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Enhanced anthraquinone production in presence of silver nitrate in suspension culture of *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae), a traditional medicinal plant mentioned in Hortus Malabaricus

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Abstract

Silver nitrate ($AgNO_3$) enhanced production of anthraquinone was standardized in cell suspension cultures of *Gynochthodes umbellata*, a plant mentioned in the Hortus Malabaricus. The present research investigates the effect of silver nitrate, an abiotic elicitor on production of anthraquinone in *in vitro* cell suspension cultures of *G. umbellata*. Friable callus culture was established using *in vitro* derived leaf segment obtained from the nodal explant culture maintained in Murashige and Skoog (MS) medium containing 2 mg/l benzyl amino purine (BAP) and 3% sucrose. The *in vitro* derived leaf segments ($0.5cm^2$) were cultured on MS medium containing 1 mg/l 2,4-D and 2% glucose for the production of friable callus. After 30 days of culture, uniform yellow friable callus was inoculated into MS liquid medium containing 1 mg/l 2,4-D and 2 % glucose for raising suspension culture. Uniform cell suspension was transferred to same media constituents and treated with different concentrations of $AgNO_3$ on 25^{th} day of culture. Fresh weight, dry weight and accumulation of anthraquinone content was studied and found that $AgNO_3$ caused a marginal increase in biomass and anthraquinone based on the concentration and duration of $AgNO_3$ treatment. A maximum fresh weight (19.48 g/fwt) dry weight (1.92g/dwt) and highest amount of anthraquinone content (48.62 mg/gdwt) were recorded in MS medium supplemented with 1 mg/l 2,4-D, 2%glucose and 3.5µM $AgNO_3$ after 72 hrs of incubation.

Keywords: *Gynochthodes umbellata*, *S*ilver nitrate, Suspension culture, Anthraquinone, Elicitor

1. Introduction

Plants are important source of secondary compounds and produce more number of secondary metabolites than animals. The secondary products have prime importance for plant survival as well as defense mechanism of both plants and animals (Wink, 2003). It also imparts protection against pests and pathogen attack. Secondary metabolites are economically important and the reason for plants having particular taste, odour and colour are due to the presence of certain compounds. Due to the medicinal properties of these compounds, secondary metabolites have been used in traditional medicine. Anthraquinones are important group of secondary metabolites found in bacteria, fungi, lichens and higher plants. Anthraquinones are used as antibacterial, antiviral, antifungal, anthelminthic, anti-inflammatory, antitumour and immune enhancing agents (Biswas *et.al*., 2017). Anthraquinones have industrial and medicinal applications and are widely used as colorants in drugs, food, hair dye, cosmetics and textile industry (Mori *et.al*., 1990; Butterworth *et.al*., 2001). In paper and pulp industry, it is used as a catalyst for

the treatment of wood pulp (Nelson and Cietek, 1983). Anthraquinones are the second largest group of natural dyes used in textile industry for colouring textiles (Morshed and Rahman, 2015). The emodin and alizarin types of anthrquinone are generally distributed among plants. Among the flowering plant families, plants belonging to Rubiaceae family are the main source of anthraquinone derivatives like purpurin, alizarin, manjistinetc and these compounds are mostly accumulated in roots. *Gynochthodes umbellata* (Syn. *Morinda umbellata*) belonging to the family Rubiaceae is a woody climber used in the traditional system of medicine for treating dysentery and diarrhea (Vijayaraghavan, 2011). This plant was mentioned by Hendrick van Rheede, the Dutch commander of Malabar in his monumental work Hortus Malabaricus, a compendium of the economic and medicinal values in the Malabar region during the 17th century (Van Rheede, 1688;Vijayaraghavan, 2011).

Secondary metabolites are commonly extracted from naturally growing plants. Plant cell and tissue culture are employed as an alternative method for the production of secondary metabolites (Hussain *et.al*., 2012). Plant cell cultures are recognized as a promising alternative source for the production of these high value compounds of economic importance independently of seasonal variation over the past decades (Bizarri *et.al*., 2009). There are a few reports on *in vitro* and callus cultures of *G. umbellata* for the tissue culture production of anthraquinones (Anjusha and Gangaprasad, 2016;Anjusha and Gangaprasad, 2017). Recently, several studies have been carried out for the enhanced secondary metabolite production in cell cultures through optimization of culture conditions, high yielding cell lines and precursor addition (Rhee *et.al*., 2010). One of the important methods for the enhanced production of secondary metabolites production in culture system is the addition of elicitors (Murthy, 2014) and received wide acceptance because of the enhanced synthesis of secondary metabolites (Smestanska, 2008). There are a number of reports on the enhanced production of secondary metabolites in various culture system using elicitors (Zafer *et. al*., 2015). Inorganic chemicals such as calcium chloride, silver nitrate, mercuric chloride, aluminum chloride etc., have been widely used as elicitors in a variety of medicinal plants to enhance the production of secondary metabolites by altering the secondary metabolism (Verpoorte *et.al*., 2002).

