Plant Archives Vol. 18 No. 2, 2018 pp. 1251-1257 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ESTABLISHMENT OF *IN VITRO* CALLUS CULTURES AND COMPARATIVE PHYTOCHEMICAL STUDY *OF IN VITRO* CALLUS CULTURES AND FIELD GROWN PLANTS OF *OCIMUM TENUIFLORUM* L.

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Abstract

The present study deals with callus induction from explants along with the comparative analysis of content of eugenol and ursolic acid present in aerial parts of field grown plants and *in vitro* callus cultures of *Ocimum tenuiflorum* L. Leaf explants exhibited higher callus induction, followed by stem explants when inoculated onto MS medium containing α-naphthalene acetic acid (0.25mg/l) and 6-benzyl amino purine (0.5mg/l). The content of eugenol and ursolic acid was found to be higher in the aerial parts (leaf and stem) of the field grown plants in comparison with that of *in vitro* callus cultures.

Key words: *Ocimum tenuiflorum* L., callus, plant growth regulators, eugenol, ursolic acid.

Introduction

Plants have been used as important sources of pharmaceuticals, foods, flavors, insect deterrents, ornamentals, fumigants, spices, cosmetics and, dyes and oil for centuries (Pieroni *et al.,* 2004). These medicinal plants have been used for curing various ailments since consuming the plants or plant extracts are safe as well as economical, effective and easily available when compared to modern medicines (Ranganatha *et al.*, 2012). The secondary metabolites are produced in ethno-botanical valued plants against biotic as well as abiotic stresses thereby protecting plants (Rejeb *et al.*, 2014). These active constituents impart various pharmacological bioactivities to the medicinal plants (Tiwari *et al.*, 2014).

Among medicinally valued plants, those belonging to the genus *Ocimum* (of family Labiatae) are well known for having enormous therapeutic potentials for various ailments. It includes more than 100 species of herbs and shrubs (Labra *et al.*, 2004). The common known species of *Ocimum* are *Ocimum sanctum* L. (syn: *O. tenuiflorum* L. Tulsi), *Ocimum basilicum, Ocimum gratissium*, *Ocimum canum*, *Ocimum ammericanum, Ocimum camphora, Ocimum kilimandscharicum* and *Ocimum micranthum* which can be found in different regions of the world (Joshi *et al.*, 2017).

Ocimum tenuiflorum L. (family Lamiaceae, syn: *Ocimum sanctum* L., the Queen of herbs, elixir of life) is one of the commonly found aromatic herbs in temples and homes as shown in fig 1. It is a highly branched aromatic, erected, bitter herb/undershrub of height 30-60 cm and native throughout the world tropics. It is widely distributed as a cultivated plant and an escaped weed (Singh *et al.*, 2010, Pattanayak *et al.*, 2010, Bano *et al.*, 2017). Its several pharmaceutical potentials have been documented in traditional and Unani system of the East (Kayastha, 2014). Several workers have reported its various pharmacological activities *viz.* antiinflammatory, analgesic, antipyretic, anticancer, anti-oxidant, antidiabetic, hepatoprotective, hypolipidemic, antistress, and immunomodulatory activities (Pattanayak *et al.* 2010). It can also used for curing cold, cough, fever, arthritis, bronchitis and convulsions etc (Prakash and Gupta, 2005). Eugenol, linalool, rosmarinic acid, and ursolic acid (UA) are major active constituents of *O. tenuiflorum* L. responsible for enormous therapeutic potentials (Pattanayak *et al.*, 2010; Verma, 2016). Eugenol (1-

