Research artical Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Artemisinin production by callus and cell suspension culture of *Artemisia nilagarica* (c.b. clarke) pamp.

Nganthoi Mayengbam and Sanatombi Keithellakpam*

Department of Biotechnology, Manipur University, Canchipur, Imphal – 795003.
*Corresponding author's Email: ksanatombi@rediffmail.com

ABSTRACT

The present study, for the first time, reports the establishment of cell suspension cultures from callus induced using leaf explants of the highly medicinal plant, *Artemisia nilagarica* (C.B. Clarke) Pamp. for artemisinin production. The influence of different growth regulators on callus texture, biomass yield and artemisinin production was investigated. Maximum biomass (4.807 g Fresh weight) was obtained in Murashige & Skoog, 1962 (MS) medium containing 1 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D) in combination with 0.5 mg L^{-1} Kinetin (Kin) and highest artemisinin content (0.028 % on dry weight basis) was obtained in the calli induced on MS media containing 5 mg L^{-1} 2, 4-D and 1 mg L^{-1} Kin. Friable callus suitable for suspension culture with optimum cell biomass (3.463 g FW) and artemisinin content (0.027 %) was induced in MS media supplemented with 3mg L^{-1} 2, 4-D and 1 mg L^{-1} Kin. Cell suspension cultures were successfully established from the friable callus on liquid MS medium containing the same combination of growth regulators. Complete growth kinetics and artemisinin production profile of cell suspension as well as liquid media were studied for every 5 days interval during a 45 days growth cycle. The maximum cell biomass of 3.206 g fresh weight and the maximum artemisinin accumulation (0.013 %) were obtained on the 25th day of the growth cycle of the cell suspension cultures.

KEYWORDS: *Artemisia nilagarica* (C.B. Clarke) Pamp., leaf explants, cell culture, artemisinin, HPLC.

***Corresponding Author:-**

Mayengbam Nganthoi

Department of Biotechnology,

Manipur University, Canchipur,

Imphal – 795003.

Email: ksanatombi@rediffmail.com

INTRODUCTION

The genus *Artemisia* possesses diverse medicinal and economic importance as sources of a large number of essential oils, secondary metabolites, pharmaceutically important medicinal compounds, food, forage, ornamentals or soil stabilizers in disturbed habitats[1]. In India, about 45 species of the genus *Artemisia* has been reported till date[2]. In Manipur also, four species of *Artemisia* including *A. nilagarica* (C.B. Clarke) Pamp., have been reported[3, 4] and these plants have a long history of being used by local practitioners for treatment of several diseases such as wounds on skin, mouth sores and also as a tonic, antiseptic, analgesic, stomachic and insect repellent or as an anti-diabetic[5, 6, 7]. Besides these, *A. nilagarica* has also been reported to possess activities against the malaria parasite, *Plasmodium falciparum*[8]. A significant amount of artemisinin, the most effective antimalarial drug, has also been reportedly quantified from leaves of field grown *A.nilagarica*, micro-propagated plants induced from shoot tip explants and callus induced from leaf explants of *A.nilagarica* in our previous study[9]. Because of its diverse medicinal importance, *A. nilagarica* is over-exploited[10] and has been listed in the threatened medicinal plant category[11]. Therefore, it is the need of the hour to conserve the plant and to develop an alternative way for sustainable production of its various important secondary metabolites and other medicinally important components. Plant cell culture technology has been the focus of plant biotechnological research as an alternative method for production of medicinally important natural plant products without much exploitation of the plants from nature[12]. Earlier, several studies reported the accumulation of artemisinin in the shoot[13]; callus[12], cell suspension[14, 15] and hairy root[16] cultures of *A. annua* L, the most important source of commercial artemisnin. Although there have been few reports on micro-propagation and callus induction in *A.nilagarica*[11], there is no report on induction of cell suspension cultures of *A.nilagarica* till date. Therefore, the present study was undertaken to investigate the artemisinin production potential of cell suspension cultures through induction of callus from the leaf explants of the highly medicinal plant, *A. nilagarica*, with a view to provide an alternative and a consistent source for artemisinin production.

