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ORIGINAL ARTICLE

Enhanced Synthesis of Curculigoside by Stress and Amino Acids in Static Culture of *Curculigo orchioides* Gaertn (Kali Musli)

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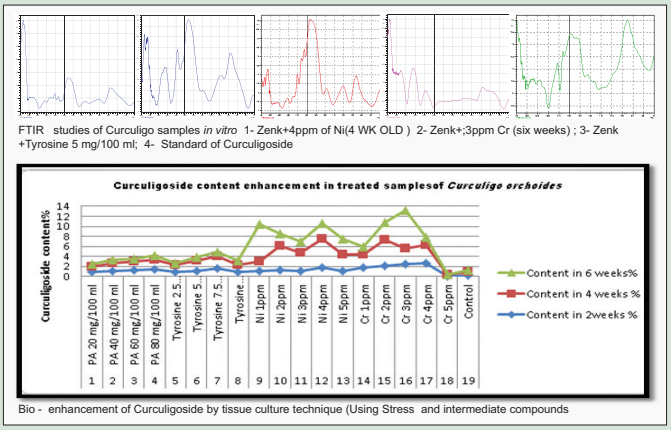
ABSTRACT

Background: *Curculigo orchioides* Gaertn (Kali musli; Family: Hypoxidaceae) is an endangered medicinal plant used for many medicinal purposes such as impotency, aphrodisiac, tonic, jaundice, and skin ailments. Its hepatoprotective, antioxidant, and anti-cancerous potential have also been evaluated by many scientists. **Objective:** The objective of this study is to enhance the curculigoside content in tissue culture of *C. orchioides*. **Materials and Methods:** The present study deals with the enhancement of an active compound of *C. orchioides* by incorporating various concentration of phenylalanine (Phe), tyrosine, (20, 40, 60, and 80 mg/100 ml), chromium (Cr) and nickel (Ni) (1, 2, 3, 4, and 5 ppm) into Zenk media in controlled and aseptic conditions. **Results:** Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. A significantly remarkable enhancement in all induced samples was noted. Curculigoside content was maximum in the 6-week-old tissue induced with 3 ppm of Cr (7.63%) followed by 4 weeks tissue of tissue fed with 4 ppm of Ni (5.66%) and 4-week-old tissue fed with tyrosine 7.5 mg/100 ml (2.38%) among all samples used. These results suggest that tyrosine is better enhancer than Phe in the biosynthetic pathway of curculigoside. The presence of curculigoside in all extracts was confirmed by Fourier transform infrared spectroscopy, high-performance thin layer chromatography analysis with standard compound of curculigoside and histology of treated samples. **Conclusion:** This investigation was carried out for the 1st time, and it is a significant step in understanding the biochemistry of curculigoside. The developed protocol will be beneficial for marketing in pharmaceutical industries.

Key words: *Curculigo orchioides,* curculigoside, enhancement, intermediate compound, medicinal plant, metal stress, phenolics

SUMMARY

- *Curculigo orchioides* Gaertn (Kali musli; Family: Hypoxidaceae) is an endangered medicinal plant used for many medicinal purposes such as impotency, aphrodisiac, tonic, jaundice, and skin ailments
- It was observed that dry matter % was maximum in 6-week-old tissue fed with 2.5 mg/100 ml of tyrosine and diminished beyond this concentration among all samples used
- The nickel (Ni) and chromium (Cr) stress has enhanced the curculigoside in considerable amount in nontoxic range, in tissue culture of *C. orchioides*
- Curculigoside content was maximum in 6-week-old tissue induced with 3 ppm of Cr (7.63%; 11-fold enhancement) followed by 4 weeks tissue of tissue fed with 4 ppm of Ni (5.66%) and 4-week-old tissue fed with tyrosine 7.5 mg/100 ml (2.38%) among all samples used. Histological studies confirmed the enhanced production of curculigoside.