In the present study, the influence of $AgNO_3$, an abiotic elicitor, on the yield of anthraquinone was evaluated in suspension culture of *G. umbellata*. Friable yellow callus was successfully established using *in vitro* derived leaf explants from the nodal cultures of *G. umbellata*. In order to enhance the production of anthraquinone in *G. umbellata*, callus derived suspension cultures were treated with different concentrations of $AgNO_3$ and its effect on the anthraquinone production.

2. Materials and methods

Young shoot cuttings of *G. umbellata* were excised from field grown plants growing in the University of Kerala, Kariavattom Campus, Kerala. The plant was identified with the help of authentic literature and a voucher specimen was deposited in the herbarium of Department of Botany, University of Kerala, Kariavattom (KUBH 7000). After defoliation, the 4th node from the shoot tip was selected for the initiation of *in vitro* culture. The nodal explants was washed and surface sterilized and inoculated into Murashige and Skoog medium (Murashige and Skoog, 1962) containing

2 mg/l BAP and 3%sucrose and 0.8% agar (Fig.1) as per the protocol developed in our laboratory (Anjusha and Gangaprasad, 2016). *In vitro* leaf explants derived from multiple shoots (Figs. 2 & 3) from the nodal culture was used as the explants source for raising callus induction. Leaf segment ($0.5cm^2$) excised from *in vitro* culture after 30 days of culture and were inoculated into MS medium containing 1 mg/l 2, 4-D and 2% glucose. This is the standardized medium for the development of friable yellow callus (Anjusha, 2016). The callus tissues were transferred to MS medium containing the same media constituents at a regular interval of 30 days for 3-4 subcultures to establish callus cultures and were used for raising cell suspension culture. The cultures were maintained in a culture room at 25±2°C under 12 hrs photoperiod at 50 $\mu molm^{-2}$ s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India Ltd.) and 60-65% relative humidity.

2.1 Establishment of cell suspension culture

About 1 gm fresh weight of yellow friable callus tissue were inoculated into 50 ml of MS liquid medium containing 2% glucose, 1 mg/l 2,4-Din 250 ml Erlenmeyer conical flask. All the cultures were kept under 12 hr. photoperiod using cool white florescent tube light of 40 W (Philips India Ltd.) and 60-65 % relative humidity. The cell suspensions were kept in a gyratory shaker under continuous agitation (90 rpm). After 30 days of culture, these were subcultured into fresh medium with same hormonal concentrations. After the second subculture, uniform cell suspension cultures were obtained. These cell suspension cultures were used for further experiments. Cell suspensions (20ml) measuring approximately 2gm fresh weight (fwt) of the cells were transferred into fresh medium containing same media constituents. Growth of the cells in cell suspension cultures for a period of 28 days was studied starting from 25th day. On 26th, 27th and 28th days, cells were harvested and washed in sterile distilled water and blotted in filter paper and determined the fresh weight (fwt) and dry weight (dwt). For determining the dwt of the cells, the cells were kept in a hot air oven at 60°C for 24 hrs and the anthraquinone content was determined.

2.2 Effect of abiotic elicitor silver nitrate on cell suspension culture

$AgNO_3$ was dissolved in double distilled sterile water and is filter sterilized before use. Silver nitrate, different concentrations *viz*. 2, 3.5, 5 and 7.5µm were used. The above mentioned final concentrations of silver nitrate were added separately to the cell suspension on 25th day of culture period. In the control, MS medium containing 1 mg/l 2,4-D and 2% glucose were used as control and on 25th day equal volume of sterile distilled water was added. For each treatment, 3 replicates were kept and were repeated thrice.