MATERIALS AND METHODS

Callus culture and cell suspension culture

Young and healthy leaves of *Artemisia nilagarica* (C.B. Clarke) Pamp., collected from the experimental fields, were first washed thoroughly with tap water followed by distilled water. Leaves were then surface sterilized under aseptic conditions with 0.05 % mercuric chloride solution for 1 min and rinsed 4-5 times with sterile distilled water. The surface-sterilised leaves were then

inoculated in culture tubes containing Murashige & Skoog (MS)[17] medium supplemented with 1 mg L^{-1} 2, 4-D (2,4-dichlorophenoxyacetic acid) alone or in combination with 0.5 mgL^{-1} Kin (Kinetin), 3 or 5 mg L^{-1} 2, 4-D alone or in combination with 1 mg L^{-1} Kin, 1 mg L^{-1} NAA (α-Naphthalene acetic acid) in combination with 0.5 mg L^{-1} BAP (6-Benzylaminopurine), and 3 or 5 mg L^{-1} NAA in combination with 1 mgL^{-1} BAP. The culture tubes were incubated at 25±2°C under complete darkness. The cultures were maintained on fresh medium of same composition by regular sub-culturing at two weeks interval using about 500 mg of callus as initial inoculum. To study the effects of light on callus texture, biomass yield and artemisinin production, cultures were kept under 16 hr photoperiod. Callus was also cultured in four other different types of media supplemented with 3 mg L^{-1} 2, 4-D and 1 mg L^{-1} Kin viz., LS (Linsmaier and Skoog medium)[18], B5 (Gamborg medium)[19], Nitsch (Nitsch and Nitsch)[20] and White (White)[21] media, for determination of the best media type for artemisinin production. Artemisinin contents of the four weeks old calli were determined and biomass production of calli was measured in terms of fresh weight (FW) and dry weight (DW).

Initial inoculum size of cell suspension culture was optimized by using three different inoculums sizes, i.e., 250 mg, 500 mg and 1 g. Further, cell suspension cultures were established by sub-culturing 500 mg of callus in 100 ml Erlenmeyer flasks containing 25 ml of liquid MS medium supplemented with 3 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin. The cell suspension cultures were incubated on a rotary shaker at 120 rpm at 25 ± 2 °C under complete dark condition and were maintained by sub-culturing at 2 weeks interval. The growth of the cell suspension culture was measured in terms of settled cell volume (SCV) (ml), FW (g) and DW (g). The growth of cell suspensions and accumulation of artemisinin were analysed by sampling of both the suspended cells and liquid media at 5 days regular interval during the 45 days period of growth cycle.

Determination of artemisinin content

Calli and cell suspensions were oven dried at 50°C and powdered. The dried cells were then extracted with toluene while the liquid media of cell suspension cultures were extracted with equal volume of methanol for 48 hours at room temperature. The artemisinin content of the extracts were quantified using UV/Vis Spectrophotometer (model -118 from Systronics) at 263 nm and High Performance Liquid Chromatography (HPLC) (Aligent Technologies ChemStation LC System) at 254 nm using methanol: H_2O (40:60) as mobile phase on Zorbax Eclipse Plus C18, 4.6 m x 100 mm with 3.50 micron column and at1 ml min^{-1} flow rate.

Statistical analysis

All the experiments were repeated thrice and each experiment for callus induction consisted of ten replicates, while each experiment for cell suspension had three replicates. Data was analysed using analysis of variance (ANOVA) followed by Duncan's multiple range test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Effects of different plant growth regulators on callus induction and artemisinin content in callus cultures of A.nilagarica

Among the five different types of media (i.e., MS, LS, Nitsch, B5 and White), MS medium was found to be the most suitable for callus induction producing friable calli with optimum biomass (4.4 g FW) and artemisinin production (0.028 %) (Table 1). Therefore, further experiment for *in vitro* artemisinin production was carried out in MS media.