Abbreviations Used: Phe: Phenylalanine; PAL: Phenylalanine ammonia-lyase; mM: mille Molar; Cr: Chromium; Ni: Nickel; HPTLC: High-performance thin layer chromatography

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INTRODUCTION

Curculigo orchioides Gaertn (Kali musli; Family: Hypoxidaceae) is an endangered medicinal plant used for many medicinal purposes such as impotency, aphrodisiac, tonic, jaundice, and skin ailments. Its hepatoprotective, antioxidant, and anti-cancerous potential have been evaluated by many scientists.[1] This tiny herbal plant widely distributed in China, India, Malaya, Japan, and Australia. In Chinese medicine system, the experimental plant is used for the treatment of many diseases. Its rhizomes have the properties used for warming kidney, invigorating yang, expelling cold, and eliminating dampness.[2] Previous phytochemical investigations of rhizomes (principal active organ) revealed the presence of curculigoside (Phenolic glycoside).[3] In addition, some other major chemical constituents of the experimental plant are cellulose, hemicellulose, and calcium oxalate. Its medicinal values are due to the presence of different secondary metabolites such as triterpenoid, saponins, flavones, and curculigoside. Plant tissue culture has given a new approach to exploit its technology for production of natural products. It provides a new platform to study and explore the field of transcriptomics, proteomics, and biochemistry. The experimental medicinal plant is becoming endangered due to its overexploitation,

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so there is a requirement to explore biotechnological strategies to increase its number as also to enhance its tuber's quality in a limited period. Some reports related to micro propagation of *C. orchioides* in tissue culture have been well documented.[4] However, the research regarding the bioenhancement of active principal, i.e., curculigoside (polyphenolics) is not well documented and almost untouched; hence, in the present investigation, an attempt was made to optimize the tissue culture parameters for bioenhancement in the production of chief active principal, curculigoside. The development of appropriate protocol for optimum production of curculigoside is essential for exploiting the tissue culture system and for relevant advantage. Many pharmaceutical industries are engaged with the exploitation of the active principal (curculigoside) of Kali Musli; therefore, the technology development in this area is a revolutionary step to enhance the usefulness of plant.[5]

Curculigoside (C22H26O12) is identified as colorless, needle-shaped polyphenolic compound. ($C_{22}H_{26}O_{11}$), mp 159–161°C. Infrared (IR) (KBr) max cm^{-1}: 3370 (OH), 2922, 1724 (ester), 1598 (aromatic ring). ESI-MS m/z: 484 ($M + NH_4$) +, 489 (M + Na).[6]

The capacity for plant cells, tissue, and organ cultures to produce and accumulate many of the valuable chemical compounds as the parent plant in nature has been recognized almost since the commencement of *in vitro* technology. Enhancement of polyphenolic compounds has been well documented in many medicinal plants using tissue culture strategies. Chaturvedi *et al.* 2014[7] have developed an economically feasible technology to optimize the production of curcumin in tissue culture of *Curcuma longa*. In the same way, Chaturvedi and Chowdhary, in 2013,[8] have observed the enhancement in flavonoids content of *Allium cepa*, *Trachyspermum ammi*, *Tylophora indica,* and *Helipterum roseum* separately in *in vitro* condition. Palacio *et al.*, in 2013,[9] have depicted the production of phenolic compounds in *in vitro* and *in vivo* studies. Al-Amier *et al.*, in 1999,[10] have screened different clones of lavender for their phenolic production in tissue culture system. Published documents reveal that phenylalanine (Phe), an aromatic amino acid, is the substrate of phenylalanine ammonia-lyase and catalyzes the reductive deamination of L-phe into trans-cinnamic acid as the first step of the biosynthesis of plant phenolic compound.[11] Bemani *et al.*, in 2012,[12] found that Phe up to 3 mM had no significant effect on the growth of hazel cells and at 6 mM, it affected the dry weight adversely. In the search for alternatives for production of desirable medicinal compounds from plants, biotechnological approaches, especially plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites.[13] Plant cell and tissue cultures hold great promise for controlled production of useful secondary metabolites; on demand, they are responsible for its therapeutic effect and are often produced as a result of adaptation to biotic and abiotic stress. Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures,[14] and DUAL culture is one of them. Dual culture system involves biomass production in a medium optimum for cell proliferation followed by transfer of healthy cells to a different medium which is favorable for product yield. This strategy was used by Zenk *et al.*, in 1977,[15] for the production of indole alkaloids by *Catharanthus roseus* cells. Curculigoside is a significantly valuable bioactive principal of *C. orchioides*; hence, there is a need to enhance its content to improve tubers quality. In the present investigation, Zenk media was used to enhance the curculigoside in *C. orchioides* tissue culture. Plant tissue culture has been proven as an efficient mean to enhance the medicinally valuable compounds by manipulating the media components (Chaturvedi *et al.*, 2011, 2011a, 2011b, 2012, 2012a, 2013a, 2014, and 2015).[16-24] Keeping in perspective all these facts, the experiment regarding the bioenhancement of curculigoside content in tissue culture of *C. orchioides* was carried out. This was attempted by feeding the tissue with various concentrations of Phe and tyrosine (Precursor and intermediate compounds) and by giving heavy metal stress (chromium [Cr] and nickel [Ni] stress) to already maintained *C. orchioides* cultures. An important approach toward enhanced production of curculigoside in tissue culture of *C. orchioides* has been established.