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hydroxy-2-methoxy-4-allylbenzene) and UA (pentacyclic triterpenoid) possesses diverse pharmacological activities such as anti-oxidant, antidengue, anti-inflammatory, antitumor, hypoglycemic, antiulcer, anti-HIV etc. (Vetal *et al.*, 2012;Pattanayak *et al.*, 2010). However, the environmental factors are responsible for variation in the content of medicinally valued secondary metabolites present in the plants. Hence, there is need of alternate approaches for production of secondary metabolites under controlled conditions to fulfill demands. Application of plant tissue culture techniques and biotechnological approaches has been reported to be effective for the production of secondary metabolites under controlled conditions. These secondary metabolites can be obtained season free throughout the year, thereby fulfilling the demand of pharmaceutical companies (Murthy *et al.*, 2014). However, there are few reports on comparison of the amount of secondary metabolites isolated from field grown plants and *in vitro* callus cultures (Besher *et al.*, 2014). The present study involves the callus induction responses from different explants in the presence of different combination of plant growth regulators and the comparative analysis of eugenol and ursolic acid production from the aerial parts of field grown plants (leave, stem) and *in vitro* callus of leaf as well as stem explants of *O.tenuiflorum* L. The chemical structures of eugenol and ursolic acid are depicted in fig 2a & 2b respectively.

Fig 1: Photo of *O. tenuiflorum* L. grown in the nursery of School of Life Sciences, JNU, New Delhi (India)

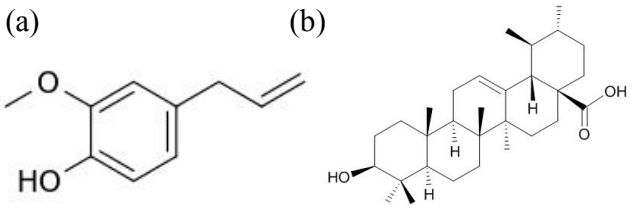

Fig 2: Chemical structures of **(a)** eugenol and, **(b)** ursolic acid present in *O. tenuiflorum* L.

Materials and method

Plant materials and induction of callus

The seeds of *O.tenuiflorum* L. were washed with detergent (Triton X-100) to remove dust particles for 20 min and then rinsed with sterilized double distilled water 5-6 times to remove excess detergent completely. After treatment of seeds with 0.1% Bavistin (fungicide) for 20 min, seeds were rinsed with sterilised double distilled water 4-5 times. The seeds were rinsed again with autoclaved double distilled water in the hood. After pretreatment with 0.1% $HgCl_2$ for 2 min, the seeds were rinsed with sterilised double distilled water. Seeds were placed on autoclaved Whatmann filter paper to dry and were transferred into autoclaved MS (Murashige and Skoog, 1962) media containing 0.8% agar in test tubes/ jam bottles. The test tubes/bottles were kept in culture room under the conditions of room temperature of 25±2°C and 16 hours illumination of 33,500 lux.

The leaf and stem explants of one-month-old *in vitro* plantlets were cut into pieces of equal size and placed onto autoclaved MS media supplemented with hormones in different combinations with various concentrations ranging for α-naphthalene acetic (NAA; 0.25, 0.5, 1.0 and 1.5 mg/l), and 2, 4-dichlorophenoxy acetic acid (2, 4D; 0.25, 0.5, 1.0 and 1.5 mg/l) and N6-benzylaminopurine (BAP; 0.25, 0.5, 1.0 and 1.5 mg/l). The cultures were kept in the culture room under fluorescent light with 16 h photoperiod at 25±2°C. The best proliferating calli were harvested after 20 days.

Solvent extraction techniques for extract preparation from callus and aerial parts of field grown plants of *O.tenuiflorum* L.

The best calli obtained from leaf as well as stem explants of *in vitro* seedlings and leaf and stem explants of field grown plants (grown in the nursery of School of Life Sciences, JNU, New Delhi, India) were kept at 45° C in an oven and dry weight was determined. The dried samples were ground to a fine power with liquid nitrogen. The powdered samples were extracted with methanol after ultra-sonication for 15 min and vigorous shaking at 220 rpm on an orbital shaker for one hour. Supernatant

was collected after centrifuging the plant extract and dried with nitrogen gas. 500µl methanol was added to the residue which was filter sterilized for further study.