Table 1. Biomass yield of cell and artemisinin content in calli of *A. Nilagarica* induced in different media types supplemented with 3 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin

Types of media	Fresh weight (g) ± SE	Dry weight (g) ± SE	Mean artemisinin content (% dry weight) ± SE
MS	4.400 ± 0.207^{a}	0.507 ± 0.377^{a}	0.028 ± 0.001^{a}
LS	3.705 ± 0.732^{a}	0.380 ± 0.006^{ab}	0.025 ± 0.003^{abc}
B5	2.965 ± 0.155^{ab}	0.304 ± 0.013^{ab}	0.026 ± 0.002^{abc}
Nitsch	2.004 ± 0.026^{ab}	0.209 ± 0.005^{abc}	0.027 ± 0.001^{ab}
White	1.496 ± 0.051^{bc}	0.156 ± 0.007^{bc}	0.027 ± 0.001^{ab}

* Superscript letters represent significance level at $p \leq 0.05$ (Duncan's test).

The nature of calli induced, texture, degree of proliferation, biomass production of callus as well as artemisinin accumulation in the cell culture of *A.nilagarica* were influenced by the types and concentrations of plant growth regulators used. Our results are in agreement with previous reports that the type and concentration of growth regulators play a crucial role in altering both the growth and accumulation of secondary products in cultured plant cells[22]. Friable calli with different colour and texture were induced within 17 to 21 days in all the media except in MS media containing 5 mg L^{-1} NAA in combination with 1 mg L^{-1} BAP, in which compact calli were induced. The percentage of callogenesis was highest in MS media containing 3 mg L^{-1} 2, 4-D (93 %) and was more than 60 %

in the rest (Table 2). The average yield of callus biomass was highest in MS media containing 1 mg L^{-1} 2, 4-D in combination with 0.5 mg L^{-1} Kin (4.807 g FW) and the lowest biomass was obtained in MS media containing 5 mg L^{-1} 2, 4-D in combination with 1 mg L^{-1} Kin (1.574 g FW) (Table 2). Artemisinin was detected in callus culture of the *A. nilagarica* cultured on MS medium supplemented with 2, 4-D, NAA or BAP. Earlier, accumulation of significant amount of artemisinin in *A. annua* calli cultured on MS media supplemented with different concentrations of BAP, NAA and 2, 4-D has also been reported[23]. The artemisinin contents in the calli of *A. nilagarica*, induced when Kin was added in combination with 2, 4-D to MS media, were comparatively higher than the calli induced in 2, 4-D alone (Table 2). Our results are in agreement with the earlier reports that inclusion of BAP or Kinetin increased artemisinin concentration in callus culture[24, 25]. The artemisinin content was highest in the calli obtained from MS media containing 5 mg L^{-1} 2, 4-D with 1 mg L^{-1} Kin (0.028 %) and lowest in the calli induced in MS media containing 1 mg L^{-1} 2, 4-D (0.011 %) (Table 2). Artemisinin content in callus of *A. nilagarica* (0.028 %), obtained in this study, is higher than earlier reports from callus of *A. absinthium* (0.022 %)[25], *A. scoparia* (0.001 %)[26] and *A. annua* (0.006 %)[12].