MATERIALS AND METHODS

Plants were initially obtained from the nursery at Dediapada village of district Dang, Gujarat. The germplasm was maintained at the Greenhouse of Loyola Centre for Research and Development (LCRD), St Xavier's College campus, Ahmedabad, Gujarat, India. *C. orchioides* is an endangered medicinal plant (IUCN) and grows only for 3 months of the monsoon; therefore, there is a need to grow rapidly and to increase its quality in a limited period using different strategies in tissue culture. The cultures for the present study were taken from already established cultures of *C. orchioides* in our Research Centre LCRD. Described study depicts the yield enhancement of curculigoside in tissue culture of *C. orchioides*. The study covers the incorporation of intermediate compounds (in various concentrations) into Zenk media with 20, 40, 60, and 80 mg/100 ml of Phe and 2.5, 5, 7.5, and 10 mg/100 ml of tyrosine separately. The other experiment regarding the addition of various concentrations of Cr and Ni (1, 2, 3, 4, and 5 ppm) separately into Zenk (Zenk 1975) basal media was also carried out, to see its effect on the production of active principle. Five percent sucrose and 0.8% agar were used and 5.6 pH was set before auto cleaving. For stress experiments, potassium dichromate and sodium sulfate were used for preparing stock solutions. After aseptic inoculation, the cultures were incubated for 2, 4, and 6 weeks in 16 h (1000 lux of light) of photoperiod and 8 h of dark period with 70% humidity and 25–27°C temperature. All samples were harvested at the time interval of 2, 4, and 6 weeks separately, dried, and weighed and dry matter % was calculated [Table 1]. The dried powder of different samples was subjected for cold extraction with methanol at room temperature. After 36 h, the extracts were filtered, dried in vacuo, and weighed separately. All extracts were subjected to high-performance thin layer chromatography (HPTLC) analysis and IR spectral studies with standard reference compound of curculigoside.

High-performance thin layer chromatography analysis

HPTLC (CAMAG) of different samples was carried out with standard compound of curculigoside. Mobile phase was ethyl acetate: ethanol: water (5:1:5). The scanning was done at 285 nm. Rf = 0.3 as noted. We

Table 1: Effect of nickel and chromium stress on dry matter % in *Curculigo orchioides* tissue culture