2.3 Determination of fwt / dwt and anthraquinone production

The fresh weight and dry weight and anthraquinone content was estimated in cultures treated with $AgNO_3$ and in the control cultures on 26th, 27th and 28th day of inoculation. Quantification of anthraquinone was determined by the method of Hagendrone *et.al*., (1994). For intracellular anthraquinone determination, 100mg dried cells were taken and extracted with 80% 20ml ethanol for 45 minutes in a boiling water bath at 60°C. This was repeated until the yellow colour of the dried cells disappeared. The ethanolic extraction was then subjected to centrifugation at 1500rpm

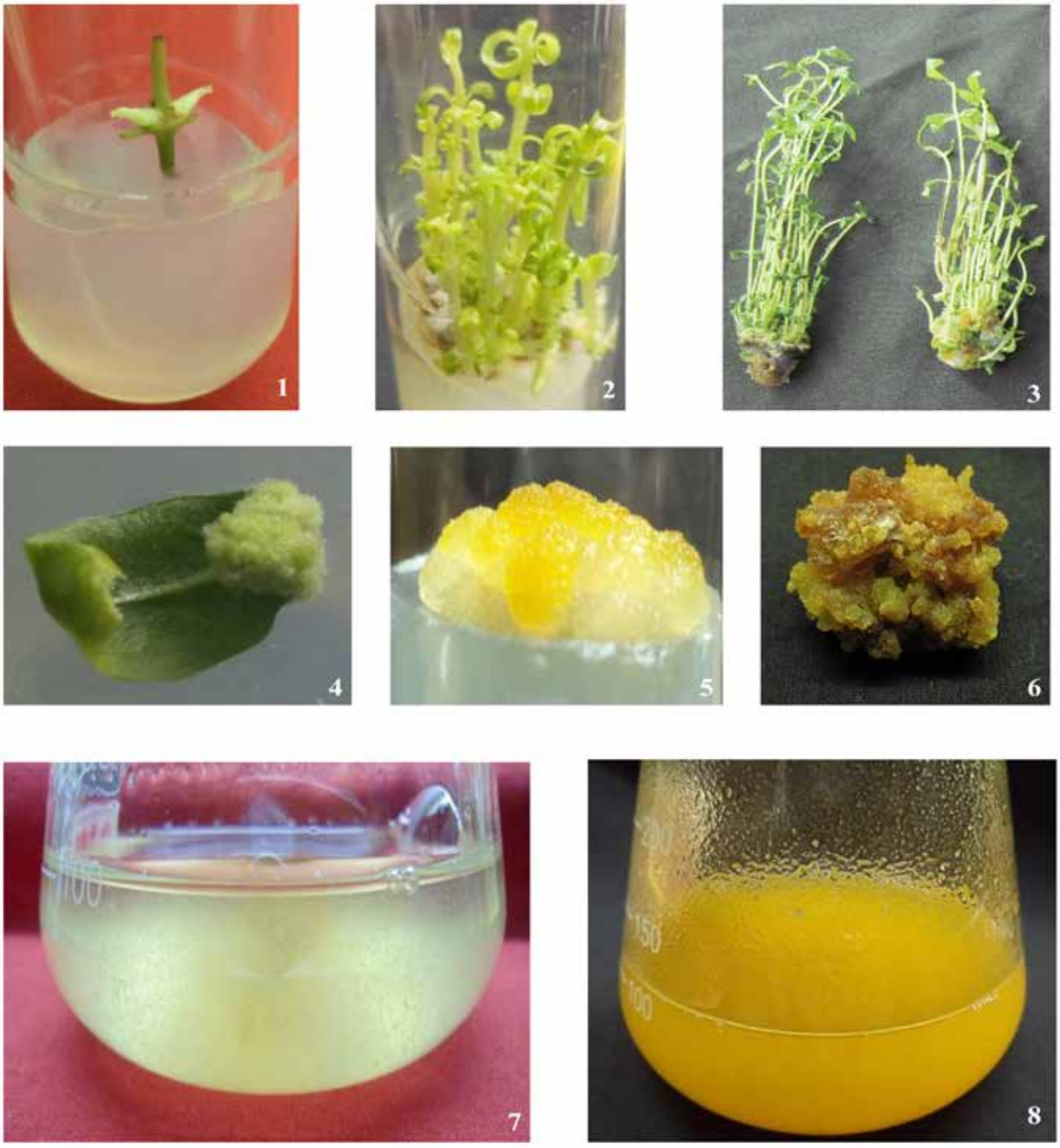

Fig. 1. Initiation of shoot from the nodal explants of *G. umbellata* in MS medium containing 2mg/l BAP and 3% sucrose. **Fig. 2. & 3**. Multiple shoot formation from nodal explants in 2mg/l BAP and 3% sucrose. **Fig. 4.** Callus initiation in presence of 1mg/l 2, 4-D and 2% glucose. **Fig. 5 & 6.** Yellow friable callus in presence of 1mg/l 2, 4-D and 2% glucose after 30 and 40 days of culture. **Fig. 7.** Initiation of suspension culture in presence of 1mg/l 2, 4-D and 2% glucose. **Fig.8.** 25 day old suspension culture in presence of 1mg/l 2, 4-D and 2% glucose.