Table 2. Effect of different concentrations and combinations of growth regulators supplemented to MS media on induction of calli from leaf explants of *A. nilagarica*

Plant growth regulator combinations (mg L^{-1})				% of callus induction	Fresh weight (g) ± SE	Dry weight (g) ± SE	Artemisinin content (% dry weight) ± SE
2, 4-D	Kin	NAA	BAP				
1	-	-	-	90	3.295±0.126^{c}	0.344±0.016^{c}	0.011 ± 0.001^{d}
3	-	-	-	93	4.400±0.206^{ab}	0.452±0.023^{ab}	0.018 ± 0.009^{abcd}
5	-	-	-	70	3.082±0.105^{c}	0.324±0.005^{c}	0.020 ± 0.001^{abcd}
1	0.5	-	-	66.66	4.807±0.242^{a}	0.494±0.022^{a}	0.016 ± 0.002^{bcd}
3	1	-	-	70	3.463±0.452^{bc}	0.358±0.043^{bc}	0.027 ± 0.002^{ab}
5	1	-	-	70	1.574±0.113^{e}	0.166±0.009^{e}	0.028 ± 0.002^{a}
-	-	1	0.5	73.33	1.957±0.174^{de}	0.208±0.017^{de}	0.012 ± 0.001^{bcd}
-	-	3	1	83.33	2.876±0.372^{c}	0.301±0.038^{c}	0.026 ± 0.002^{abc}
-	-	5	1	60	2.839±0.212^{c}	0.297±0.021^{c}	0.024 ± 0.002^{abcd}

* Superscript letters represent significance level at $p \leq 0.05$ (Duncan's test).

Both the biomass yield of cell and artemisinin production increased with increasing concentration of 2,4-D or NAA with 0.028 % and 0.026 % for 5 mg L^{-1} 2,4-D with Kin and 5 mg L^{-1} NAA with BAP, respectively. on the other hand, the artemisinin content decreased to 0.012 % when 5 mg L^{-1} 2, 4-D was used alone and the biomass yield also decreased when 5 mg L^{-1} 2, 4-D was used alone (3.082 g FW) or in combination with 1 mg L^{-1} Kin (1.574 g FW) or 5 mg L^{-1} NAA in combination with 1 mg L^{-1} BAP (2.839 g FW) (Table 2).

Attempt has also been made to investigate the influence of irradiation on the growth and accumulation of artemisinin in callus cultures of *A. nilagarica*. Friable calli were induced both under light and dark conditions whereas, the colour of the calli induced under light conditions was greener compared to dark grown callus (Figs. 1a and 1b).