Treatment (ppm)	Dry matter % in 2 weeks old	Dry matter % in 4 weeks old	Dry matter % in 6 weeks old
Ni 1	11.91	12.4	12.56
Ni 2	13.24	15.76	17.67
Ni 3	12.99	19.45	15.59
Ni 4	13.31	16.58	14.07
Ni 5	13.99	15.15	14.19
Cr 1	11.65	12.66	14.84
Cr 2	8.91	11.76	15.44
Cr 3	11.34	13.71	15.89
Cr 4	13.88	15.71	17.72
Cr 5	14,22	15.9	14.17
Control	13.107	18.74	16.81

Results show the mean values of triplicates. Dry matter %: Tissue dry weight/ tissue fresh weight ×10. Ni: Nickel; Cr: Chromium

also have used the other solvent system such as ethyl acetate: n-butanol: water (2:3:5). n-butanol: water (1:1), ethyl acetate: methanol: water (4:1:4), but the separation was observed better in ethyl acetate: ethanol: water (5:1:5). Detection was done by spraying developed plates with reagent mixture (1:1) of 0.02% potassium ferrocyanide and 0.02% $FeCl_3$ solution. Dark blue color was observed. The quantitative estimation was done using peak area mentioned in the given results of HPTLC graphs.

Infrared spectral studies

IR spectral studies of all used samples were carried out with standard compound of curculigoside, using equipment Buck scientific 500.

Histological studies

Histological studies of *in vitro* treated samples of *C. orchioides* were carried out by cross sectioning. The samples were stained with safranin previously and afterward spraying reagent of curculigoside (mentioned above).[25] The sections were observed in microscope in ×40 power [Figure 1].

Statistical analysis

Statistical analysis of obtained data (triplicate) was performed using Microsoft Excel and standard deviation was obtained.

RESULTS AND DISCUSSIONS

Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. Environmental factors, namely, temperature, humidity, light intensity, the supply of water, minerals, and CO_2 influence the growth of a plant and secondary metabolite production. Drought, high salinity, and freezing temperatures are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops.[26]

The stimulation of phenolic compound biosynthesis was noted in wheat in response to Ni toxicity. Insoluble and soluble phenolic were produced in response to Cd^{2+}.[27] One of the most important groups of secondary metabolites is phenolic compound. They are characterized by at least one aromatic ring having one or more hydroxyl groups and are mainly synthesized from cinnamic acid, which is formed from Phe by the action of L-phenylalanine ammonia-lyase. Plants accumulate ultraviolet (UV)-absorbing flavonoids and other phenolic compounds mostly in vacuoles of epidermal cells, to prevent the penetration of UV-B into the deeper tissues of the plant.[28]

In the present study, we have given treatment of intermediate compounds such as Phe (20, 40, 60, and 80 mg/100 ml) and tyrosine (2.5, 5, 7.5, and 10 mg/100 ml) in various concentrations and also Cr and Ni stress to *C. orchioides*. It was observed that dry matter % was maximum in 6-week-old tissue fed with 2.5 mg/100 ml of tyrosine and diminished beyond this concentration [Table 2]. Among all samples used, this may be due to lethal

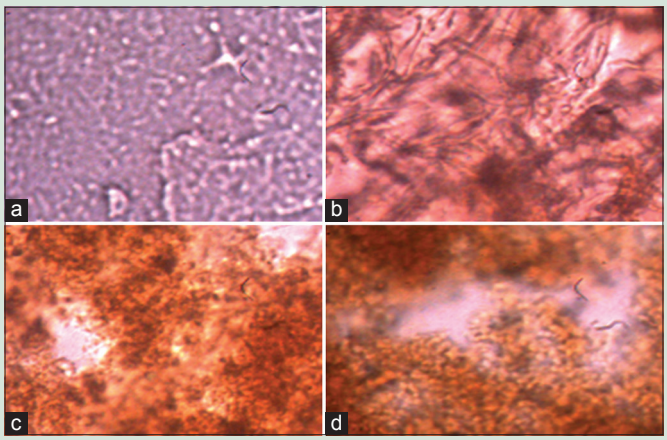

Figure 1: Histological studies of treated tissue of *Curculigo orchioides* static culture with intermediate and stress giving compounds. (a) Control, (b) tyrosine 7.5 mg/100 ml (4-week-old tissue), (c) nickel 4 ppm (4-week-old tissue), and (d) chromium 3 ppm (6-week-old tissue). The phenolic compound can be determined in the form of black spots. It is observed that black spots are increased according to the enhancement of curculigoside in various treated samples

