Advances in Life Sciences 2012, 2(4): 108-111
DOI: 10.5923/j.als.20120204.05

In Vitro Production of Cucurbitacins From *Trichosanthes cucumerina* L. var. *cucumerina*

Devendra N. K[1,*], Everaldo G. Attard[2], Raghunandan D[3], Seetharam Y. N[1]

[1]Plant systematics and Medicinal Plant Laboratory Department of P.G. Studies and research in Botany Gulbarga University, GULBARGA-585 106, India
[2]Institute of Agriculture, University of Malta, Msida MSD06, Malta
[3]H.K.E's Matoshree Taradevi Institute of Pharmaceutical Sciences, Sedam Road, Gulbarga, Karnataka, India

Abstract The objectives of this study were to investigate the effect of growth regulators in callus induction, increase of biomass and to the yield more of cucurbitacin and cucurbitacin-E in leaf explants of *Trichosanthes cucumerina* L. var. *cucumerina*. The explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of auxins like 2,4-dichlorophenoxy acetic acid (2,4-D), [alpha]-naphthalene acetic acid (NAA), Indole butyric acid (IBA), Indole acetic acid (IAA), cytokinins like Kinetin (kn) and Benzyl adenine (BA). The different concentrations and combinations of BAP + IBA, 2, 4-D + BAP and 2, 4-D + kn increased the callus of fresh weight and dry weight. Among all concentrations and combinations, the best results revealed that leaf-derived callus cultured on 2,4-D (3.0mg^{-1}) + kin (1.0 mg^{-1}) produced the highest total cucurbitacins content with an optimum yield 4.9% w/w and cucurbitacin-E 2.75% w/w at third week.

Keywords *Trichosanthes Cucumerina* L. Var. *Cucumerina*, Cell Culture, Cucurbitacins

1. Introduction

The plant *Trichosanthes cucumerina* L. var. *cucumerina* (in English Chinese cucumber or wild snake gourd) belongs to family cucurbitaceae. The fruit is known to contain many form of cucurbitacins, (1, 2, 3) and due to its bitterness the fruit is been using in many ayurvedic preparations (4) and also in Indian folk medicine to cure jaundice (5), to reduce congestion on congestive cardiac failure (6).

These days many people are interested in the beneficial effects of food on health, and cucurbitacins have been studied because of the wide range of biological activities they exhibit in living beings. They are predominantly found in the Cucurbitaceae and several other families of the plant kingdom. A number of compounds of this group have been investigated for their cytotoxic (7), hepatoprotective (8), cardiovascular (9), antidiabetic (10) Antibacterial (11), anti- inflammatory (12-13) and antioxidant activity of cucurbitacins B and I and the glucosides of cucurbitacin I and L (14). Additionally, several studies indicated that different cucurbitacin species inhibit the proliferation of cancer cells through different mechanisms (15-19)

The members of cucurbitaceae has gained increasing attention as a natural insecticide and its activity has been evaluated against many economically important insect species. Cucurbitta spp. are deterrent, antifeedant, growth-regulating and fertility – reducing properties on insects (20-21) Also, it is used as an abortifacient, cathartic, purgative and vermifuse, and for the treatment of fever, cancer, amenorrhea, jaundice, leukemia, rheumatism, tumour and as an insect repellant (22).The content of cucurbitacins in various organs of *Trichosanthes cucumerina* L. var. *cucumerina* has been investigated (23).

The present study was carried out to develop an efficient protocol for callus induction, proliferation, total cucurbitacins and cucurbitacin-E accumulation under the effect of different growth hormones with leaf explant of *Trichosanthes cucumerina* L. var. *cucumerina* to study various pharmacological effects.