Quantification of eugenol and ursolic acid contents by HPLC

Samples were analyzed by HPLC system (Agilent 1200 series) consisting of binary pump, UV detector and the column (Agilent Zorbax 300SB-C18,150 × 4.6 mm). The mobile phase for eugenol was methanol: water: acetonitrile (70:20:10) whereas mobile phase for ursolic acid was acetonitrile: methanol: water (90:5:5). Eugenol and ursolic acid were detected at wavelength of 280 nm and 210 nm respectively at flow rate of 0.2 ml/min. The quantification of eugenol and ursolic acid was done by comparing with the standard curve plotted using different concentrations of eugenol and ursolic acid standards respectively.

Statistical analysis

All the experiments were done thrice, and data are mean values of three samples ± standard deviation (SD). The error bars in the figures are standard deviation.

Results and discussion

Callus induction

Callus induction was reported from the wounded ends of leaf and stem explants of *in vitro* grown seedlings of *O. tenuiflorum* when inoculated onto MS media containing 2, 4-D, NAA and BAP.

The highest callus induction frequency of 71.6 % was reported from leaf explants when cultured on MS medium containing with 2, 4-D (0.25 mg/l) and BAP (0.25 mg/l) (Fig 3b). However, the frequency of callus formation decreased from 71.16 % to 38.88 with increasing concentration of 2, 4-D upto 1.0 mg/l and BAP upto 0.5 mg/l (fig 3c). However, no callus was formed at 2, 4-D (1.0 mg/l) with increased concentration of BAP beyond 0.5 mg/l Furthermore, no callus was formed on leaf explants when the concentration of 2, 4-D was increased upto 1.5 mg/l with any concentration of BAP (fig 3d). Inclusion of 2, 4-D and BAP in different concentrations in MS media resulted in callus induction in stem explants with varying frequency (43.32 and 25.53%). The stem explants exhibited 43.32% of callus induction at low concentration of plant growth regulators, 2, 4-D (0.25 mg/l) and BAP (0.25 mg/l). No callus formation was observed at 2, 4-D (1.0 mg/l) with increased concentration of BAP beyond 0.5 mg/l. However, the suppression of callus formation from stem explants was reported when concentration of 2, 4-D was increased upto 1.5 mg/l with any concentration of BAP which is shown in fig 3. The findings revealed that the combination of 2, 4-D (0.25 mg/l) and BAP (0.25 mg/l) was the best for good callus induction frequency in leaf as well as stem explants as shown in table 1. The hormonal combination of 2, 4-D and BAP was also previously reported to be effective in producing optimum callus induction in garlic (Luciani *et al.*, 2006).

In addition to 2, 4-D and BAP, good frequency of callus induction was also observed in leaf and stem explants of *O.tenuiflorum* when inoculated on MS

Table 1: Effect of different concentrations of plant growth regulators auxins (2, 4-D and NAA) and cytokinin (BAP) for induction of callus in leaf and stem explants of *O. tenuiflorum* L. .

Plant growth regulators (mg/l)			Callus induction (%)	
Auxins		Cytokinin	Explants	
2,4-D	NAA	BAP	Leaf	Stem
0.25	-	0.25	71.6±3.72	43.32±5.77
0.25	-	0.5	69.99±3.33	41.11±1.92
0.25	-	1.0	62.22±1.92	36.66±3.33
0.25	-	1.5	57.77±6.93	33.33±3.33
0.5	-	0.25	56.66±3.33	32.22±1.92
0.5	-	0.5	51.1±6.93	28.88±6.93
0.5	-	1.0	47.7±5.09	27.77±9.62
0.5	-	1.5	46.66±3.35	26.66 ±3.33
1.0	-	0.25	44.44±3.84	26.66±3.35
1.0	-	0.5	38.88±1.92	25.53±5.09
1.0	-	1.0	-	-
1.0	-	1.5	-	-
1.5	-	0.25	-	-
1.5	-	0.5	-	-
1.5	-	1.0	-	-
1.5	-	1.5	-	-
-	0.25	0.25	72.08±5.03	58.86±5.09
-	0.25	0.5	86.64±5.79	68.88±1.92
-	0.25	1.0	77.77±5.09	61.11±1.92
-	0.25	1.5	73.33±3.33	59.99±3.33
-	0.5	0.25	68.9±7.12	55.55±1.92
-	0.5	0.5	63.33±3.33	47.77±6.93
-	0.5	1.0	56.66±5.73	42.21±5.09
-	0.5	1.5	41.46±4.49	38.88±3.85
-	1.0	0.25	31.87±2.25	33.32±5.77
-	1.0	0.5	29.99±3.35	31.1±6.93
-	1.0	1.0	27.72±4.03	28.88±6.93
-	1.0	1.5	26.29±5.14	24.44±3.84
-	1.5	0.25	-	-
-	1.5	0.5	-	-
-	1.5	1.0	-	-
-	1.5	1.5	-	-