Table 2: The dry matter % of treated samples with various concentrations of precursor compounds in *Curculigo orchioides*

Treatment (mg/100 ml)	Dry matter % (2 weeks)	Dry matter % (4 weeks)	Dry matter % (6 weeks)
PA 20	20.855	11.2	18.1
PA 40	19.203	14.32	10.99
PA 60	12.167	20.08	13.47
PA 80	10.236	12.5	16.35
Tyrosine 2.5	17.224	13.84	18.47
Tyrosine 5	7.917	12.39	10.15
Tyrosine 7.5	16.311	20.52	17.4
Tyrosine 10	9.375	13.4	17.68
Control	13.107	18.74	16.81

Results showing the mean values of triplicates. Dry matter %: Tissue dry weight/tissue fresh weight × 10. PA: Phenylalanine

Table 3: The effect of precursor compounds and heavy metal stress on production of curculigoside in tissue culture of *Curculigo orchioides*

Treatment	Content in 2 weeks %	Content in 4 weeks %	Content in 6 weeks %
PA 20 mg/100 ml	0.91±0.032	1.1±0.025	0.453±0.014
PA 40 mg/100 ml	1.11±0.021	1.55±0.042	0.69±0.016
PA 60 mg/100 ml	1.31±0.031	1.7±0.051	0.47±0.018
PA 80 mg/100 ml	1.41±0.021	1.9±0.072	0.94±0.019
Tyrosine 2.5 mg /100 ml	1.01±0.036	1.32±0.025	0.37±0.017
Tyrosine 5 mg /100 ml	1.05±0.041	2.07±0.042	0.79±0.018
Tyrosine 7.5 mg /100 ml	1.61±0.045	2.38±0.038	0.86±0.014
Tyrosine 10 mg /100 ml	1.01±0.012	1.33±0.028	0.86±0.018
Ni 1 ppm	1.14±0.021	1.8±0.038	7.54±0.015
Ni 2 ppm	1.21±0.037	4.9±0.039	2.5±0.018
Ni 3 ppm	1.09±0.047	3.67±0.006	2.27±0.014
Ni 4 ppm	1.88±0.051	5.66±0.016	3.1±0.018
Ni 5 ppm	1.05±0.047	3.39±0.041	3.1±0.017
Cr 1 ppm	1.76±0.017	2.7±0.032	1.4±0.013
Cr 2 ppm	2.11±0.021	5.3±0.081	3.4±0.017
Cr 3 ppm	2.55±0.021	3.1±0.037	7.63±0.014
Cr 4 ppm	2.73±0.016	3.64±0.68	1.55±0.018
Cr 5 ppm	0.14±0.017	0.22±0.031	0.11±0.015
Control	0.21±0.019	0.66±0.042	0.34±0.031

The results are mean±SD values in triplicates. SD: Standard deviation; Ni: Nickel; Cr: Chromium; PA: Phenylalanine

effect of higher concentrations of these compounds on growth of tissue. Curculigoside content was maximum in 6-week-old tissue induced with 3 ppm of Cr (7.63%; 11-fold enhancement) followed by 4 weeks tissue of tissue fed with 4 ppm of Ni (5.66%) and 4-week-old tissue fed with tyrosine 7.5 mg/100 ml (2.38%) among all samples used. These results suggest that tyrosine is better precursor than Phe in the biosynthetic pathway of curculigoside [Table 3]. The presence of curculigoside in all extracts was confirmed by histological studies [Figure 1], IR, HPTLC analysis with standard compound of curculigoside [Figures 2 and 3].

