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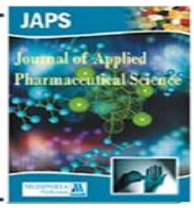

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Evaluation of certain flavonoids of medicinal importance in the wild and micropropagated plants of the endangered medicinal species, *Exacum bicolor* Roxb.

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ABSTRACT

An efficient micropropagation protocol was developed for the endangered medicinal plant species, *Exacum bicolor* Roxb. (Gentianaceae) using nodal segments by producing axillary shoots. Quantitative estimation of active compounds of medicinal properties such as luteolin and chlorogenic acid (flavonoids) was made in *in vitro* regenerated plantlets and compared to that of the wild intact plant by HPLC analysis. The results of the study revealed that the luteolin content was determined to be significantly higher in the wild plant tissues than the plantlets regenerated. However, this trend was reverse for the content of chlorogenic acid.

Key words: *Exacum bicolor*, Micropropagation, Luteolin, Chlorogenic acid.

INTRODUCTION

Exacum bicolor (Gentianaceae), a popular medicinal herb included in endangered category and generally distributed in northern part of Kerala state, India has been used to treat a variety of diseases (Anonymous,1966 and 1998). In recent times, pharmacological studies of this species are of growing interest due to its usage as stimulant to diabetics, stomachic and antifungal agent in traditional medicinal practices in Kerala (Reddi *et al*., 2005; Pullaiah, 2006; Khare, 2007; Shiddamallayya *et al*., 2010). Due to this demand, over exploitation by man resulted in less population size of this species (Jeeshna, 2011). Very less rate of seed germination (< 5 %) also keeps the population size of *E. bicolor* at low level in its grassland habitats at northern Kerala (Jeeshna, 2011). Attempts made for propagation by stem cuttings are also not effective (Sreelatha *et al*., 2007). Hence, it is difficult to restore the population of this species through conventional propagation methods. Therefore, in the present study to safeguard this species by increasing its population, an attempt has been made via *in vitro* regeneration by employing tissue culture technology. In addition, in order to know the content of the important active principle compounds such as luteolin and chlorogenic acid (flavonoids) in regenerated plantlets, phytochemical studies were carried out in the plantlets produced and it was compared to that of the intact wild plant tissues.

MATERIALS AND METHODS

Nodes from the young and healthy branches of the individuals of the species, *E. bicolor* present in the grasslands of Wayanad district of Kerala, India were collected and used as explants. Effective callus production and organogenesis followed by elite plantlet production by proper

acclimatization were achieved after proper sterilization and subsequent culturing onto the MS medium supplemented with appropriate concentration of certain growth regulators.

Extraction of plant materials

The species, *E. bicolor* was collected from the grasslands of Taliparamba, Kannur district, Kerala, India and the whole plant was dried for 20 days at room temperature. They were kept away from high temperatures and direct sun light to avoid the denaturation of active compounds. The dried plants were ground to fine powder with an electric grinder, packaged in airtight glass jar and stored at room temperature until further analysis was carried out. Hundred gram of powdered plant material was exhaustively extracted using methanol in soxhlet apparatus for 24 hr in order to get maximum yield of soluble compounds. After extraction, the crude extracts were filtered and concentrated under vacuum and controlled temperature with a rotary evaporator and residues were freeze dried. The extracts were stored at -8°C in deep freezer until further use. In the similar way, dried *in vitro* regenerated plantlets of 90 days old were subjected to extraction in soxhlet apparatus by using methanol solvent, followed by drying in deep freezer.

High performance liquid chromatography (HPLC) analysis

Sample preparation

5 ml of methanol was added to 2 g of whole plant powdered sample. The mixture was heated for 30 min at 40 ^{0}C. The residue was removed by centrifugation and the supernatant was diluted to 50 ml by adding methanol then the solution was filtered through 0.45 µm filter paper. In a similar fashion, samples were prepared for *in vitro* regenerated plantlets.

Chromatography

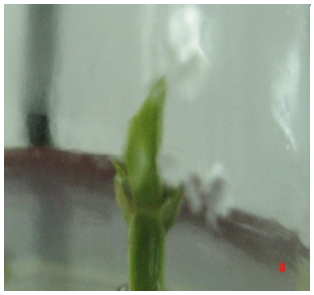
Samples of intact plant tissue and *in vitro* regenerated plantlets were analyzed separately with Hitachi-6200 HPLC equipped with a L-1000 UV spectrometric detector and the separation was carried out on a Pursuit Column (250 x 4.6 mm, 5 - µm particle, symmetry C 18). The mobile phase gradient was prepared from 100: 0.1 (v/v) water: formic acid (component A) and 100: 0.1 (v/v) acetonitrite :formic acid (component B); the gradient programme was from 84 – 60% for compound A in 30 min and from 16 - 60 % for compound B in 30 min. The injection volume was 10 µl, the mobile phase flow rate was 0.7 ml/mi and the detection wavelength was 255 nm.