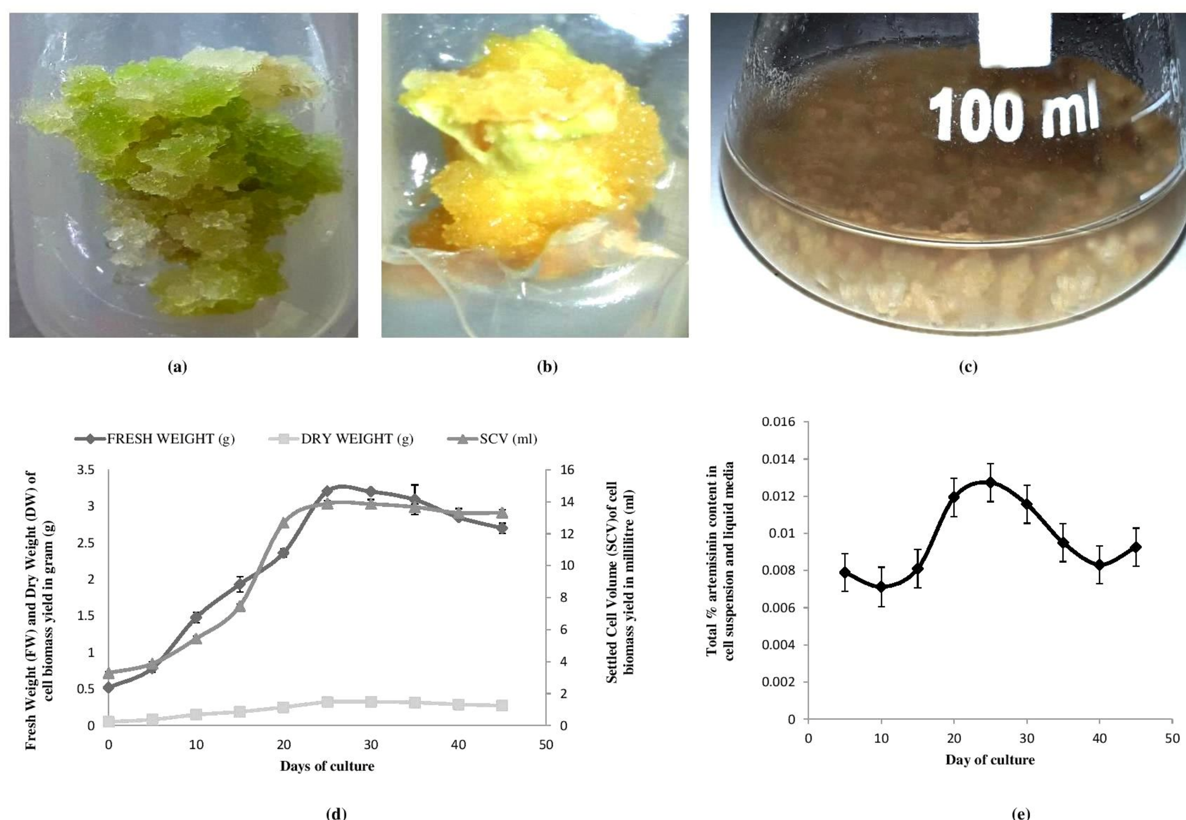

Figure1. Callus induced from leaf explants under (a) 16 hr photoperiod irradiation, (b) complete dark conditions; (c) a well suspended cells; (d) Time course for growth in terms of fresh weight, dry weight and settled cell volume (ml) of cell biomass; and (e) Profile of total percentage artemisinin content in cells suspension and liquid media over 45 days growth cycle of cell suspension culture of *A. Nilagarica* in MS medium supplemented with 3 mg L^{-1} 2, 4-D, 1 mg L^{-1} Kin and 30 gL^{-1} sucrose. Bars show standard errors.

The biomass production as well as artemisinin content was more in the calli induced under light conditions. Our results are in agreement with earlier studies, which reported green callus with higher biomass yield in calli cultured under light conditions [27]. However, Mohammad *et al*. (2014)[12] reported higher artemisinin content in the calli grown in dark condition.

Growth kinetics and artemisinin production in cell suspension cultures of A. nilagarica

Suitable initial inoculum size has been shown to influence the accumulation of secondary metabolites and cell biomass in cell cultures of many other plant species[28]. Earlier, an inoculum size of 500 mg has been reported to be the most suitable initial inoculum for production of artemisinin in cell suspension culture of *A. annua*[15]. In our study, the maximum growth index was attained with an inoculum density of 250 mg, followed by 500 mg and 1 g while the maximum artemisinin production was obtained with 500 mg initial inoculum.

Cell suspension culture of *A. nilagarica* was successfully established in MS media containing 3 mg L^{-1} 2, 4-D in combination with 1 mg L^{-1} Kin. The growth kinetics of the well suspended cells (Fig. 1c) in cell suspension culture of *A. nilagarica* exhibited regular sigmoid growth curves when FW, DW and SCV of cell biomass were plotted against a 45-day culture period (Fig. 1d). The growth rate of cells were slower at the beginning, but at the end of culture, a significantly higher biomass accumulation was achieved. After a prolonged lag phase of 10 days, the cells were dividing and grew vigorously entering their exponential phase. The growth rate of cells continued to increase until it reached a stationary phase on 25th day of culture when the peak of growth with maximum fresh weight biomass of an average of 3.206 g FW or 0.323 g DW or 13.889 ml SCV (v/v) culture^{-1}as well as maximum accumulation of total artemisinin in cell suspension and in liquid media (0.013 %) was obtained (Figs. 1c and 1d).