The values within a column are mean ± standard deviation.

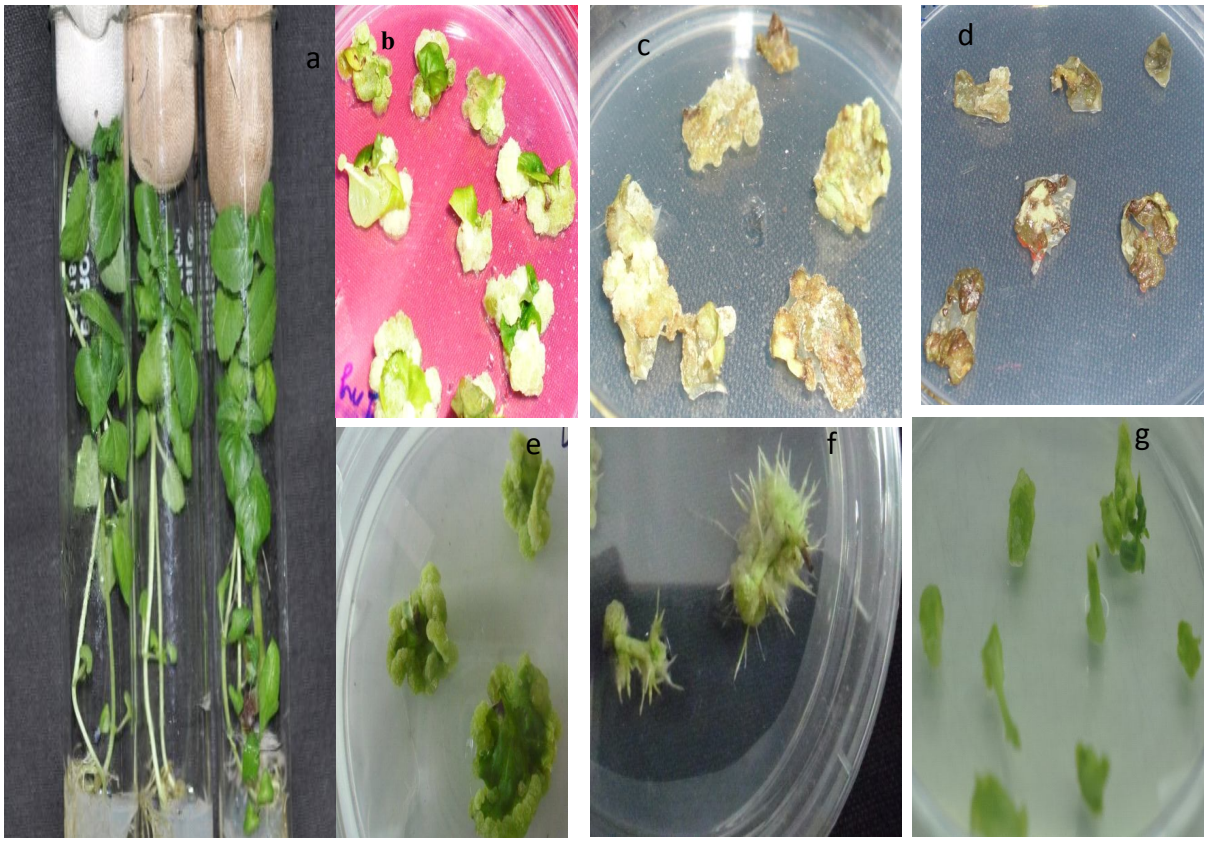

Fig 3*:* **(a)** 30-days- old *in vitro* seedlings of *O. tenuiflorum* L. used for callus induction in presence of different plant growth regulators 2, 4-D, NAA and BAP; calli induction from different explants of *in vitro* seedlings of *O. tenuiflorum* L. **(b)** Leaf explants inoculated onto MS media containing 2, 4-D (0.25 mg/l) and BAP (0.25 mg/l), **(c)** Leaf explants inoculated onto MS media containing (2, 4-D 1mg/l) and BAP (0.25 mg/l), **(d)** Leaf explants inoculated onto MS media containing 2, 4-D (1.0 mg/l) and BAP (1.5 mg/l), **(e)** Leaf explants inoculated onto MS media containing NAA (0.25 mg/l) and BAP (0.5 mg/l), **(f)** stem explants inoculated onto MS media containing NAA (0.25 mg/l) and BAP (0.5 mg/l), **(g)** stem explants inoculated onto MS media containing NAA (1.0 mg/l) and BAP (0.5 mg/l).

medium supplemented with NAA and BAP. The callus induction frequency of leaf explants was reported to vary between 86.66 and 26.29 % in presence of different concentrations of NAA and BAP whereas in stem explants it varied between 68.88 and 24.44 %. In the presence of low concentrations of NAA (0.25 mg/l) and BAP (0.25 mg/l) in MS medium, the callus induction frequency of 72.08% was reported in leaf explants while in stem explants the callus induction frequency was 58.86%. The MS medium supplemented with NAA (0.25 mg/l) and BAP (0.5 mg/l) was the best for callogenesis and showed 86.66% callus response in leaf explants (fig 3e). Increasing the concentrations of NAA upto 1 mg/l as well as BAP upto 1.5 mg/l, resulted in decrease in callus