The cytological studies of cross section of treated samples were carried out [Figure 1]. The stained sections were showing the black spot (due to derivatization) in ×40 power, which were seen maximum in 6-week-old tissue fed with 3 ppm/100 ml of Cr followed by 4-week-old tissue fed with 4 ppm of Ni/100 ml and 4-week-old tissue fed with 7.5 mg/100 ml tyrosine in ×40 in microscope [Table 3 and Figure 1].

Infrared spectral studies

IR spectral studies of all extracts with standard compound of curculigoside showing the presence of curculigoside are shown in Table 4.

Melato *et al*., in 2012,[29] have investigated the positive effect of metal stress (Ni and Cr) on production of phenolics in Vetiver grass. Plants may

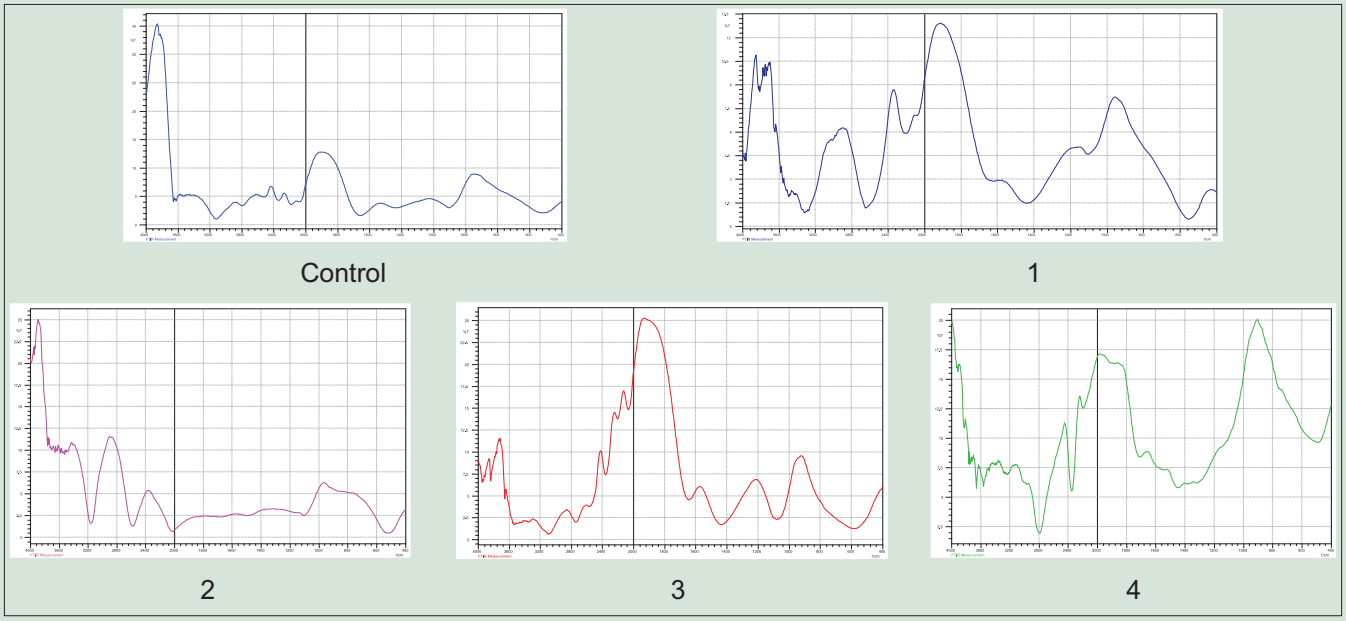

Figure 2: Infrared studies of curculigo samples *in vitro* 1-Zenk + 4 ppm of nickel (4-week-old) 2- Zenk +; 3 ppm chromium (6 weeks); 3-Zenk + tyrosine 5 mg/100 ml; 4- standard of curculigoside. Graphs showing the similar pattern of peaks in all samples and comparable to standard compound of *Curculigo orchioides*