Intracellular artemisinin in cell suspension culture of *A. nilagarica* (0.013 %) was significantly lower than the calli (0.028 %) induced in solid media. In the earlier reports also, artemisinin production was found to be quite low or sometimes even not detectable in the dedifferentiated cells, such as, callus or cell suspension cultures[29, 30]. However, several studies have reported the successful enhancement of artemisinin production by elicitors such as fungal elicitors, (22S, 23S)-homobrassinolide, methyl jasmonate, abscisic acid, metal ion like calcium, blooming agent (gibberellic acid), chitosan and yeast extract in cell suspension cultures and hairy root cultures of *A. annua*[14, 31, 32]. Therefore, enhancement of artemisinin accumulation in cell suspension culture of

A. nilagarica using various elicitors and precursors specific to artemisinin biosynthetic pathways could be a future perspective for research.

CONCLUSION

Friable callus suitable for cell suspension culture of *A. nilagarica* was induced and cell suspension culture with significant artemisinin accumulation was successfully established, for the first time. Combination of 2, 4- D and Kin were found to be more effective than NAA and BAP for production of artemisinin *in vitro*. The present report thus provides an initiative for future application of various enhancement techniques of *in vitro* plant metabolic engineering using recent biotechnological methods.

ACKNOWLEDGMENTS

Mayengbam Nganthoi is thankful to University Grant Commission (UGC) New Delhi, India, for providing Junior Research Fellowship (JRF) and Senior Research Fellowship (SRF) *vide* grant number 17-31/08 (SA-I).

REFERENCES

1. Valles J, Torrell M, Garnatje T, Garcia-Jacas N, Vilatersanaand R, *et al.* Genus *Artemisia* and its allies, phylogeny of the sub-tribe *Artemisiinae* (*Asteraceae*, *Anthemadea*) based on nucleotide sequences of nuclear ribosomal DNA internal transcribed spacers (ITS). Plant Biol. 2003; 5: 274-284.
2. Shah NC. The economic and Medicinal Artemisia species in India. The Scitech. Journal. 2014; 1(1): 29-38.
3. Sinha SC.1996. Medicinal Plants of Manipur, (Mass and Sinha, Imphal, Manipur, India) 795001.
4. Nganthoi M, Sanatombi K. *Artemisia myriantha* ex Bess var. *pleiocephala* (Pamp.) Y.R. Ling (Asteraceae) – A new floral record of Manipur, India. Indian J. Plant Sci. 2016; 5(2): 12-15.
5. Devi WI, Devi GS, Singh CB. Traditional herbal medicine used for the treatment of diabetes in Manipur. Indian Res. J. Pharm. Biol. Chem. Sci. 2011; 2: 709-715.
6. Singh LW. Traditional medicinal plants of Manipur as anti-diabetics. J. Med. Plants Res. 2011; 5: 667-687.
7. Lokho A. The folk medicinal plants of the Mao Naga in Manipur, North East India. *Int. J. Sci. Res. Pub.* 2012; 2: 1-8.
8. Devmurari VP, Pandey S, Goyani MB, Jivani NP. Phytochemical screening of ethanolic extract of *Artemisia nilagarica. Int. J. Chem. Sci.* 2010; 8: 2099-2104.
9. Nganthoi M, Heikrujam J, Kabita KC, Sanatombi K. Screening and Quantification of Artemisinin and Phytochemicals Content in *Artemisia nilagarica* (C.B. Clarke) Pamp. J. Plant Biol. Soil Health. 2016; 3(1): 4.