formation. No callus induction was reported at NAA (1.5 mg/l) with any concentration of BAP used. Similar results were also reported by Holme and Petersen (1996) in *Miscanthus ogiformis*. The MS medium containing NAA (0.25 mg/l) and BAP (0.5 mg/l) favoured best callus induction with 68.88 % from stem explants as shown in table 1 and fig 3f. Moreover, increasing the concentration of NAA upto 1 mg/l as well as BAP upto 1.5 mg/l, resulted in decrease in callus formation (fig 3g). The treatment with NAA (1.5 mg/l) tested with any concentration of BAP did not favor callus induction, while NAA (0.25mg/l) and BAP (0.5 mg/l) resulted in the formation of light green friable callus in leaf and stem explants of *O. tenuiflorum* L. There was no sign of callus induction in leaf and stem explants of *O.tenuiflorum L.* in controls (untreated with growth regulators). Leaf explants proved to be the best for callus induction when compared to that of stem explants which is clear from Table 1.

Comparison of level of eugenol and ursolic acid in leaf and stem explants of field grown plants and calli obtained from leaf and stem explants of *in vitro* seedlings of *O. tenuiflorum* L. respectively

It was observed that amongst all the samples, leaves from field grown plants accumulated higher level of eugenol (53.29 µg/g DW) which was 1.74 folds higher

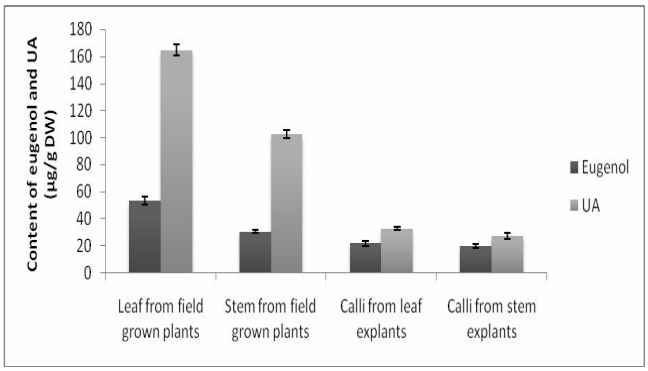

Fig 4: Comparison of content of eugenol and UA from leaf and stem explants of field grown plants and *in vitro* callus cultures derived from leaf and stem explants of *O. tenuiflorum* L. The values are mean ± standard deviation. The error bars in the figures represent standard deviation.

