IJPSR (2016), Vol. 7, Issue 9 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 April, 2016; received in revised form, 24 May, 2016; accepted, 13 June, 2016; published 01 September, 2016

ELICITOR AND PRECURSOR MEDIATED ANTHRAQUINONE PRODUCTION FROM CELL SUSPENSION CULTURES OF *OLDENLANDIA UMBELLATA* L.

S. R. S. Krishnan* and E. A. Siril

Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, 695581, Kerala, India

Keywords:

Anthraquinones, cell suspension cultures, elicitors, HPLC analysis, *Oldenlandia umbellata*, precursors

Correspondence to Author:
S. R. S. Krishnan

Department of Botany,
University of Kerala, Kariavattom,
Thiruvananthapuram, 695 581,
Kerala, India.

E-mail: saranyakrishnansr@gmail.com

ABSTRACT: Enhanced production of anthraquinones from cell suspension cultures of *Oldenlandia umbellata* were established through elicitor or precursor treatment. Stock cell suspension cultures were developed in liquid MS medium supplemented with 2.5 µM NAA using friable calli. Standardization experiments of suspension cultures revealed that 10 µM NAA added liquid MS medium was optimum for the growth and AQ accumulation from *O. umbellata* suspension cultures. 10 µM NAA produced 9.96 mg g^{-1}dw AQ on 30th day of incubation. Later enhanced AQ production was achieved by the addition of elicitors or precursors into the standardized media on 25th day of incubation and AQ quantification was done after 5 days of incubation. Addition of elicitors or precursors resulted in a sudden increase in AQ content. 25 mg L^{-1} pectinadded suspension cultures produced 35.67 mg g^{-1}dw AQ and precursor feeding with 50 mg L^{-1}α-keto glutaric acid resulted in the accumulation of 42.63 mg g^{-1}dw AQ. HPLC analysis of elicitor or precursor mediated suspension cultures revealed the presence of alizarin and purpurin in samples. The overall AQ production by the addition of elicitors or precursors showed an increment when compared to that of control (9.44 mg g^{-1}dw AQ).

INTRODUCTION: Anthraquinones are a group of secondary metabolites comes under the quinones category. They are widely used in industrial and medicinal fields for various purposes. Industrially anthraquinones are used in the paper industry [1, 2], cosmetics and food processing industry [3]. Apart from the above uses, AQs are good dye stuffs and used in dying cloths since ancient times. AQs are the second largest groups of natural dyes used in textile industries [4].

Therapeutic application of AQ is well known. AQs are used as antibacterial, antiviral, anti-fungal, anthelminthic, analgesic, hypotensive, anti-inflammatory, anti-genotoxic, antitumor and immune enhancing agents [5, 6, 7].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(9).3649-57

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (9).3649-57

AQ constituents are clinically important molecules especially rubiadin, damnacanthal, alizarin, and purpurin were used in the formulation of chemotherapeutical drugs. They were also used treatment various ailments to kidney and bladder stones, as a laxative mixture and as a mild sedative[8, 9]. Anthraquinone compounds, with great medicinal value, also have widespread use in different industries. Therefore, the anthraquinone industry is of great promise. China is the largest producers and exporters of AQs in industrial scale[10].

Oldenlandia umbellata L., is a potent AQ source from the family rubiaceae. The plant is valued in various traditional Indian (Ayurveda and Siddha) and Chinese systems of medicine due to various pharmacological properties like styptic, expectorant and chalagogue action [11, 12]. Fresh extract of the whole plant is used to cure bleeding conditions like menorrhagia. The decoction prepared from its roots and leaves are used externally as a wash for toxic bites and ulcers [13, 14]. Decoction of roots

administered to relive cough, bronchial asthma and bronchitis [15]. Gupta et al.,[14] reported the hepato-protective and anti-oxidant activity of methanolic extracts of *O. umbellata* in carbon tetrachloride induced hepatotoxicity in rats. Ethnic community in China used *Oldenlandia* extract as first aid to snake bites, particularly for pit vipers [15]. *Oldenlandia* has also been employed to treat sores and carbuncles on the skin, appendicitis, sore throats, urinary tract infections and hepatitis. Methanolic extracts of the plant has anti-bacterial activity against gram positive and gram negative bacteria which cause respiratory tract infection [16].

Root paste of *O. umbellata* is taken orally for worm infections [17]. The root powder has been subjected to clinical trials and it has been proved to be an efficacious remedy for blood, particularly in the condition of tuberculosis [18]. Ethno-pharmaceutical value of *Oldenlandia umbellata* is very high among several tribal groups in India [11]. Tribal groups like 'Valaiyans' of Tamil Nadu, 'Malasar' tribes of Coimbatore, Tamil Nadu, tribal and non-tribal inhabitants of Andhra Pradesh and traditional healers in Kancheepuram make use of this plant in the treatments of various ailments [18, 19, 20].

Many workers studied the chemical composition and *in vitro* AQ production from *O. umbellata*. GC-MS analysis of ethanolic extract of roots revealed the presence of alizarin, ruberithryc acid, and ursolic acid [21]. HPLC analysis of ethanol-water extract of *O. umbellata* contain AQ derivatives like 1, 2, 3-trimethoxy anthraquinone, 1, 3-dimethoxy-2-hydroxy anthraquinone, 1, 2-dimethoxy anthraquinone, 1-methoxy - 2 - hydroxy anthraquinone and 1, 2-dihydroxy anthraquinone [22]. Mahibalan et al., [23] recently identified a new compound oledicoumarin and 11 new compounds having strong cytotoxic activity against several types of cancers like colon cancer HT-29, lung epithelial A549 and breast adenocarcinoma MDA-MB-231 cell lines showed HUM-E to be significantly cytotoxic with lower IC_{50} values.

The present study focus on the *in vitro* production of AQs from cell suspension cultures of *O. umbellata* by the usage of elicitors or precursors.

MATERIALS AND METHODS:

Development of stock suspension culture:

Friable callus produced in MS medium fortified with 2.5 µM NAA derived from internodal explants was used for the initiation of cell suspension cultures. Approximately 200 mg friable piece of callus was transferred to 100mL Erlenmeyer flask containing 30mL MS liquid medium. The stock suspension culture was developed in liquid MS medium supplemented with 2.5 µM NAA. When friable callus were transferred to liquid medium, the cells from callus mass started to get dispersed into the medium. To get a fine stock suspension culture, unbroken callus material and large clumps from the suspension were removed through filtration using a pre-autoclaved, cell dissociation sieve (mesh diameter 100 mm and opening size 140 mm, Sigma, St. Louis, US).