for 10 minutes. Anthraquinone content was estimated using the graph of alizarin (Sigma Aldrich, US) by calculating the absorption of the ethanolic extracts were determined at 434 nm on a UV - visible spectrophotometer (Shimadzu, Japan).

2.4 Statistical analysis

All the experiments were repeated thrice and each data point reported is the mean of three replicate measurements. The data was subjected to statistical analysis by using analysis of variance (ANOVA). It was performed using SPSS software version 1990 (SPSS. Inc. Chicago, US).

3. Results

3.1 Callus induction and proliferation

The *in vitro* derived leaf explants were cultured on MS medium augmented with 1 mg/l 2, 4-D and 2 % glucose in solid medium produced yellow friable callus (Figs. 4, 5 and 6). From these friable callus, cell suspension was raised in liquid MS medium containing 1 mg/l 2, 4-D and 2% glucose in MS medium (Figs. 7 and 8). $AgNO_3$ was added to the cultures on 25th day of culture and determined the fwt, dwt and anthraquinone content after 24, 48 and 72 hrs of incubation. Elicitation with $AgNO_3$ in medium resulted in improved biomass in terms of fwt, dwt and anthraquinone accumulation compared to the control in the cell suspension culture of *G. umbellata* (Table 1). Out of the different concentrations of $AgNO_3$ tested, the optimum concentration of $AgNO_3$ was 3.5µm. 3.5 µm concentration of $AgNO_3$ on the 48 and 72 hrs of incubation produced a marginal increase in biomass (16.66 g/fwt and 19.48 g/fwt) and anthraquinone content (46.16 mg/g dwt and 48.62 mg/g dwt) respectively.When compared to the control, a marginal increase of anthraquinone production and biomass were recorded with $AgNO_3$ elicitation. A change in anthraquinone and biomass production in presence of $AgNO_3$ was on their concentration and the incubation period. In all the concentration tried, the biomass and anthraquinone content were marginally increased (Table 1).

Table 1. Effect of different concentrations of $AgNO_3$ elicitation and incubation period on biomass and anthraquinone (AQ) content in cell suspension culture of *G. umbellata* in MS liquid medium containing 1mg/l 2,4-D and 2% glucose

$AgNO_3$ (Conc. µM)	Biomass Fwt (g/flask)			Biomass Dwt (g/flask)			AQ content mg/g dw		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
0.0	10.37±0.23^{e}	12.36±0.21^{d}	13.54±0.31^{d}	0.72±0.33^{d}	0.85±0.39^{c}	1.02±0.10^{d}	38.33±0.22^{d}	38.79±0.20^{d}	39.57±0.31^{e}
2.0	13.43±0.10^{b}	14.56±0.32^{b}	15.87±0.24^{b}	1.03±0.60^{b}	1.16±0.62^{b}	1.72±0.22^{b}	44.04±0.34^{b}	45.45±0.21^{a}	46.69±0.21^{b}
3.5	13.72±0.20^{a}	16.66±0.22^{a}	19.48±0.22^{a}	1.34±0.67^{a}	1.44±0.34^{a}	1.92±0.20^{a}	45.22±0.04^{a}	46.16±0.43^{a}	48.62±0.20^{a}
5.0	12.83±0.10^{c}	14.45±0.31^{b}	15.77±0.20^{b}	1.43±0.33^{b}	1.32±0.20^{a}	1.43±0.20^{c}	44.32±0.30^{b}	43.11±0.22^{b}	44.02±0.58^{c}
7.5	11.91±0.20^{d}	14.04±0.20^{c}	15.33±.31^{c}	1.11±0.00^{c}	1.10±0.20^{b}	1.45±0.21^{c}	42.44±0.30^{c}	41.32±0.20^{c}	42.91±0.61^{d}
	265.2***	242.3***	908.3***	45.5***	12.6***	29.8***	97.7***	108.9***	683.7***