when compared to that of stems of field grown plants (30.53 µg/g DW). However, callus obtained from leaf and stem explants showed lower accumulation of eugenol of 21.79 µg/g DW and 19.95 µg/g DW respectively when compared with field grown plants (fig 4).

Similarly, the content of UA in leaf and stem explants from field grown plants was found to be maximum which was 164.63 µg/g DW in leaves and 102.78 µg/g DW in stem explants (fig 4). The content of UA in leaf explants was 1.60-fold higher than that of stem explants of field grown plants. The content of UA was 32.6 µg/g DW in callus extracts obtained from leaf explants whereas its amount was 27.49 µg/g DW in callus cultures of stem explants. From the data obtained it is clear that leaves and stems of field grown plants have higher amount of eugenol and UA when compared to that of callus cultures. Lim *et al.*, (2009) found total flavonoids content was higher *in vivo* leaf explants of *O. sanctum* when compared to that of leaf-derived callus. The result is also consistent with observations made by Yoshimatsu (2008) and Wilson

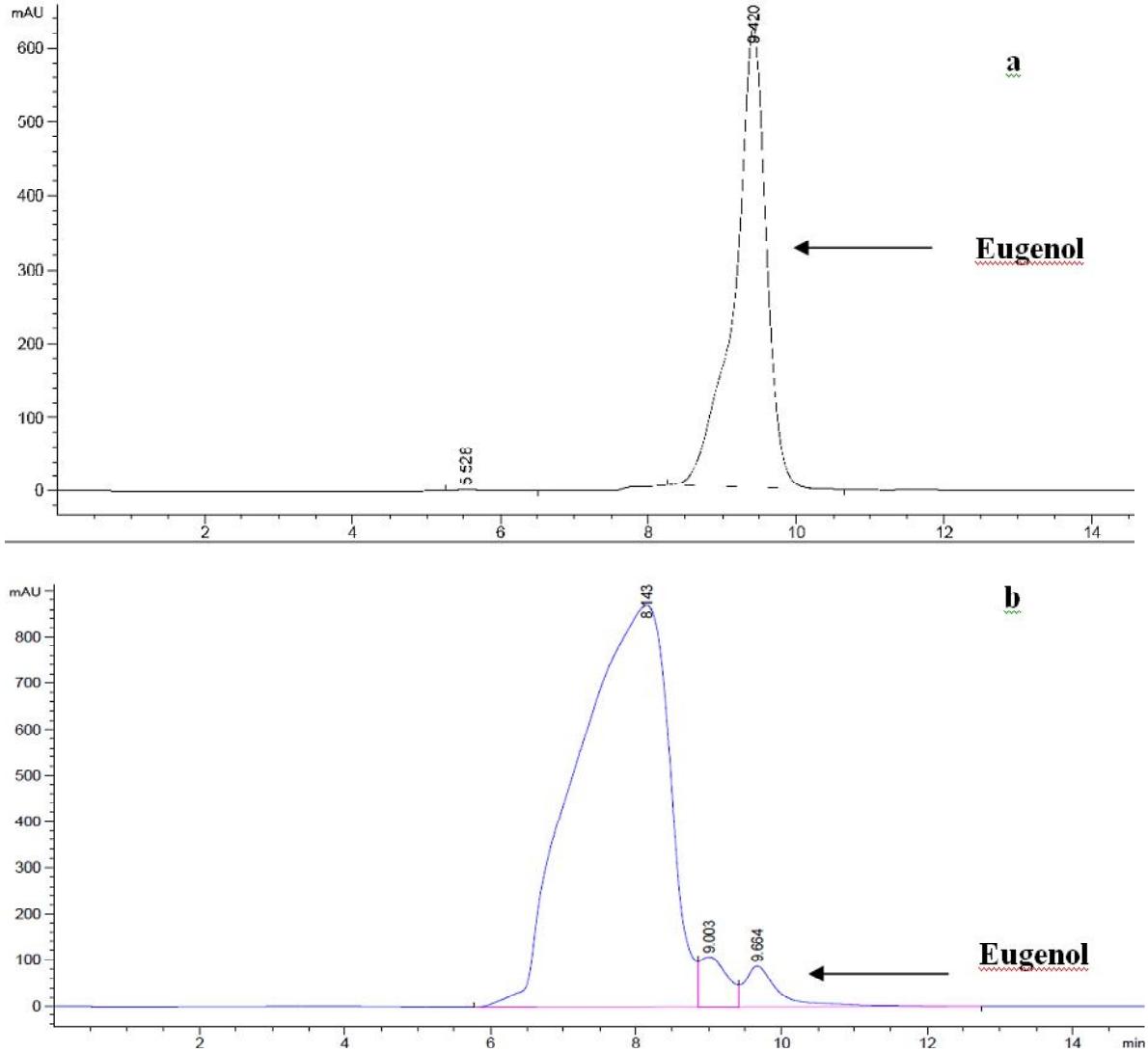

Fig. 5: HPLC profiles of eugenol **(a)** Standard of eugenol **(b)** eugenol content in leaf of field grown plant of *O. tenuiflorum* L.