The suspension cultures were then incubated on a gyratory shaker (Orbital shaking incubator, RIS 24-BL, REMI, India) at 100 rpm at 25±2^{o}C under 16 h photoperiod at 50 µmol $m^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) and 60-65% relative humidity. The suspension raised in liquid medium was repeatedly sub-cultured for six times to fresh medium in every two weeks interval. Approximately, 200mg fresh weight of cell suspension was inoculated to fresh medium on every 15 day interval and thus developed stock suspension culture. The developed stock culture was then used for following experiments;

Influence of different auxins on suspension cultures:

Influence of MS liquid medium (3% sucrose, pH 5.8) supplemented with different types of auxins (IBA, NAA, IAA or 2, 4-D) at varying concentrations (1.0, 2.5, 5.0, or 10.0 µM) on growth and AQ production from suspension cultures were tested. An approximate weight of 200 mg cells from stock suspension culture was transferred to new media with different auxin concentrations. Cells inoculated in growth regulator free MS medium served as control. Parameters like packed cell volume (PCV), fresh weight, dry weight, AQ content and growth index were recorded for 60 days at 10 days interval. After the standardization of optimum hormonal

concentration, the cell suspension cultures were subjected to elicitor or precursor treatment.

Preparation of elicitors or precursors:

Elicitors *viz.*, pectin (PE) (Sd Fine-chem., Mumbai, India), yeast extract (YE) and xylan (XY) (SRL, Mumbai, India) and precursors *viz.* ,α-keto glutaric acid (KGA) and phenyl alanine (PAL) (SRL, Mumbai, India) and an inhibitor piroxicam (PM) (Sigma-Aldrich, St. Louis, US) were used for the experiment. The elicitors and precursors were dissolved in double distilled water and prepared the stock solutions in gL^{-1}. The stock solution of the inhibitor piroxicam was prepared by dissolving it in hot ethanol. All the additives were filter sterilized by using disposable syringe filter unit (AXIVA syringe filter; 25 mm diameter, 0.2 µm pore size) and added to the medium at the following concentrations 10, 25, or 50 mg L^{-1}. These substances were added to suspension cultures and examined level of AQ production.

Effect of elicitors or precursors in suspension culture:

To study the effect of elicitors in suspension cultures, standardized suspension cultures of 25 days old, were incubated with different concentrations (10, 25 and 50 mg L^{-1}) of filtered elicitors *viz.*, yeast extract, pectin or xylan. Filter sterilized precursors *viz.*, α-keto glutaric acid or phenylalanine were tested for AQ production and response of precursors were compared with an inhibitor piroxicam. Elicitor or precursor omitted cultures were served as control. After five days of incubation (30^{th} day) cells were harvested and parameters like fresh weight, dry weight and total AQ content were determined.

AQ quantification from cell suspension cultures:

AQ quantification was based on the procedure of Hagendoorn et al. [24]. Dried cells (100 mg) from suspension cultures were collected and were boiled in 80 % ethanol for 45 minutes in a water bath (KEMI water bath incubator shaker) at 80°C. The extracts were then collected and the process was repeated thrice. The extract was concentrated in a rotary evaporator (Super fit Rotavap, PBU-6, Super fit Continental Private Limited, Mumbai) at 60°C and centrifuged at 1500 rpm for 10 minutes. The absorption was determined at434 nm on UV visible spectrophotometer (Shimadzu; Model No.UV-1700), and anthraquinones was estimated using the standard graph of alizarin (Fluka Analytical, US).

HPLC analysis for the detection of alizarin and purpurin:

Extracts from elicitor or precursor treated suspension cultures were subjected to HPLC analysis. Ethanolic extracts from the samples were filtered (AXIVA syringe filter, 25 mm diameter, 0.2 µm pore size) and used for the analysis. The analysis was performed in Agilent 1100 system (Model No. G1310A) connected with DAD-UV detector (Diode Array UV Detector). The chromatographic conditions were as follows; SpherisorbeS5 ODS column (12 9 0.58 cm); mobile phase of triethylamine (0.5 %) in water: acetonitrile, 50:50 (v/v) with flow rate of 1 mL min^{-1} and detection wavelength at 250 nm. Alizarin (Fluka Analytical Chemical Co., St. Louis, US) and purpurin (Sigma Chemical Co. Ltd., St. Louis, USA) were used as standard. Alizarin and purpurin quantification assay was done by applying the following equation [25];

$$Assay\ of\ Sample = \frac{Standard\ Weight}{Sample\ Weight} * \frac{Sample\ Area}{Standard\ Area} * Assay\ of\ Standard$$

Experimental design and statistical analysis:

All the experiments were conducted using a completely randomized block design (CRBD) method. Each treatment composed of three replications and each replication block was represented by three conical flasks per treatment. For analyzing growth characteristics of cell suspension, fresh weight, dry weight and PCV in 10 mL culture per three graduated centrifuge tubes for each replica were used. One way to two way ANOVA, appropriately was performed to determine significance of treatments and also to determine interaction of factors [26]. The mean separation was done according to Duncan's multiple Range Test ($P<0.05$) [27].

RESULTS AND DISCUSSION:

Initiation of cell suspension cultures:

Suspension cultures were initiated by transferring approximately 200 mg friable callus produced on 2.5µM NAA supplemented medium to full strength MS liquid medium. Continuous, vigorous agitation

of the friable callus in an orbital shaker caused dispersal of the cell mass from callus to the liquid medium. A fine suspension culture was produced by repeated subculture of suspension to new media up to six times at 15 days interval. The fine suspension developed was maintained on an orbital shaker (120 rpm) for further experiments. Effect of different concentrations of auxins *viz.*, NAA, IBA, IAA, or 2, 4-D were compared for the growth and AQ accumulation in suspension cultures of *O. umbellata*.