2. Materials and Methods

Quantitative determination of cucurbitacins from *in vitro* callus culture:

Plant material

Trichosanthes cucumerina L. var. *cucumerina* seeds were obtained from mature fruits collected from Khanapur forest Bhalki taluka, Bidar District India. The collected seed materials were botanically authenticated by the Botany department, Gulbarga University, Gulbarga (Voucher No. HGUG-804). Also, the plant was confirmed with authenticated herbariums at the Centre for Ecological Studies, IISc, Bangalore, and Botanical Survey of India, Pune. The col-

* Corresponding author:
dnkage@rediffmail.com (Devendra N. K)
Published online at http://journal.sapub.org/als

lected seeds preserved in amber bottles under normal lab conditions until used.

Media preparation

The basal medium described by Murashige and Skoog (MS) (24) was used. Deferent concentrations of plant growth regulators were added to the MS medium. The media were sterilized by autoclaving at 121℃ for 15 min.

Callus induction

The seeds were surface sterilized with 2% mercuric chloride for 1 min. then washed thrice with distilled water and soaked for 24 hrs in a beaker. The sterilized seeds were used for germination on MS hormone free medium. After few days, the seedlings were excised to yield explants for callus production. The initiated callus was then maintained on MS medium supplemented with BAP 1.0mg^{-1} and IBA 0.5mg^{-1} at 24±2℃ in continuous light (2400 lux) and maintained by transferring approximately 1 g of callus every 4 weeks.

Callus propagation

First experiment

Approximately 1 g of initiated callus material was cultured, on different media, to select the best plant growth regulator combination.

Second experiment

1-1.5 g aliquots of callus were cultured in conical flask containing 100 ml MS medium supplemented with Kn/2,4-D combinations with hormone concentrations of 0.0, 0.5, 1.0, 2.0 and 3.0 mg^{-1} to determine the best callus proliferation and yield of cucurbitacins and cucurbitacin E.

Fresh and dry weight measurement

For the first experiment the callus samples were sacrificed on week 3, while for the second one, samples were collected at weekly intervals for a maximum of 5 weeks. After obtaining the fresh weights, the samples were then dried at 40℃ and the dry weights obtained after 24 h.

Determination of cucurbitacins

Solvents and reagents

Absolute ethanol, petroleum ether 30-40℃, chloroform and phosphomolybdic acid (all at analar grade). A cucurbitacin E reference standard was used.

Sample solutions

For total cucurbitacin assay, dried callus material (100-200 mg per sample) was extracted with absolute ethanol (5ml) for 2 h, after centrifugation (2000 rpm for 3 min), the supernatant was mixed with an equal volume of petroleum ether, the precipitate obtained was filtered and dissolved in absolute ethanol (5ml), and then reduced to a volume of 2 ml as above.

Reference solution

The reference standard cucurbitacin E was dissolved in ethanol and serial dilutions (0.01-1.0 mg/ml) were prepared.

Assey

All samples (100µl, in duplicate), together with various concentrations of cucurbitacin E standard as per (25) at room temperature. The absorbance was measured at 492 nm after 5 min on a MTP reader STATFAX2100, USA. The results were expressed as w/w% calculated from dry callus weight and then analyzed statistically by ANOVA.

3. Results and Discussion

Quantitative determination cucurbitacins from in vitro callus culture:

Experiment 1 with mixed PGRs grid

Biomass accumulation:

Table-1 indicated that BAP was the best cytokinin in combination with IBA as regards callus accumulation. Calluses on these plant growth regulators were friable and white in colour, while with kinetin and 2,4-D, these were relatively hard and brown in colour, and showed a slow rate of accumulation. Secondary metabolite accumulation:

On other hand 2,4-D and Kn gave the most significant Cu and CuE accumulation, especially when compared to IBA and BAP (Table-2). Overall, an inverse proportionality was observed between callus weight and cucurbitacin yield. This was clearly shown in the combination of 2,4-D and IBA with BAP, having dry weights of 187 and 258 mg, respectively, and corresponding Cu contents of 0.359 and 0.256% w/w. similar results were observed in *Ecballium elaterium* (26).