*** Significant at p <0.001; Means within a column followed by different letters are significantly (p<0.05) different as determined by DMRT

4. Discussion

Abiotic and biotic elicitors, the signalizing compounds trigger and activate the genes responsible for the defence system of plants which leads to the enhanced production of secondary metabolites and elicitation is used as an *in vitro* method for the enhanced production of secondary compound in cell suspension cultures (Zafar *et*. *al* ., 2017). The present study is aimed to explore the possibility of the enhanced production of biomass and anthraquinone accumulation in *G. umbellata*, a natural dye yielding plant. The experiment started with the induction of yellow friable callus from *in vitro* derived leaf explants. In *G. umbellata*, *in vitro* derived leaf is the best explants for the production of yellow friable callus (Anjusha and Gangaprasad, 2016). In this study, $AgNO_3$ was used at varying concentrations, of which 3.5 µm was found to be the best concentration for the highest accumulation of anthraquinone in cell suspension culture of *G. umbellata*. Elicitation studies in cell suspension culture using abiotic and biotic elicitors were successfully reported in cell suspension cultures earlier (Veerashree*et.al*., 2012; Kubes *et.al*., 2014).

The amendment of elicitor into the nutrient medium and the correct stage of the culture for elicitation are considered as an important factor in the production of secondary metabolites (Namdeo, 2007). But it may differ between various cell culture systems. $AgNO_3$ elicitation, during the late phase of the exponential phase as in the case of *G. umbellata* is effective for the enhanced production of biomass and anthraquinone content. Elicitation with $AgNO_3$ marginally increased the anthraquinone accumulation, fwt and dwt of the cell suspension culture of *G. umbellata*. Of the different concentration of $AgNO_3$ tested, 3.5 µm was the optimum concentration for the enhanced production of anthraquinone. Heavy metal induced secondary metabolite production has been reported in plant tissue culture by earlier workers (Kim *et.al*., 1991., Zhao*et*. *al*., 2010., De Debjani and De Bratati, 2011). $AgNO_3$ elicitation has been used for the increased production of secondary compounds in cell cultures of *Genista tinctoria* (Kubes *et.al*., 2014), tropane alkaloid production in *Brugmansia canlida* (Pitta- Alvarez *et.al*., 2000) and flavonolignan production in cell suspension culture of *Silybum marianum* (Vildova *et.al*., 2014). The biomass production and accumulation of anthraquinone induced by elicitation of $AgNO_3$ was dependent on the concentration of $AgNO_3$ and period of incubation. Thus, the results obtained in the present experimentation indicates, $AgNO_3$ elicitation could be a promising approach for the enhanced production of anthraquinone in a short period of 28 days. The present investigation could be utilized for large scale production of this pharmaceutically and industrially important natural compound without disturbing the natural population.

5. Conclusion

The study has proven that silver nitrate is a promising abiotic elicitor for the enhanced production of anthraquinone in cell suspension culture of *Gynochthodes umbellata*, a natural dye yielding species mentioned in the famous book Van Rheed's *Hortus Malabaricus*. Silver nitrate in lower concentrations is more effective for the highest production of anthraquinone in *G. umbellata*.