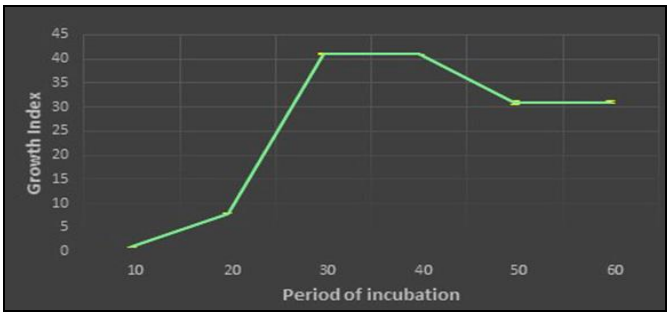

FIG. 1: GROWTH CURVES OF 10 µM NAA, PLOTTED BY GROWTH INDEX AGAINST PERIOD OF INCUBATION

Effect of auxins in of suspension cultures of *O. umbellata*:

Suspension culture was subcultured in different concentrations (1.0, 2.5, 5.0 and 10 µM) of four auxins (NAA, IBA, IAA, or 2, 4-D) to evaluate the influence of auxin type and concentration on growth and AQ accumulation. Two way ANOVA on various growth parameters reveal that auxin type and concentration had significant ($p < 0.001$) influence in the growth parameters of cell suspension cultures of *O. umbellata*.

The concentration of NAA and duration of incubation had significant ($p < 0.001$) influence on the growth parameters like PCV, fresh weight and dry weight. When suspension culture was incubated in four different concentrations of NAA, a significant ($p < 0.001$) increase in PCV was observed from 30th day onwards. Highest PCV were noticed on 40th day of incubation (7.83) (**Table 1**). After 40 days of incubation there were slight decline in PCV. NAA concentration had significant ($p < 0.001$) role in biomass accumulation in suspension cultures of *O. umbellata*. Up to 20 days of incubation fresh weight biomass showed slow rate of increase, after that there were a sudden hike in fresh weight biomass accumulation. On 40th day of incubation 10 µM NAA recorded the highest fresh weight biomass accumulation (9.68 g) (**Table 2**). Further incubation on the same media showed a decline in fresh weight biomass.

TABLE 1: PACKED CELL VOLUME OF SUSPENSION CULTURES OF *O. UMBELLATA* L. DEVELOPED IN DIFFERENT AUXIN SUPPLEMENTED MEDIUM

Auxin type	Conc (µM)	Period of incubation (Days)					
		10	20	30	40	50	60
NAA	1.0	10.00±1.15^{abc}	20.33±1.20^{abc}	26.67±1.20^{a}	30.00±0.58^{a}	32.67±0.88^{bc}	33.33±0.88^{bcdef}
	2.5	13.00±1.15^{a}	21.00±2.52^{abc}	28.00±1.53^{a}	30.33±0.67^{a}	32.00±0.58^{bcd}	33.67±1.20^{bcde}
	5.0	11.00±2.00^{ab}	22.67±2.33^{a}	27.33±1.20^{a}	28.33±1.20^{ab}	34.00±0.58^{ab}	36.00±0.58^{ab}
	10.0	9.67±1.45^{abc}	20.67±2.60^{abc}	**29.33±0.33^{a}**	**30.67±0.88^{a}**	**35.33±0.33^{a}**	**38.00±0.58^{a}**
IBA	1.0	6.33±0.88^{c}	19.33±3.53^{abcd}	23.33±0.88^{b}	29.33±0.88^{a}	31.33±0.88^{cd}	33.67±0.33^{bcde}
	2.5	8.00±0.58^{bc}	15.33±1.45^{bcde}	21.00±0.58^{bcd}	26.33±0.67^{bc}	32.33±0.67^{bc}	35.67±0.88^{abc}
	5.0	11.67±1.20^{ab}	15.00±1.53^{cde}	17.67±0.88^{d}	25.00±0.58^{cde}	28.67±0.33^{e}	32.00±0.58^{def}
	10.0	10.00±0.58^{abc}	12.00±0.58^{e}	17.67±0.88^{d}	24.67±0.88^{cde}	31.00±1.00^{cd}	34.33±0.88^{bcd}
IAA	1.0	6.00±1.15^{c}	13.00±1.53^{e}	18.67±0.88^{d}	22.33±0.88^{efg}	28.00±1.00^{e}	31.33±0.88^{ef}
	2.5	6.33±1.45^{c}	14.00±2.00d^{e}	19.33±1.20^{cd}	25.33±0.33^{cd}	30.00±0.58^{de}	33.00±0.58^{cdef}
	5.0	8.33±0.88^{bc}	15.33±1.45^{bcde}	17.67±0.88^{d}	25.33±0.67^{cd}	28.00±0.58^{e}	30.67±0.67^{fg}
	10.0	7.67±1.86^{bc}	16.00±2.08^{bcde}	18.33±2.19^{d}	21.00±0.58^{fg}	25.00±0.58^{fg}	27.67±0.88^{hi}
2,4-D	1.0	10.00±1.53^{abc}	21.00±1.15^{abc}	18.00±1.53^{d}	21.00±0.58^{g}	23.33±0.67^{gh}	27.67±0.88^{hi}
	2.5	12.00±1.53^{ab}	20.67±2.19^{abc}	18.00±0.58^{d}	23.33±0.88^{def}	25.67±0.88^{f}	28.33±0.33^{gh}
	5.0	9.67±1.20^{abc}	21.33±1.45^{ab}	22.67±1.20^{bc}	20.33±0.67^{g}	24.00±1.00^{fgh}	27.33±0.88^{hi}
	10.0	12.00±1.53^{ab}	20.00±0.58^{abcd}	21.33±0.88^{bcd}	22.33±1.86^{efg}	24.33±0.33^{fg}	25.67±1.20^{hi}
Control	0	5.67±0.33^{c}	10.00±0.58^{e}	13.33±0.88^{e}	20.33±0.33^{g}	22.00±0.58^{h}	25.00±1.53^{i}
Treatment Df(n-1)=16		3.255**	4.339***	16.070***	18.428***	32.854***	19.850***
Auxin type Df (n-1)= 3		7.759***	13.092***	55.590***	66.577***	121.707***	79.208***
Auxin ConcDf (n-1)= 3		2.052^{NS}	0.462^{NS}	0.077^{NS}	3.367**	2.860^{NS}	2.219^{NS}
Auxin Type X Conc Df (n-1)= 9		1.323^{NS}	0.963^{NS}	3.862**	4.289***	5.407***	6.147***

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level.