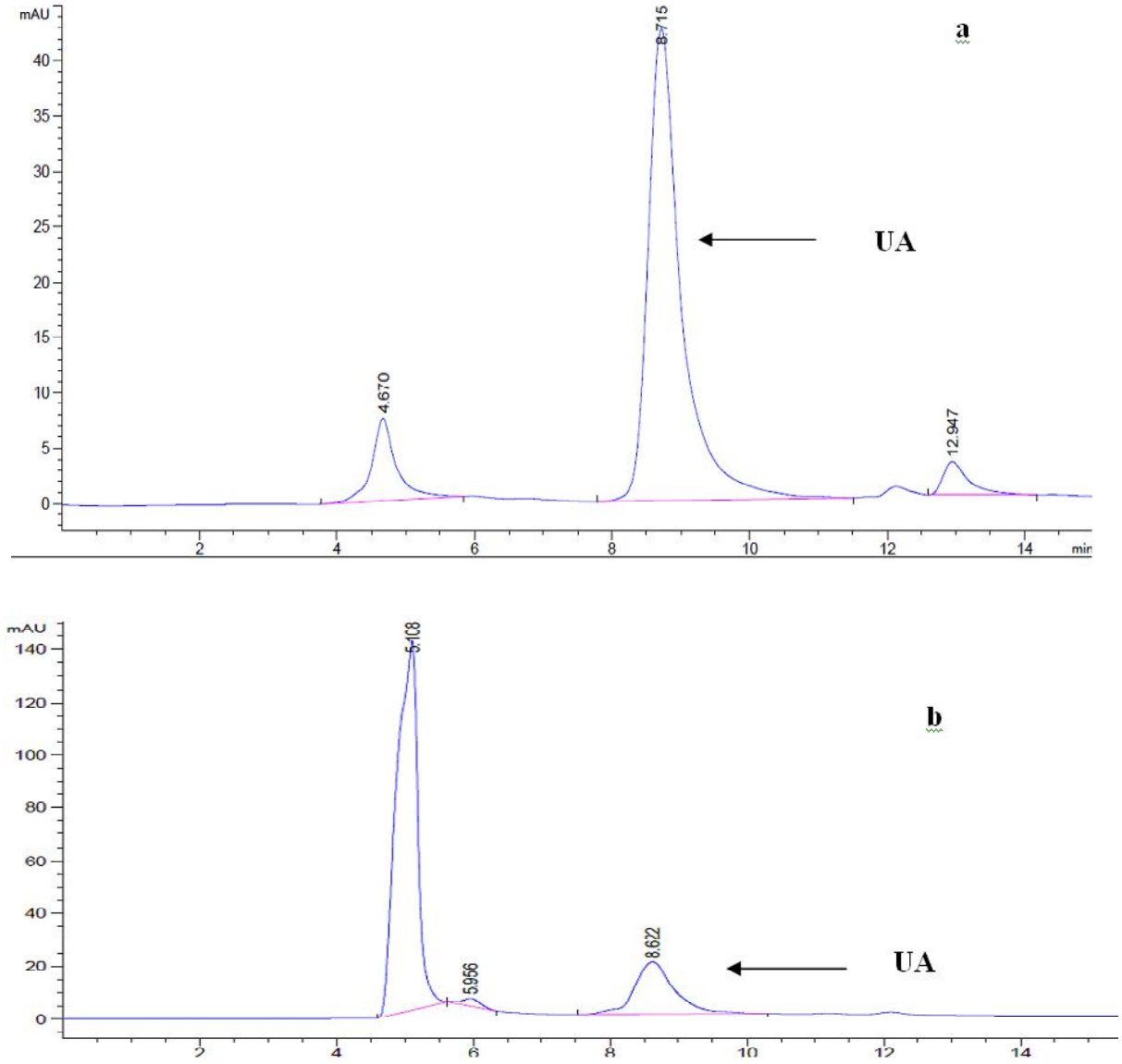

Fig. 6: HPLC profiles of UA production **(a)** Standard of UA, **(b)** UA content in stem of field grown plant of *O. tenuiflorum* L**.**

and Robert (2012) who found lower accumulation of secondary metabolites in dedifferentiated cell cultures *viz.* callus or suspension cultures than differentiated cells *viz*. leaf, shoots or roots. The reason of lower accumulation of active constituents in callus culture when compared to field grown plant could be that the differentiated cells contain enzymes responsible for biosynthesis of secondary metabolites whereas de-differentiated cells lacking differentiation and/or organisation may not produce as much enzymes like the *in vivo* tissues, thereby restricting secondary metabolite biosynthesis (Lim *et al.*, 2009, Yusuf *et al.*, 2013; Abou Zid 2014; Ng *et al.*, 2016). However, Ng *et al.*, (2016) reported that low accumulation of flavonoid was due to lack of activity of the flavonoid biosynthetic pathways in callus cultures of finger root ginger. In this investigation the leaf and stem explants of field grown plants of *O. tenuiflorum* L. were found to contain higher amount of UA followed by eugenol. The HPLC chromatograms of eugenol and UA of standard and plant extracts with retention time of 9.3 and 8.6 min respectively are shown in fig 5 and 6 respectively.

Conclusions

Although good callus was obtained by including NAA (0.25 mg/l) and BAP (0.5 mg/l) in MS medium, high amounts of secondary metabolites was not obtained from these cultures when compared with leaf explants and stem explants of field grown plants of *O. tenuiflorum* plants. The variation in activation of metabolic pathway of secondary metabolite biosynthesis might be responsible for the production of secondary metabolite in different

types of cell cultures. Understanding the biosynthesis mechanism at molecular level and applying newly developed treatment strategies can help in achieving enhanced production of secondary metabolites in cell cultures. As callus can be a continuous source of producing secondary metabolites irrespective of seasonal changes, taken in totality, it may yield an accumulated amount of secondary metabolites in a sustained manner.

Acknowledgements

The authors thank University Grants Commission (UGC), New Delhi through UPE/II & UGC Networking and Department of Science and Technology (Government of India) through DST-FIST & DST PURSE for partial financial support and facilities. Thanks to Dr. Adetunji Charles Oluwaseun (Landmark University, Nigeria) for his suggestions on the manuscript.

Conflict of interest: Authors declared that they have no conflict of interest.

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