenoxyacetic acid + 0.3 mg/L kinetin within 90 days. (a) Ammonium sulfate (50 mg/L), (b) ammonium sulfate (100 mg/L), and (c) ammonium sulfate (150 mg/L)

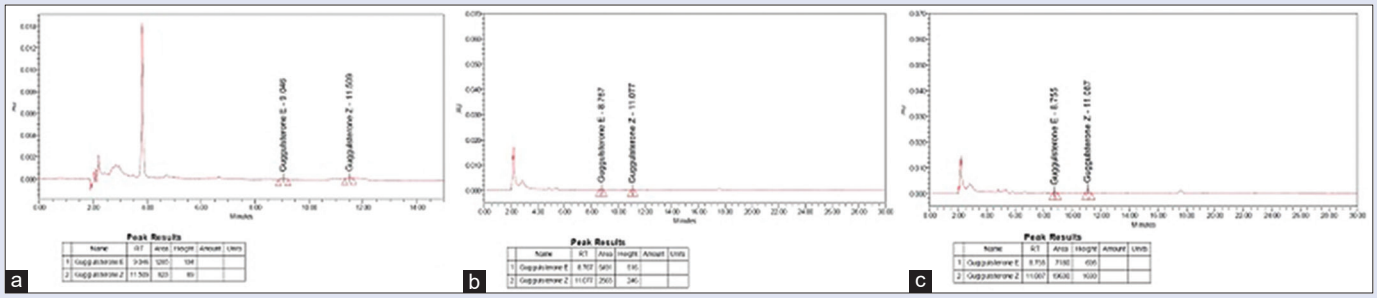

Figure 5: High-performance liquid chromatography profile of callus grown on 50 mg/L calcium chloride induced media showing the presence of guggulsterone (E and Z) at (a) 9 months; (b) 12 months, and (c) 15 months

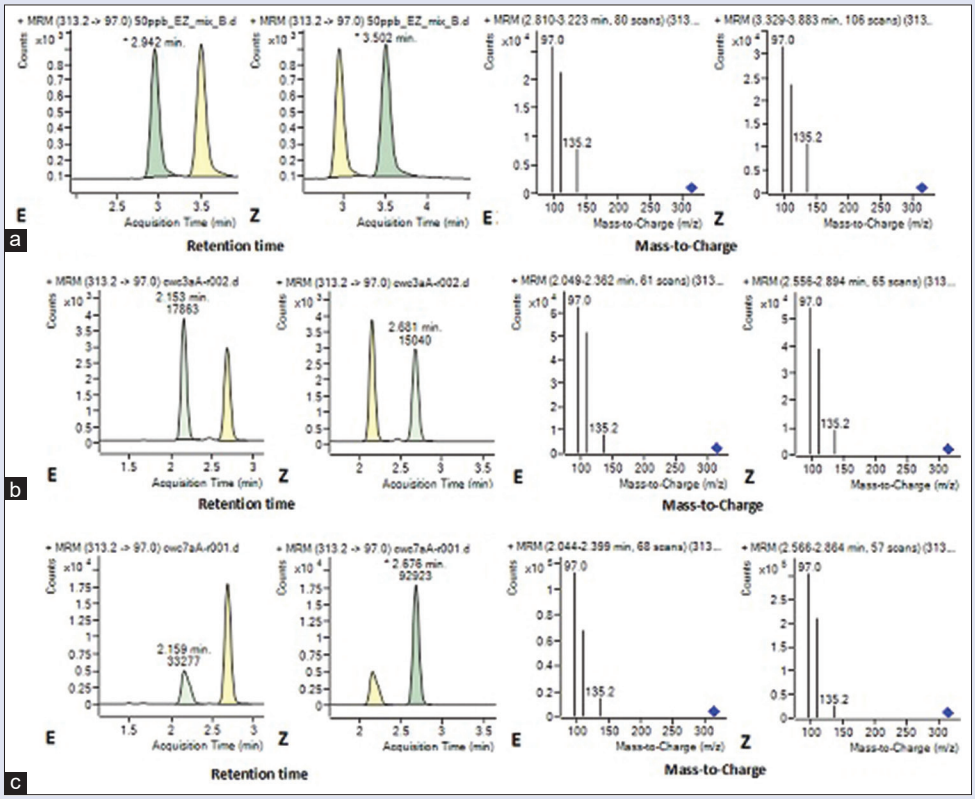

Figure 6: Liquid chromatography-mass spectrometry/mass spectrometry profile of callus grown on 50 mg/L calcium chloride induced media showing the presence of guggulsterone (E and Z) at (a) 9 months; (b) 12 months, and (c) 15 months

previous findings that there is a direct relationship between salt tolerance and callus induction capacity.[79] Our results suggest that the callus growth and water content are indirectly related; and are in agreement with the previous observations recorded in Sugarcane variety cvs. R570 where mannitol-induced calli showed the highest growth of the callus and the lowest water content.[70]