TABLE 2: FRESH WEIGHT BIOMASS ACCUMULATION IN SUSPENSION CULTURES OF *O. UMBELLATA* L. IN RESPONSE TO DIFFERENT AUXINS

Auxin type	Conc (µM)	Period of incubation in days					
		10	20	30	40	50	60
	1.0	0.29±0.01^{cd}	1.08±0.02^{ef}	3.70±0.05^{e}	3.80±0.11^{e}	3.47±0.11^{e}	3.33±0.16^{e}
NAA	2.5	0.28±0.00^{d}	2.29±0.11^{a}	4.21±0.26^{de}	4.20±0.04^{d}	3.95±0.05^{de}	3.74±0.13^{de}
	5.0	0.24±0.00^{ef}	1.76±0.07^{b}	5.22±0.40^{c}	5.12±0.07^{c}	4.78±0.07^{c}	4.64±0.13^{c}
	10.0	0.23±0.01^{efg}	1.50±0.01^{c}	8.69±0.67^{a}	9.68±0.04^{a}	9.21±0.35^{a}	8.27±0.40^{a}
IBA	1.0	0.18±0.00^{hi}	0.47±0.05^{h}	1.26±0.05^{g}	1.32±0.01^{i}	1.00±0.02^{g}	1.01±0.01^{gh}
	2.5	0.50±0.01^{a}	1.31±0.03^{d}	2.29±0.14^{f}	2.61±0.05^{f}	1.97±0.37^{f}	1.98±0.29^{f}
	5.0	0.34±0.01^{b}	0.98±0.01^{f}	2.17±0.05^{f}	2.08±0.07^{g}	1.55±0.17^{f}	1.58±0.19^{fg}
	10.0	0.32±0.01^{bc}	1.30±0.01^{d}	1.26±0.10^{g}	1.32±0.00^{i}	0.99±0.01^{g}	0.89±0.07^{i}
IAA	1.0	0.14±0.01^{k}	0.37±0.04^{hi}	2.28±0.07^{f}	2.11±0.01^{g}	2.07±0.07^{f}	2.13±0.13^{f}
	2.5	0.18±0.01^{ij}	0.75±0.05^{g}	2.11±0.06^{f}	1.83±0.15^{h}	1.65±0.22^{f}	1.62±0.33^{fg}
	5.0	0.15±0.01^{jk}	1.12±0.06^{ef}	0.99±0.04^{g}	0.95±0.05^{j}	0.89±0.02^{g}	0.58±0.24^{ij}
	10.0	0.15±0.02^{k}	0.49±0.03^{h}	0.94±0.03^{g}	0.98±0.01^{j}	0.08±0.00^{h}	0.09±0.01^{j}
2,4-D	1.0	0.20±0.01^{ghi}	1.18±0.04^{de}	9.32±0.30^{a}	8.03±0.10^{b}	6.82±0.31^{b}	6.72±0.42^{b}
	2.5	0.21±0.01^{fgh}	1.62±0.04^{bc}	6.33±0.22^{b}	4.22±0.10^{d}	4.07±0.08^{d}	3.71±0.23^{de}
	5.0	0.24±0.01^{ef}	1.73±0.04^{b}	4.72±0.07^{cd}	4.07±0.06^{d}	3.97±0.03^{de}	3.89±0.16^{de}
	10.0	0.25±0.01^{e}	0.20±0.00^{j}	4.09±0.03^{de}	4.14±0.10^{d}	3.91±0.16^{de}	4.20±0.20^{cd}
Control	0	0.06±0.02^{l}	0.26±0.04^{ij}	1.05±0.04^{g}	1.15±0.06^{ij}	1.05±0.05^{g}	1.04±0.04^{gh}
Treatment Df(n-1)=16		98.788***	167.863***	134.294***	1169.750***	195.355***	104.340***
Auxin type Df (n-1)= 3		293.197***	306.143***	424.572***	3569.379***	618.885***	339.137***
Auxin ConcDf (n-1)= 3		66.288***	248.797***	9.159***	163.421***	16.261***	9.937***
Auxin Type X Conc Df (n-1)= 9		58.159***	74.753***	66.697***	684.404***	101.963***	50.392***

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level.

TABLE 3: DRY WEIGHT BIOMASS ACCUMULATION IN SUSPENSION CULTURES OF *O. UMBELLATA* L. FORTIFIED WITH DIFFERENT AUXINS

Auxin type	Conc (µM)	Period of incubation in days					
		10	20	30	40	50	60
	1.0	0.02±0.00^{c}	0.07±0.00^{f}	0.14±0.00^{c}	0.12±0.00^{cd}	0.10±0.00^{def}	0.11±0.00^{cde}
NAA	2.5	0.02±0.00^{c}	0.13±0.01^{b}	0.14±0.03^{c}	0.13±0.01^{cd}	0.09±0.00^{efg}	0.10±0.00^{cdef}
	5.0	0.03±0.00^{a}	0.10±0.00^{c}	0.42±0.29^{b}	0.24±0.01^{bcd}	0.12±0.01^{d}	0.12±0.00^{c}
	10.0	0.02±0.00^{c}	0.09±0.00^{d}	**0.42±0.03^{b}**	**0.42±0.01^{ab}**	0.32±0.01^{a}	0.32±0.01^{a}
IBA	1.0	0.01±0.00^{d}	0.03±0.00^{i}	0.09±0.00^{c}	0.40±0.31^{ab}	0.08±0.00^{fg}	0.06±0.01^{g}
	2.5	0.03±0.00^{a}	0.08±0.00^{e}	0.09±0.00^{c}	0.08±0.00^{cd}	0.09±0.00^{fg}	0.08±0.00^{efg}
	5.0	0.02±0.00^{b}	0.05±0.00^{g}	0.12±0.01^{c}	0.09±0.00^{cd}	0.08±0.00^{fg}	0.09±0.01^{defg}
	10.0	0.02±0.00^{b}	0.08±0.00^{de}	0.06±0.02^{c}	0.08±0.01^{cd}	0.08±0.00^{g}	0.08±0.01^{efg}
IAA	1.0	0.01±0.00^{f}	0.02±0.00^{j}	0.03±0.00^{c}	0.02±0.00^{d}	0.01±0.00^{h}	0.02±0.00^{h}
	2.5	0.01±0.00^{e}	0.04±0.00^{h}	0.43±0.02^{b}	0.28±0.02^{abc}	0.15±0.02^{c}	0.02±0.01^{h}
	5.0	0.01±0.00^{d}	0.06±0.00^{f}	0.07±0.00^{c}	0.08±0.00^{cd}	0.07±0.00^{g}	0.08±0.00^{efg}
	10.0	0.01±0.00^{e}	0.23±0.00^{a}	0.06±0.00^{c}	0.08±0.00^{cd}	0.08±0.00^{g}	0.06±0.01^{g}
2,4-D	1.0	0.01±0.00^{de}	0.08±0.00^{de}	0.54±0.02^{b}	0.39±0.03^{ab}	0.23±0.01^{b}	0.22±0.02^{b}
	2.5	0.01±0.00^{e}	0.10±0.00^{c}	0.95±0.01^{a}	0.51±0.07^{a}	0.32±0.00^{a}	0.30±0.01^{a}
	5.0	0.01±0.00^{e}	0.02±0.00^{j}	0.17±0.06^{c}	0.19±0.00^{bcd}	0.09±0.00^{efg}	0.12±0.01^{cd}
	10.0	0.01±0.00^{d}	0.01±0.00^{k}	0.15±0.01^{c}	0.11±0.00^{cd}	0.11±0.01^{de}	0.10±0.01^{cde}
Control	0	0.01±0.00^{f}	0.01±0.00^{k}	0.12±0.00^{c}	0.10±0.00^{cd}	0.09±0.00^{efg}	0.07±0.01^{fg}
Treatment Df(n-1)=16		138.955***	389760***	11.287***	3.780***	138.930***	89.099***
Auxin type Df (n-1)= 3		370.078***	249.017***	18.631***	4.060*	221.263***	237.321***
Auxin ConcDf (n-1)= 3		97.179***	309.108***	8.203***	1.391^{NS}	75.870***	20.983***
Auxin Type X Conc Df (n-1)= 9		68.975***	429.638***	9.659***	4.332***	132.559***	83.508***