Table 1. The effects of plant growth regulators 1.0mg^{-1} of medium on growth of *T. cucumerina* L. var. *cucumerina* callus after 3 weeks in culture

	None		IBA		2,4-D		NAA		IAA	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
None	1040 ± 13.5	84 ±1.87	1570 ±14.14	112 ±8.64	1620 ±7.48	124 ±5.12	1228 ±14.28	103 ±20.5	1002 ±11.57	92.8 ±4.53
BAP	1020 ±12.24	81 ±5.09	3108 ±12.42	258 ±5.93	2636 ±12.08	187 ±40.52	1440 ±11.40	114 ±6.92	1140 ±9.48	85 ±1.63
Kn	1152 ±12.8	106 ±1.87	2008 ±13.92	199 ±4.21	2032 ±10.68	234 ±10.77	1238 ±11.58	121 ±4.40	1320 ±7.07	124 ±2.98

Table 2. The effects of plant growth regulators (1.0 mg^{-1} of medium) on the production of Cu and CuE (%w/w, on dry weight) after 3 weeks

		None	Ki	BAP
None	Cu	0.398	0.584	0.201
	CuE	0.218	0.398	0.099
2, 4-D	Cu	0.543	0.604	0.256
	CuE	0.271	0.223	0.162
NAA	Cu	0.469	0.461	0.282
	CuE	0.212	0.303	0.094
IAA	Cu	0.496	0.341	0.359
	CuE	0.278	0.273	0.104
IBA	Cu	0.411	0.523	0.278
	CuE	0.152	0.312	0.086

cucurbitacin (Cu), cucurbitacin E (CuE)

Table 3. Accumulation of cucurbitacins Cu and CuE with different combinations of plant growth regulators at week 3 for leaf

	2, 4-D									
	0.0 mg^{-1}		0.5 mg^{-1}		1.0 mg^{-1}		2.0 mg^{-1}		3.0 mg^{-1}	
	Cu	CuE	Cu	CuE	Cu	CuE	Cu	CuE	Cu	CuE
Kn 0.0	0.39	0.21	1.55	0.16	1.75	0.40	2.04	0.46	2.62	1.74
0.5	0.61	0.23	0.80	0.28	0.60	0.22	1.30	0.12	2.43	1.90
1.0	1.30	0.14	1.48	0.16	1.60	0.15	1.98	0.25	4.90	2.75
2.0	0.82	0.34	0.62	0.34	1.55	0.13	1.56	0.16	1.81	1.18
3.0	0.52	0.30	0.70	0.17	1.34	0.09	1.27	0.14	0.89	0.19

Experiment 2 with 2,4-D/Kn grid

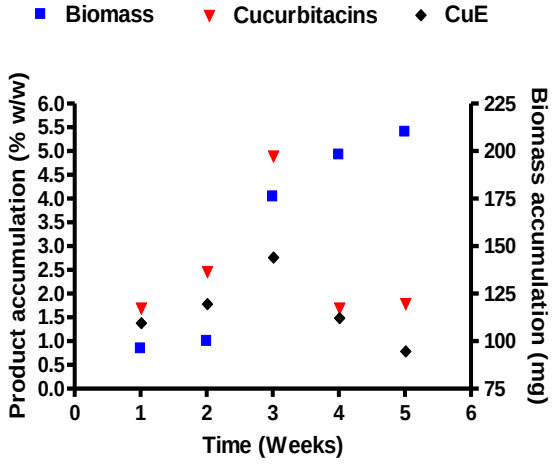

Figure 1. The pattern of growth linked accumulation of cucurbitacins (Cu) and cucurbitacin E (CuE) in *T. cucumerina* L. var. *cucumerina* (treatment: 2,4-D 3.0mg^{-1} + Kn 1.0mg^{-1}