Secondary metabolites are accumulated in low concentration in plant cell cultures but could be enhanced by elicitation.[83] Tissue differentiation, growth, and development in plants determine the site and accumulation of secondary metabolites in plant cell and tissue cultures.[84] Therefore, the reaction to specific stress may vary from plant to plant and different cell lines; thus, it becomes critical to determine suitable concentrations of elicitors for product optimization.[85] The stress inducers/elicitors have different mechanisms of elicitations and, when used in combination, synergistically enhance secondary metabolite production *in vitro* cultures.[86]

Our study showed that the increase of basal $CaCl_2$ concentration by 18% promoted the accumulation of guggulsterone (E and Z) when kept for 15 months; contrasting cellular responses with the same elicitor, calcium deprivation was found to be potent for guggulsterone production.[21] This result suggests that calcium plays a role in guggulsterone production, but the effect is dose dependent. Our results corroborated with the previous observations of calcium involvement in the production of anthocyanin in *Vitis vinifera* cells,[87] callose in liquid culture of *Glycine max*,[88] cholecalciferol in *Solanum malacoxylon*,[89] risithin in Solanum tuberosum tubers, sanguinarine and chelerythrine in liquid culture of Sanguinaria canadensis, and anthraquinones, chrysophanol, and emodin in *Rhubarb cells*.[91]

Calcium acts as a secondary messenger in metabolic activities such as cell division[93] and increases accumulation of secondary metabolites.[94] Elicitors of latter biosynthesis result in a transient increase in cytosolic calcium level[93-97] which are strongly linked to increased oxygen species[91] (known to act as a second messenger).[93] The calcium transient is controlled by overall downstream mechanisms, especially gene expression.[15,98-101] A change in cytosolic calcium represents a signal which either directly or via calcium-binding proteins regulates the activities of the targeted enzymes;[102] therefore, it may be inferred that instead of calcium channels, calcium-dependent enzymes are affected by calcium enhancement and thus resulted in enhanced guggulsterone synthesis.

It has been reported through *in vitro* and *in vivo* studies that the accumulation of secondary metabolites is related to the age of callus/plant as at a later stage of the growth cycle,[103] the growth of the callus slows down or reaches a plateau and results in more production of secondary metabolites, example, in *Morinda citrifolia*[104] and cryptotanshinone production in *Salvia miltiorrhiza*.[105] Agarwal and Kamal, 2007[106] reported maximum flavonoids content in 6-week-old callus (2.90 mg/g DW) while the minimum was 2 weeks old (1.83 mg/g DW). Therefore, our results concur with previous observations and suggest that there exists a direct correlation between the age of the plant/callus and the accumulation of secondary metabolites. Interestingly, our studies indicated that NaCl-induced calli failed to promote guggulsterone production. This effect was also observed by Hagimori *et al*., 1983,[107] in which Ca^{2+} but not Cl^- was responsible for digitoxin production in *Digitalis purpurea* L.

The results suggest that salinity stress affects the growth of the callus and production of guggulsterone *in vitro* callus cultures of *C. wightii*. The elicitor, $CaCl_2$ at a concentration of 50 mg/L may cause high-stress conditions in callus cultures and triggers differential changes and promotes the biosynthesis of guggulsterone. The present result also suggests a strong positive correlation between the age of the callus and the accumulation of guggulsterone.

CONCLUSION

In this study, the effect of salinity stress on the growth of the callus and production of guggulsterone *in vitro* callus cultures of *C. wightii* was investigated, which was favored by the calcium elicitation. The results indicated that 100 mg/L NaCl was most suitable for the growth of callus; therefore, it may be concluded that *C. wightii* is a salt-tolerant medicinal plant that may be intolerant to salinity stress above a concentration of 100 mg/L NaCl in *in vitro* system. The study also reported that the accumulation of guggulsterone in the callus cultures was enhanced under stress condition, primarily by $CaCl_2$ at a concentration of 50 mg/L incubated for 15 months, thus suggesting a positive correlation between the callus age and guggulsterone produced. The positive effect of calcium on the biosynthesis of guggulsterone in *C. wightii* may be due to direct elicitation of the steroid production or deviation of the biosynthetic pathway and stimulation of any other metabolic mechanisms ensuing indirectly in the enhancement of guggulsterone production; however, further investigations are required to assess the gamut of this physiological and biological basis of ion effects on stressed cell metabolism, before any irrefutable conclusion regarding the precise nature of salt-tolerant mechanisms.

Due to the increasing demand for therapeutically important plant *C. wightii* in pharmaceutical and nutraceutical industries, this study will be a beneficial basis for large-scale production of guggulsterone and germplasm conservation and also encouraged studies on other stimuli such as light, injury, and temperature to improve the phytochemical contents.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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