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level.

Dry weight accumulation was also highest (0.42 g) in 10 µM NAA from 30th day onwards (**Table 3**). The growth index of cell suspension cultures in 10 µM NAA supplemented media showed a steep rise in between 20 to 30 days of incubation followed by a steady phase from 30 to 40 days of incubation and then showed declined growth rate after 40 days (**Fig. 1**). Growth parameters, at different concentrations (1.0, 2.5, 5.0 and 10 µM) of IBA, IAA and 2, 4-D were observed to be inferior to10 µM NAA.

Suspension culture in different concentrations of NAA started producing significant ($p < 0.001$) amount of AQ from 20th day onwards. Continuous monitoring of AQ content from 10th day up to 60 days revealed that the AQ production was highest on 30th day of incubation on 10µM NAA

supplemented medium (9.96 mg g -1dw) (**Fig.2 A-D**). After 30 days AQ content declined.

Effect of elicitors in suspension culture:

Type and concentrations of elicitors had significant ($p < 0.001$) influence in AQ production from cell suspension cultures of *O. umbellate* (**Table 4**). Biomass accumulation in suspension cultures were significantly higher ($p < 0.001$) when compared with that of control (8.66 g). Highest fresh weight was recorded during elicitation using 25 mg L-1 pectin added medium (12.24 g). Elicitation with 25 mg L-1 pectin also showed significant ($p < 0.001$) enhancement dry weight biomass accumulation (2.13 g) when compared to control (0.66 g). The AQ production by elicitation had significant ($p < 0.001$) influence in cell suspension cultures. Highest amount of AQ accumulated during elicitation was 35.67 mg g-1dw in 25 mg L-1 pectin added medium (**Table 4, Fig.3A**).

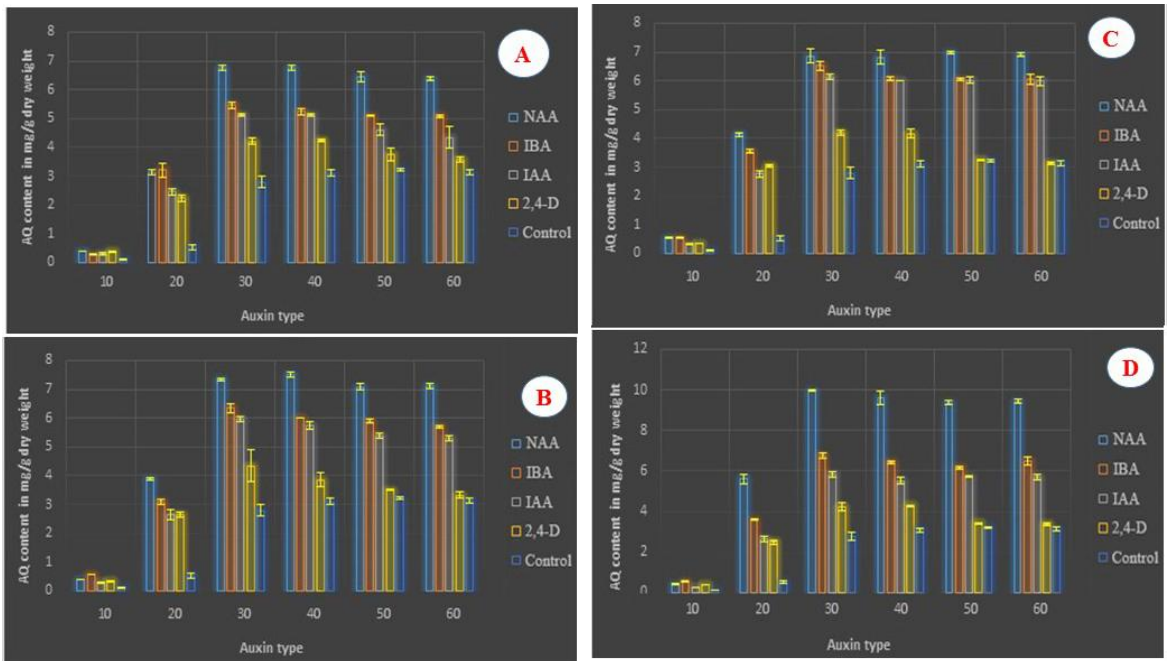

FIG. 2: AQ ACCUMULATION IN CELL SUSPENSION CULTURES OF *O. UMBELLATA* WITH RESPECT TO TYPE AND CONCENTRATION OF AUXIN AND PERIOD OF INCUBATION. A. PERIODIC EFFECT OF DIFFERENT AUXINS AT 1 µM CONCENTRATION. B. EFFECT OF 2.5 µM CONCENTRATIONS OF DIFFERENT AUXINS WITH RESPECT TO PERIOD OF INCUBATION. C. PERIODIC EFFECT OF DIFFERENT AUXINS AT 5 µM CONCENTRATION. D. EFFECT OF 10 µM CONCENTRATIONS OF DIFFERENT AUXINS WITH RESPECT TO PERIOD OF INCUBATION.