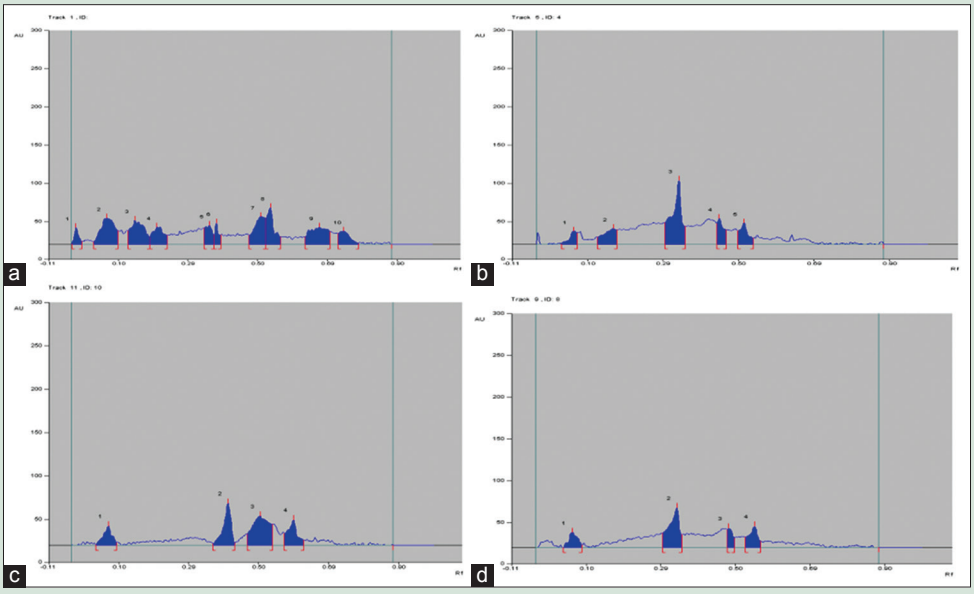

Figure 3: High-performance thin layer chromatography (a) standard, (b) 4-week-old tissue fed with 4 ppm of (nickel), (c) 6-week-old fed with 3 ppm of chromium, and (d) 4-week-old fed with 7.5 MG/100 ml tyrosine. The curculigoside content in all samples were calculated using their peak area

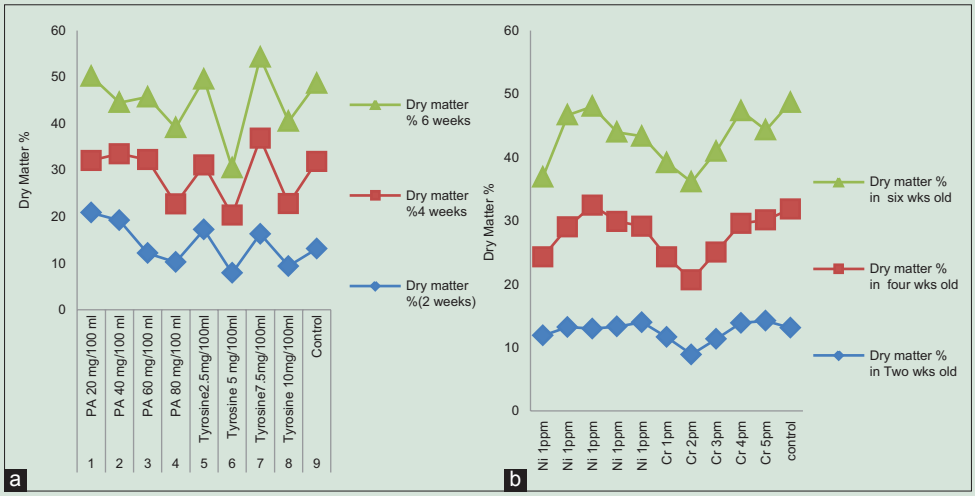

Figure 4: Effect of intermediate compounds (a) and heavy metal stress ((b) chromium and nickel) on dry matter % in *Curculigo orchioides* tissue culture reveals the higher concentration of treatment is harmful for growth of the tissues