RESULTS AND DISCUSSION

The data on effective *in vitro* regeneration of nodal explants in terms of callogenesis, shoot proliferation, multiple shoot regeneration and rooting of *E. bicolor* in standardized MS medium is given in Table 1. and the response for these attributes was shown in Fig. 1a-d. The callus formation was more pronounced (80.03 %) in the MS basal medium supplemented with the growth regulators, BAP and 2,4-D at 1.5 and 0.9 mg/l respectively. The subculturing of the callus for shoot formation

Table 1. Effective regeneration efficiency of *Exacum bicolor* by using nodal explants in MS medium fortified with certain growth regulators.

S. No.	Callus/Organogenesis	Regeneration efficiency (%)	Optimum concentration of growth regulators (mg/l)
1	Callus formation	80.03	BAP – 1.5 2,4-D – 0.9
2	Shoot proliferation	80.37	BAP - 1.0 NAA- 0.2
3	Multiple shoot regeneration	78.14	BAP- 1.5 GA_3- 0.5
4	Rooting	75.22	IBA- 1.0 NAA- 0.5



(a)

(b)

(c)

(d)

(e)

was effective (80.37%) in the medium containing BAP and NAA at 1.0 and 0.2 mg/l respectively. Multiple shooting by subculturing of the secondary explants, the shoots was highly appreciable (78.14) in the MS medium containing BAP and GA_3 at 1.5 and 0.5 mg/l respectively. The further subculturing for rooting was better (75.22%) in the medium containing IBA and NAA at 1.0 and 0.5 mg/l respectively. Plantlet survivability was well (80.0 %) in the hardening medium containing garden soil, sand and forest litter in the ratio of 1:1:1 by volume (Fig. 1e).

The plantlets were developed within 45 days from nodal segments by employing tissue culture technology and they were maintained further in the green house for another 45 days by

irrigating them with nutrient salt solution (1/2 strength of MS medium salts) to maintain the maximum growth of the plants. The two flavonoid compounds, luteolin and chlorogenic acid reported to be the medicinally important phytochemicals in *E. bicolor* elsewhere (Khare, 2007; Jeeshna,2011) have been estimated quantitatively in both intact wild plant tissue and *in vitro* cultured plants by using HPLC (Table 2 and Fig. 2-5).

Table 2. HPLC analysis of flavonoid content in *in vitro* regenerated plantlets and intact of *Exacum bicolor*.

Chemical compounds	Plant materials used	Content (mg/g of dry matter)	Relative extraction yield (%)
Luteolin	*In vitro* regenerated plantlet	0.6±2.65	6.06
	Wild plant	0.7±2.50	7.93
Chlorogenic acid	*In vitro* regenerated plantlet	0.02±0.40	0.26
	Wild plant	0.01±0.48	0.11

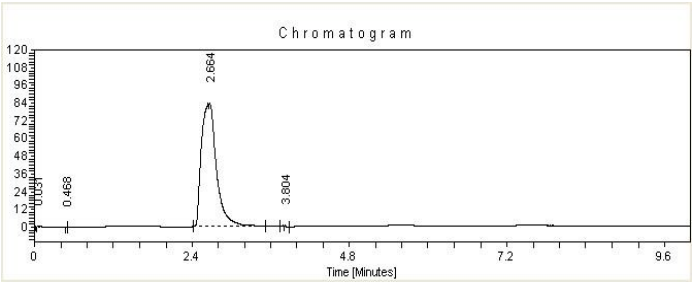


Fig. 2. HPLC chromatogram obtained from a standard containing luteolin.