Effect of precursors on suspension cultures:

Addition of precursors in cell suspension of *O. umbellata* had significant ($p < 0.001$) effect on AQ production (**Table 5**). Fresh weight biomass accumulation during precursor feeding was highest when suspension treated with 50 mg L^{-1}α-keto glutaric acid (15.54 g) (**Table 5**). When the dry weight biomass was considered, highest accumulation was reported during 50 mg L^{-1}α-keto glutaric acid addition to the suspension cultures (3.08 g). Precursor feeding with 50 mg L^{-1}α-keto glutaric acid resulted in the accumulation of 42.63 mg g^{-1}dw AQ (**Table 5**, **Fig.3B**). The overall AQ production by the addition of elicitors, precursors or inhibitors showed an increment when compared to that of control (9.44 mg g^{-1}dw AQ). The interaction of precursor type and concentration had a significant ($p< 0.001$) influence in AQ production from cell suspension cultures of *O. umbellata*.

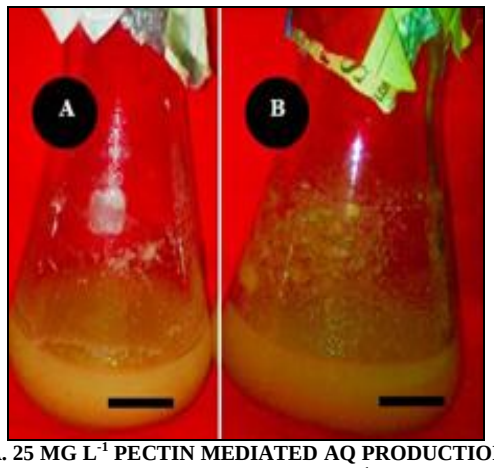

FIG. 3: A. 25 MG L^{-1} PECTIN MEDIATED AQ PRODUCTION FROM CELL SUSPENSION CULTURES. B. 50 MG L^{-1} α-KETO GLUTARIC ACID INDUCED AQ PRODUCTION FROM CELL SUSPENSION CULTURES (BAR = 0.90 CM).

TABLE 4: EFFECT OF DIFFERENT TYPES OF ELICITORS ON 25-DAYS-OLD SUSPENSION CULTURE OF *O. UMBELLATA* DEVELOPED IN MS MEDIUM FORTIFIED WITH 10 µM NAA

Elicitor	Conc. (mg L^{-1})	Fresh Weight (g)	Dry Weight (g)	Total AQ mg g^{-1}dw
Yeast Extract	10	8.80±0.23^{c}	0.77±0.06^{c}	27.20±0.68^{bcd}
	25	8.87±0.64^{c}	0.63±0.13^{c}	26.35±0.58^{cd}
	50	9.06±0.09^{c}	0.50±0.03^{c}	26.25±0.58^{cd}
Pectin	10	10.75±0.42^{b}	1.34±0.27^{b}	27.97±0.91^{bc}
	25	**12.24±0.14^{a}**	**2.13±0.32^{a}**	**35.67±1.21^{a}**
	50	11.11±0.11^{b}	1.29±0.15^{b}	28.91±0.29^{bc}
Xylan	10	8.61±0.25^{c}	0.37±0.01^{c}	24.79±1.54^{d}
	25	9.30±0.10^{c}	0.55±0.10^{c}	27.10±0.74^{bcd}
	50	10.31±0.27^{b}	0.65±0.10^{c}	29.29±0.22^{b}
Control		8.66±0.14^{c}	0.69±0.03^{c}	9.44±0.18^{e}
Treatment Df (n-1)=9		18.725***	12.458***	67.187***
Elicitor type Df (n-1)= 2		55.353***	39.687***	22.640***
Elicitor concDf (n-1)=2		6.359**	3.113^{NS}	9.697***
Elicitor type X concDf (n-1)=4		4.226*	3.489*	11.081***

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS- non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level

TABLE 5: EFFECT OF PRECURSOR OR AQ INHIBITOR PIROXICAM ON 25-DAYS-OLD CELL SUSPENSION CULTURE OF *O. UMBELLATA* DEVELOPED IN MS MEDIUM FORTIFIED WITH 10 µM NAA

Precursor or inhibitor	Conc. (mg L^{-1})	Fresh Weight (g)	Dry Weight (g)	Total AQ mg g^{-1}dw
Piroxicam	10	10.91±0.35^{d}	0.55±0.05^{ef}	24.93±1.45^{d}
	25	10.06±0.12^{e}	0.33±0.01^{ef}	25.12±0.41^{d}
	50	7.98±0.45^{f}	0.27±0.02^{f}	21.78±0.76^{e}
α-keto glutaric acid	10	12.58±0.19^{c}	1.30±0.04^{d}	35.14±0.61^{bc}
	25	14.55±0.17^{b}	2.03±0.33^{b}	37.10±0.99^{b}
	50	15.54±0.10^{a}	3.08±0.07^{a}	42.63±0.73^{a}
Phenylalanine	10	13.04±0.41^{c}	1.74±0.13^{bc}	32.85±0.81^{c}
	25	12.43±0.17^{c}	1.54±0.12^{cd}	33.14±0.53^{c}
	50	11.60±0.18^{d}	1.49±0.14^{cd}	32.30±1.59^{c}
Control		8.66±0.14^{f}	0.69±0.03^{e}	9.44±0.18^{f}
Treatment Df(n-1)=9		87.104***	45.835***	107.255***
Prec type Df (n-1)=2		220.557***	130.189***	172.124***
PrecconcDf (n-1)=2		4.548*	7.649**	1.349^{NS}
Prec type X concDf (n-1)=4		32.847***	18.816***	9.604***

Means within a column followed by same letters are not significantly ($p<0.05$) different as determined by Duncan's Multiple Range test. NS-non significant, ***F value is highly significant at $p<0.001$ level, **significant at $p<0.01$ level, *significant at $p<0.05$ level

Enhanced AQ production through elicitation or precursor feeding was also reported by a number of workers. Precursor feeding of cell suspension cultures of *Morindacitrifolia* resulted enhanced AQ accumulation [28]. El-Mawla [29] reported the influence of certain abiotic elicitors on production of anthraquinones from cell cultures of *Rubiatinctorum*. *Aloe vera* suspension cultures produced 127% higher AQ content than control when treated with chitosan [30]. Enhanced AQ production from cell suspension cultures of *O. umbellata* is supported by these reports.