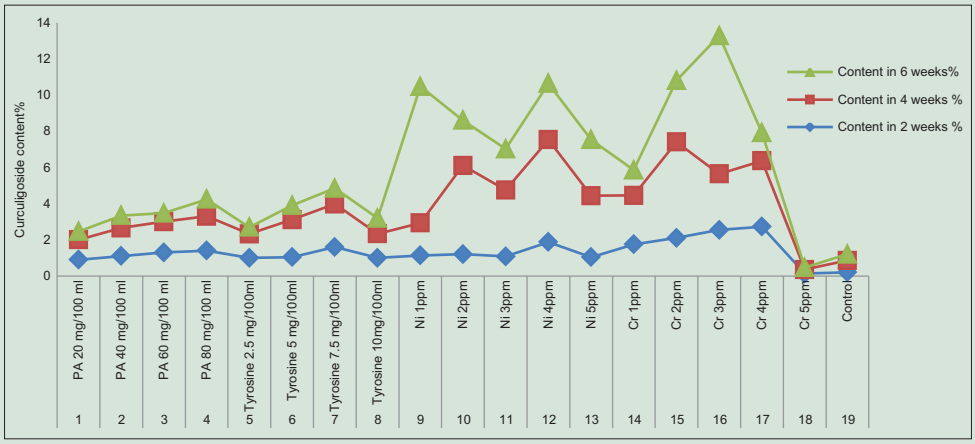

Figure 5: Graphical presentation of bioenhancement of curculigoside, a medicinally valuable phenolic compound by incorporation of intermediate compounds and heavy metal (nickel and chromium) into Zenk medium

Table 4: Infrared spectral studies showing the characteristic peaks of treated samples, corresponded with standard compound of curculigoside

Peak at wavelengt (nm)	Group denoted
3370	Alcoholic
17,242,922	Ester
1598	Aromatic

undergo significant morphological and metabolic changes in response to metal uptake. Many of these changes are believed to be adaptive responses to metal stress.[30] Additional consequences of phytotoxicity are enhanced production of reactive oxygen species and oxidative damage of important macromolecules including DNA, protein, lipids, chloroplast pigments, and enzymes.[31] However, enhancement of their metabolism was observed under different environmental factors and stress conditions.[32] An increase in phenolic content is correlated with the increase in enzymatic activity involved in phenolic metabolism,[33] suggesting synthesis of phenolic under metal stress and supported previous findings.

In sensitive species (for example, barley, water spinach, and wheat), chlorosis and necrosis of leaves can appear after plants are treated with Ni at very low concentrations (0.2 mM or 11.74 ppm) for less than a week.[34] Cr compounds are highly toxic to plants and are detrimental to their growth and development. Cr is toxic to most of higher plants at 100 μmol/kg (29.4 ppm) dry weight.[35] Hence, Ni and Cr concentrations that we have used in the present investigation are in nontoxic range for *in vitro* studies to enhance curculigoside in *C. orchioides*. Lu *et al*., 2002[36] have reported a range of the curculigoside content in 6 different samples. They reported that range varied from 0.11% to 0.35%. We have reported 0.2% curculigoside content in our findings, which supports the previous results [Table 3 and Figures 1-5].

CONCLUSION

The described study is showing the optimization of static media for production of curculigoside in tissue culture of *C. orchioides*. The Ni and Cr stress have enhanced the curculigoside in considerable amount in nontoxic range, in tissue culture of *C. orchioides*. Intermediate compounds of phenolics play a major role in the production of curculigoside. The results suggest that tyrosine is better precursor compound than Phe. Static tissue culture of *Curculigo orchioides* is suitable system to study biosynthetic pathway of phenolic active compound. The developed

technology will be useful for pharmaceutical industries. All results support the previous findings.

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Conflicts of interest

There are no conflicts of interest.

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