10. Paulsamy S, Senthilkumar P, Shivashanmugam M. Clonal multiplication strategies for medicinal plants inhabiting Nilgiri biosphere reserve, the Western Ghats, India. J. Theo. Exp. Biol. 2008; 4: 115-119.
11. Ganesan CM, Paulsamy S. Mass propagation of a threatened medicinal plant, *Artemisia nilagarica* (C.B. Clarke) Pampanini inhabiting high hills of Nilgiri, the Western Ghats. Indian J. Fund. Appl. Life Sci. 2011; 1: 14-21.
12. Mohammad A, Alam P, Ahmad MM, Ali A, Ahmad J, Abdin MZ. Impact of plant growth regulators (PGRs) on callogenesis and artemisinin content in *Artemisia annua* L. plants. Indian J. Biotechnol. 2014; 13: 26-33.
13. Woerdenbag HJ, Luers JFJ, van Uden W, Pras N, Malingre TM, Alfermann AW. Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. Plant Cell Tiss. Organ. Cult. 1993; 32: 247-257.
14. Baldi A, Dixit VK.Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua* L. *Bioresour Technol*. 2008; 99: 4609-4614.
15. Yann LK, Jelodar NB, Keng CL. Investigation on the effect of subculture frequency and inoculum size on the artemisinin content in a cell suspension culture of *Artemisia annua* L. Australian J. Crop. Sci. 2012; 6(7): 801-807.
16. Ahlawat S, Saxena P, Alam P, Wajid S, Abdib MZ. Modulation of artemisinin biosynthesis by elicitors, inhibitor and precursor in hairy root cultures of *Artemisia annua* L. J. Plant Interactions. 2014; 9(1): 811-824.
17. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol. 1962; 15: 473-497.
18. Linsmaier EM, Skoog F. Physiol. Plant.1965; 18: 100-127.
19. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research. 1968; 50: 151–158.
20. Nitsch, JP, Nitsch C. Science. 1969; 163: 85-87.
21. White PR. The cultivation of animal and plant cells. 2nd Eds, *Ronald Press, New York*. 1963.
22. Mantell SH, Smith H. Cultural factors that influence secondary metabolite accumulation in plant cell and tissue cultures. In: Plant Biotechnology. (Eds.): Mantell SH, Smith H. Cambridge Uni Press, Cambridge. 1984; 75-108.
23. Paniego NB, Giulietti AM. *Artemisia annua*; dedifferentiated and differentiated cultures. Plant Cell Tiss Org Cult. 1994; 36: 163-168.
24. Whipkey A, Simon JE, Charles DJ, Janick J. *In vitro* production of artemisinin from *Artemisia annua* L. Phytother. Res. 1992; 1: 15-25.
25. Zia M, Abdulmannan, Chaudhary MF. Effect of amino acids and plant growth regulators on artenisinin production in the callus of *Artemisia absinthium.* Pak. J. Bot. 2007; 39(2): 799-805.
26. Singh A, Sarin R. *Artemisia scoparia*- a new source of artemisinin. Bangladesh J. Pharmacol. 2010; 5(1):17-20.
27. Afshari RT, Angoshtari R, Kalantari S. Effects of light and different plant growth regulator son induction of callus growth in rapeseed (*Brassica napus* L.) genotypes. Plant Omics J. 2011; 4(2): 60-67.
28. Lee EJ, Mobin M, Hahn EJ, Paek KY. Effects of sucrose, inoculum density, auxins, and aeration volume on cell growth of *Gymnema sylvestre*. J. Plant Biol. 2006; 49 (6): 427-431.

29. Martinez BC, Staba EJ. The production of artemisinin in *Artemisia annua* L. tissue cultures. Adv. Cell Cult. 1988; 6: 69-87.
30. Brown GD. Annulide, a sesquiterpene lactone from *Artemisia annua*. *Phytochemistry*. 1993; 32: 391-393.
31. Liu C, Wang Y, Xu X, Ouyang F, Ye H, Li G. Improvement of artemisinin accumulation in hairy root cultures of *Artemisia annua* L by fungal elicitor. Bioproc. Eng. 1999; 20: 161-164.
32. Putalun W, Luealon W, De-Eknamkul, Tanka H, Shoyama Y. Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. Biotechnol. Lett. 2007; 29: 1143-1146.