HPLC analysis of samples:

HPLC analysis of *in vitro* produced AQ samples confirmed the presence of alizarin and purpurin in the extract. Alizarin was detected in samples at a retention time of 1.93 at 250 nm in standard HPLC chromatogram (**Fig.4A**) and retention time of purpurin was 1.82 (**Fig.4B**). 25 mg L^{-1}pectin treated suspension cultures produced 10.259 mg g^{-1}dw alizarin and 8.245 mg g^{-1}dw purpurin (**Fig.4C**). Precursor feeding of suspension cultures resulted in increased accumulation of alizarin and purpurin (15.24 and 10.215 mg g^{-1}dw respectively) (**Fig.4D**).

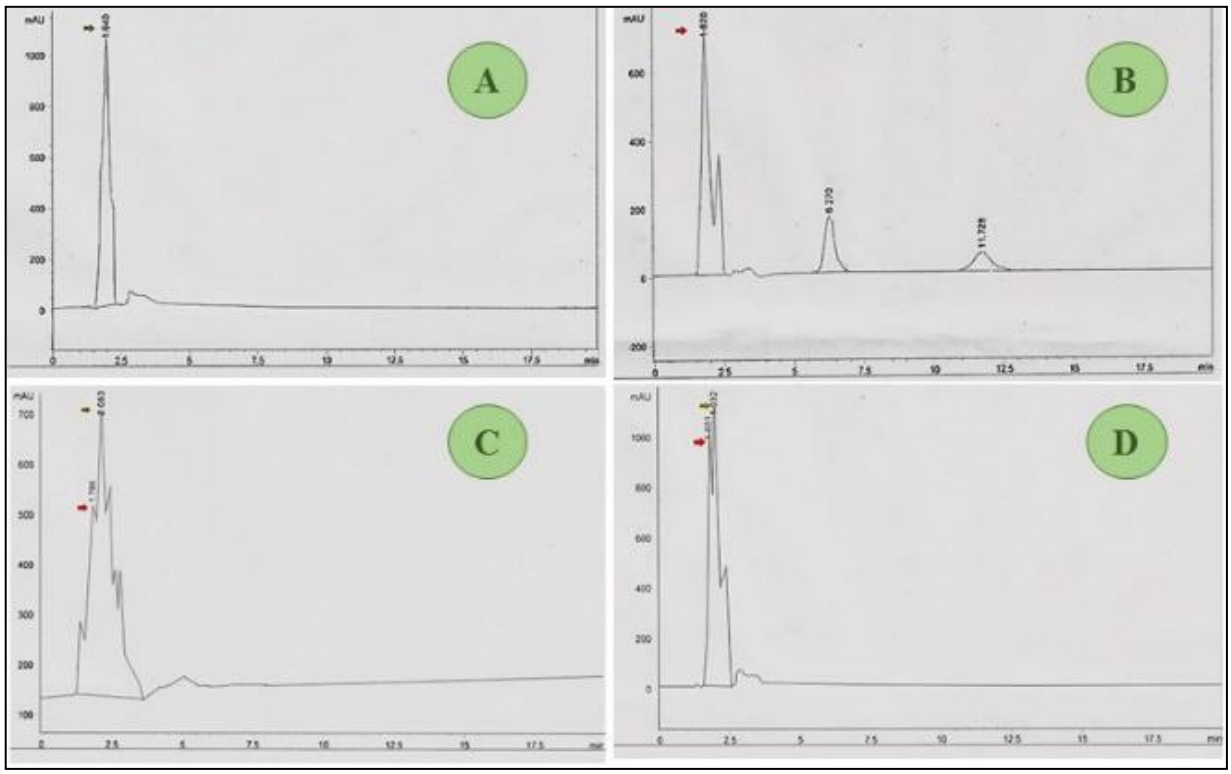

FIG 4: HPLC PROFILE OF STANDARDS A. ALIZARIN. B. PURPURIN. C AND D. HPLC PROFILE OF ETHANOLIC EXTRACTS OF ELICITOR AND PRECURSOR MEDIATED SUSPENSION CULTURE.

CONCLUSION: The present study reveals that elicitors or precursors can significantly influence AQ production from suspension cultures of *O. umbellata*. AQ accumulated in 10 µM NAA added suspension cultures (9.96mg g^{-1}dw) of *O. umbellata* were seem to be inferior to elicitation or precursor feeding of suspension cultures. Highest AQ accumulation was recorded in 50 mg L^{-1} α-keto glutaric acid added suspension cultures (42.63mg g^{-1}dw). The present protocol can be applied for large scale production of AQ to meet global needs.

ACKNOWLEDGEMENTS: The authors are thankful to Dr. P.M. Radhamany, Professor & Head, Department of Botany, University of Kerala for facilities provided. SKSR thank University of Kerala, Thiruvananthapuram, India for granting University JRF (Ac E1B1/43700/2011 dt. 26/12/11).

REFERENCES:

1. Gonza´les-Garcı´a S, Moreira MT, Artal G, Maldonado L andFeijoo G: Environmental impact assessment of non-wood based pulp production by soda-anthraquinone pulping process. Journal of Cleaner Production.2010; 18:137–145.
2. Jahan MS, Shamsuzzaman M, Rahman MM, Moeiz SI and Ni Y: Effect of pre-extraction on soda-anthraquinone (AQ) pulping of rice straw. Industrial Crops and Products. 2012; 37:164-169.
3. Ghosh P, Devi GP, Priya R, Amrita A, Sivaramakrishna A, Babu S andSiva R: Spectroscopic and in silico evaluation of interaction of DNA with six anthraquinone derivatives. Applied Biochemistry and Biotechnology. 2013; 170:1127-1137.
4. Siddiqui MF, Andleeb S, Ali N, GhumroPB and Ahmed S: Biotreatment of anthraquinone dye Drimarene Blue K 2 RL. African Journal of Environmental Science and Technology.2010; 4:45-50.
5. Hu B, Zhang H, Meng X, Wang F and Wang P: Aloe-emodin from rhubarb (Rheum rhabarbarum) inhibits lipopolysaccharide-induced inflammatory responses in RAW264. 7 macrophages. Journal of Ethnopharmacology. 2014; 153:846-853.
6. Yamazaki M, Mochida K, Asano T, Nakabayashi R, Chiba M, Udomson N, Yamazaki Y, Goodenowe DB, Sankawa U, Yoshida T and Toyoda A: Coupling deep transcriptome analysis with untargeted metabolic profiling in *Ophiorrhizapumila* to further the understanding of the biosynthesis of the anti-cancer alkaloid camptothecin and anthraquinones. Plant and Cell Physiology. 2013; doi: 10.1093/pcp/pct040.
7. Sreeranjini S and Siril EA: Production of anthraquinones from adventitious root derived callus and suspension cultures of *Morindacitrifolia* L. in response to auxins, cytokinins and sucrose levels. Asian Journal of Plant Science and Research. 2013; 3:131-138.
8. Shrimali D, Shanmugam MK, Kumar AP, Zhang J, Tan BK, Ahn KS and Sethi G: Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. Cancer Letters. 2013; 341:139-149.
9. Frackowiak A, Skibinski P, Gawel W, Zaczynska E, Czarny A and Gancarz R: Synthesis of glycoside