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Reference

1. Anjusha S 2016. Pharmacognostic evaluation and *in vitro* culture of *Gynochthodes umbellata* (L.) Razafim. & B. Bremer for propagation and exploitation of active compounds. Ph D thesis, University of Kerala.
2. Anjusha S and Gangaprasad A 2016. *In vitro* propagation and anthraquinone quantification in *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae)—a dye yielding plant. Ind. Crops Prod. 81:83–90.
3. Anjusha S and Gangaprasad A 2017. Callus culture and *in vitro* production of anthraquinone in *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae). Ind. Crops Prod. 95: 608–614.
4. Biswas I, Seth P and Laskar S 2017. Exploration of therapeutic potential of anthraquinones and flavonoids isolated from different species of *Cassia:* a promising journey from ethnomedicine to biomedicine. Indian J. Sci. Res. 13:01-08.
5. Bizarri SC, CH Da Silva Cordeiro L de Castro TC, Coutada LC Da Silva AJ Albarello, N and Mansur E 2009. Anthocyanin production in callus cultures of *Cleome rosea*: modulation by cultures conditions and characterization of pigments by means of HPLC-DAD/ESIMS. Plant Physiol. Biochem. 47 (10):895–903.
6. Butterworth B E,Mathre O B and Ballinger K 2001. The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene. Mutagenesis. 16 (2):169–177.
7. De Debjani and De Bratati 2011. Elicitation of diosgenin production in *Trigonella foenumgraecum* L. seedlings by heavy metals and signaling molecules. Acta Physiologiae Plantarum. 33:1585-1590.
8. Hagendoorn M J, van der Plas L H and Segers G J 1994. Accumulation of anthraquinones in *Morinda citrifolia* cell suspensions. Plant Cell Tiss. Org. Cult. 38 (2–3):227–234.
9. Hussain M S, Fareed S, Ansari S, Rahman M A, Ahmad I Z and Saeed M 2012. Current approaches towards production of secondary plant metabolites. J Pharm. Bioallied Sci. 4:10–20.
10. Kim D, Pederson Hand Chin C 1991. Stimulation of berberine production of *Thalictrum rugosum* suspension cultures in response to addition of cupric sulfate. Biotech Lett. 13:213-216.
11. Kubes J, Tumova L, Martin J, Vildova A, Hendrychova H and Sojkova K 2014. The producton of isoflavonoids in *Genista tinctoria* L. cell suspension culture after abiotic stressors treatment. Nat Prod Res. 28: 253-2263.
12. Mori H, Yoshimi N, Iwata H, Mori Y, Hara A, Tanaka T and Kawai K 1990. Carcinogenicity of naturally occurring 1-hydroxyl anthraquinone in rats: induction of large bowel, liver and stomach neoplasms. Carcinogenesis. 11:799–802.
13. Morshed AMA and Rahman MM 2015. Areview on the physic-chemical studies of dyeing progress and dyeing kinetics using natural dyes. IOSR JPolyand TexEngi. 2: 74-78.
14. Murthy H N, Lee E J and Paek K Y 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches

for biomass improvement and metabolite accumulation. Plant Cell Tiss Org Cult 118:1–16.

15. Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 (3) : 473–497.
16. Namdeo A G 2007. Plant cell elicitation for production of secondary metabolites: a review. Pharmacog. Rev. 1:69-79.
17. Nelson K H and Cietek D J 1983. Determination of anthraquinone in pulping liquors by using C-18 cartridges and high-performance liquid-chromotography. J. Chromatography. 281:237–244.
18. Pitta-Alvarez S I, Spolllansky T C, Giulietti A M 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Enzyme Microb Technol. 26: 252-258.
19. Rhee H S, Cho H Y, Son S Y, Yoon S Y H and Park J M 2010. Enhanced accumulation of decursin and decursinol angelate in root cultures and intact roots of *Angelica gigas* Nakai following elicitation. Plant Cell Tiss. Org. Cult. 101: 295–302.
20. Smetanska I 2008. Production of secondary metabolites using plant cell cultures. Adv. Biochem. Eng. Biotechnol. 111:187–228.
21. Van Rheede 1688. Hortus Malabaricus, vol. 7. pp. 51–52.
22. Verpoorte R, Contin A and Memelink J 2002. Biotechnology for the production of plant secondary metabolites. Phytochem. Rev. 1:13–25.
23. Veerashree V, Anuradha C M and Kumar V 2012. Elicitor enhanced production of gymnemic acid in cell suspension cultures of *Gymnema sylvestre* R. R Br. Plant Cell Tiss. Org. Cult. 108:27-35.
24. Vijayaraghavan G 2011. Comprehensive medicinal plants. Plant Monographs Alphabetically, vol. 4. Studium Press Llc, Texas, USA.
25. Vildová A, Hendrychová H, Kubes J andTumova L 2014. Influence of $AgNO_3$ treatment on the flavonolignan production in cell suspension culture of *Silybum marianum* (L.) Gaertn, Int. J. Biological, Biomolecular, Agricultural, Food and Biotec. Engineering. 8: 959-962.
26. Wink M 2003 Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry. 64 (1):3-19.
27. Zafer N, Mujib A, Ali M, Tonk D and Gulzar B 2017. Aluminium chloride elicitation (amendment) improves callus biomass growth and reserpine yield in *Rauvolfia serpentina* leaf callus. Plant Cell Tiss. Org. Cult. 357-368.
28. Zhao J L, Zhou L G and Wu J Y 2010. Effect of biotic and abiotic elicitators on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. Appl. Micro. and Biotech. 87: 137-144.