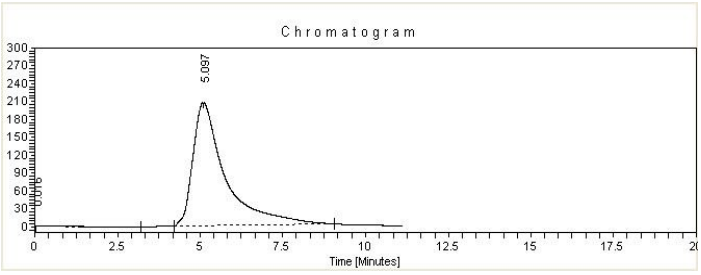


Fig.3. HPLC chromatogram obtained from a standard containing chlorogenic acid

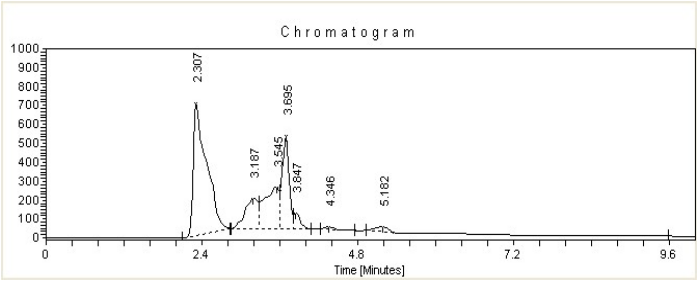


Fig. 4. HPLC chromatogram obtained from the regenerated plantlets.

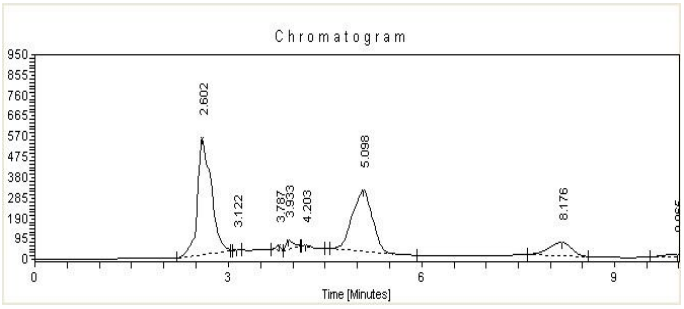


Fig. 5 HPLC chromatogram obtained from intact plant.

The components, luteolin and chlorogenic acid were identified both in wild and *in vitro* plants of *E. bicolor* by comparison with the retention time, and the UV spectra of authentic standards and quantitative data were calculated on basis of the peak area of respective compound. The content for luteolin and chlorogenic acid for the intact plant were 0.7 and 0.01 mg/g respectively. Whereas, in *in vitro* regenerated plant, they were present respectively at 0.6 and 0.02 mg/g. It indicates the presence of high amount of chlorogenic acid in *in vitro* regenerated plantlets than that of the wild. It may be explained that the hardened plantlets even after proper acclimatization have some environment sensitivity, and to adopt they produced certain variety of secondary metabolites vigorously by defense mechanism to develop resistance (Chintalwar *et al.*, 2003, Santarem *et al.*, 2003).

Further it is explained that as the *in vitro* produced plantlets growing in hardening medium containing forest litter with garden soil and sand is undoubtedly exposed to more carbon influx than the wild which may influence the metabolic flux for the biosynthesis of elevated levels of certain metabolic like chlorogenic acid (Hakkim *et al.*, 2007). On the other hand, the amount of luteolin was less in *in vitro* produced plants than that of its wild counter parts. Jones *et al.*, (2008) pointed out that the production rate of certain secondary metabolites in different populations of same species can be controlled mainly by temperature and moisture factors through altering the metabolic pathways favourably or unfavourably. Further, the populations growing under controlled conditions for some metabolites cannot respond considerably as the functions of enzymes involving in production processes are mostly temperature specific.

Therefore, higher production of certain biochemical compounds in certain conditions may not be applicable for some other compounds with respect to yield. These results highlights that for the controlled environment where the plantlets are regenerated may be favourable for the production of certain flavonoids like chlorogenic acid and unfavourable for certain other flavonoids like luteolin.

Robins (1994) also reported that production of certain compounds is possible under *in vitro* as a short – term response to an altered environment. Ranjitha Kumari *et al* .(2007) in a similar fashion reported that in *in vitro* regenerated *Chichorium intybus*, certain bioactive compounds showed variations in contents in comparison to that of its wild.

CONCLUSION

As the study species, *Exacum bicolor* is an endangered one, the *in vitro* regenerated elite plantlets can be used for the treatment of ailments and in the pharmacological industries which may help in the conservation of this valuable bioresourse and hence the germplasm as well. However, pharmacognosic studies are suggested for further confirmation of therapeutic value of the *in vitro* produced plants.

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