derivatives of hydroxylanthraquinone with ability to dissolve and inhibit formation of crystals of calcium oxalate. Potential Compounds in Kidney Stone Therapy.2010; 45:1001-1007.
10. Jie Z: Present Market Status and Development Priorities of Dyestuff and Pigment Intermediates in China [J]. Fine and Specialty Chemicals.2001; 10.
11. Singh SK, Singh S and Yadav A: A review on Cassia species: Pharmacological, traditional and medicinal aspects in various countries. American Journal of Phytomedicine and Clinical Therapeutics. 2013; 1:291-312.
12. Soundhari C and Rajarajan S: *In vitro* screening of lyophilised extracts of *Alpiniagalanga* L. and *Oldenlandia umbellata* L. for anti-mycobacterial activity. International Journal of Biological and Pharmaceutical Research.2013; 4:427-432.
13. Rekha S, Srinivasan V, Saradha V and Hamsaveni GR: The *in vitro* antibacterial activity of *Hedyotis umbellata.* Indian Journal of Pharmaceutical Science.2006; 68:236–238.
14. Gupta M, Mazumder UK, Thamilselvan V, Manikandan L, Senthilkumar GP, Suresh R and Kakotti BK: Potential hepatoprotective effect and antioxidant role of methanol extract of *Oldenlandia umbellata* in carbon tetrachloride induced hepatotoxicity in Wistar rats. Iranian Journal of Pharmacology and Therapeutics.2007; 6:5–9.
15. Siva R, Rajasekaran C and Mudgal G: Induction of somatic embryogenesis and organogenesis in *Oldenlandia umbellata* L., a dye-yielding medicinal plant. Plant Cell, Tissue and Organ Culture.2009; 98:205-211.
16. Arun P, Purushotham KG, Jayarani JJ and Kumari V: *In vitro* antibacterial activity of *Oldenlandia umbellata* an Indian medicinal plant. Journal of Pharmaceutical Science and Technology.2010; 2:198-201.
17. Somnath DE, Dey A, Babu AS and Aneela S: *In vitro* anthelmintic activity of aqueous and methanolic extracts of *Oldenlandia umbellata*. International Journal of Pharmacy and Pharmaceutical Sciences.2014; 6:94-95.
18. Muthu C, Ayyanar M, Raja NandIgnacimuthu S: Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. Journal of Ethnobiology and Ethnomedicine. 2006; doi: 10.1186/1746-4269-2-43.
19. Kumar PP, Ayyanar M and Ignacimuthu S: Medicinal plants used by Malasar tribes of Coimbatore district, Tamil Nadu. Indian journal of traditional Knowledge.2007; 6:579-582.
20. Savithramma N, Sulochanaand Rao KN: Ethnobotanical survey of plants used to treat asthma in Andhra Pradesh, India. Journal of Ethnopharmacology. 2007; 113:54-61.
21. Siva R, Mudgal G, Rajesh D, Khan NF, Vijaya Kumar V and Rajasekaran C: Characterization of novel pH indicator of natural dye *Oldenlandia umbellata* L. Natural Product Research.2009; 23:1210-1217.
22. Siva R, Mayes S, Behera SKandRajasekaran C: Anthraquinones dye production using root cultures of *Oldenlandia umbellata* L. Industrial Crops and Products. 2012; 37:415–419.
23. Mahibalan S, Rao PC, Khan R, Basha A, Siddareddy R, Masubuti H, Fujimoto Y and Begum AS: Cytotoxic constituents of *Oldenlandia umbellata* and isolation of a new symmetrical coumarin dimer. Medicinal Chemistry Research.2016; DOI 10.1007/s00044-015-1500-z.
24. Hagendoorn MJ, van der Plas LH and SegersGJ: Accumulation of anthraquinones in *Morindacitrifolia* cell suspensions. Plant Cell, Tissue and Organ Culture.1994; 38:227-234.
25. Roy A, Lakshmi T and Geeta RV: Estimation of quercetin in *Acacia catechu* ethanolic bark extracts by HPLC method. International Journal of Pharm Tech Research.2012; 4:501-505.
26. Snedecor GW and Cochran WG: Statistical methods. The Iowa State University Press, Fourth edition 1962.
27. Duncan DB: Multiple range and multiple F tests. Biometrics.1955; 11:1-42.
28. Stalman M, Koskamp AM, Luderer R, Vernooy JH, Wind JC, Wullems GJ and Croes AF: Regulation of anthraquinone biosynthesis in cell cultures of *Morindacitrifolia*. Journal of Plant Physiology.2003; 160:607-614.
29. El-Mawla AM: Influence of certain abiotic elicitors on production of anthraquinones in cell cultures of *Rubiatinctorum*. Spatula DD-Peer Reviewed Journal on Complementary Medicine and Drug Discovery.2012; 2:89-94.
30. Raei M, Angaji SA, Omidi M and Khodayari M: Effect of abiotic elicitors on tissue culture of *Aloe vera*. International Journal of Biosciences.2014; 5:74-81

How to cite this article:
Krishnan SRS and Siril EA: Elicitor and Precursor Mediated Anthraquinone Production from Cell Suspension Cultures of *Oldenlandia Umbellata* L. Int J Pharm Sci Res 2016; 7(9): 3649-57.doi: 10.13040/IJPSR.0975-8232.7(9).3649-57.