It is evident that the combination with the highest callus accumulation proved to be the 0.5mg^{-1} 2,4-D as compared to the rest. The accumulation of secondary metabolites was best at week 3 with a decline in Cu and CuE at week 4. The 1.0mg^{-1} Kn concentration in combination with different 2,4-D concentrations gave optimum metabolite accumulation 0.52– 5.0 % w/w at week 3, (Table-3, Fig-1). Increase in the lone concentration of 2,4-D and Kn showed increase in the significant accumulation of Cu and CuE, while there was a decline with an increase in 2,4-D and Kn concentration. The rate of production of CuE from the Kn 1.0mg^{-1} + 2,4-D 3.0mg^{-1} treatment was approximately one and half times higher than that from Kn 2.0mg^{-1} and 2,4-D 3.0mg^{-1}. But, at moderate concentrations 2,4-D inhibits Cu accumulation, as occurs with other metabolites (27). In the case of CuE, significant yields were obtained with higher 2,4-D concentrations. These results are in agreement with the findings of Halaweish and Tallamy, (28). They confirmed that MS-medium supplemented by 2 mg^{-1} 2,4-D in combination with 1 mg^{-1} kin produced the highest biomass of callus tissues when induced from the rootless seedlings explants *Cucurbita andreana*. The optimum medium for callus induction of *Eremochloa ophiuroides* (Munro) was MS media supplemented with 2,4-D at 1.0 mg^{-1} (29).

The leaf explants of *T. cucumerina* L. var. *cucumerina* demonstrated better callus induction and also proved to synthesize total cucurbitacins and cucurbitacin-E in undifferentiated callus. Using of 2, 4-D in combination with kn was found to be the best treatment for cucurbitacins production and accumulations of total cucurbitacins and cucurbitacin-E in callus tissues. Enhancement of callus induction and accumulation of cucurbitacins were higher than those obtained from the in vivo grown plant parts. This study suggested that in vitro secondary metabolites production by *T. cucumerina* L. var. *cucumerina* callus cultures could be considered an appropriate alternative method to whole plant extraction.

REFFERENCES

[1] Jiratchariyakul, W and Frahm, A. W (1992). Cucurbitacin B and Dihydrocucurbitacin B From *Trichosanthes cucumerina* L. Mahidol Univ. J. Pharm. Sci. *Journal of Pharmaceutical science.* 19, 5-12.

[2] Sardsengjun (1993) Sardsengjun, C (1993). Master thesis, Department of Pharmacy, Graduate School, Mahidol University

[3] Matee *et al.,* (2002) Matee, B., Sunanta, W., Anon, C and Apichart, S (2002).The chemical constituents of *Trichosanthes cucumerina.*

[4] Devendra N.K., Rajanna L, Sheetal C and Seetharam Y.N., (2008). In vitro Clonal Propagtion of *Trichosanthes cucumerina* L. var. *cucumerina*. Plant Tissue Cult. & Biotech. 18(2): 103-111

[5] Devendra N. K., Vijaykumar B. M and Seetharam Y. N. (2010). Folklore Medicinal Plants of Gulbarga District, Karnataka, India. e-*Journal of Indian Medicine.* 3(1); 23-30.

[6] Pullaih, 2006 Pullaih, T (2006). Encyclopaedia of World Medicinal Plants. Vol-IV, Regency Publication New Delhi. pp-1977.

[7] Dahai, M., Shigao, Q., Liguang, L and Weimin, Z (2008). Cytotoxic cucurbitane type triterpenoids from Elaeocarpus. *Planta Med.* 74:1741-1744.

[8] Naoki, W., Dong, Yin., James, O'Kelly., Talin, H., Beth, K., Jonathan, S., Hongtao, X. and Philip, H. K (2008). Cucurbitacin B has a potent antiproliferative effect on brest cancer cells *in vitro* and *in vivo*. *Cancer Science*. 99:1793-1797

[9] Liu-T., M. Zhang, H. Zhang, C. Sun, X. Yang, Y. Deng, and W. Ji. (2008). Combinedantitumor activity of cucurbitacin B and docetaxel in laryngeal cancer. European Journal of Pharmacology, 587, 78 84.

[10] Jayaprakasam, B., N. Seeram and M. Nair, (2003). Anticancer activities of cucurbitacins from *Cucurbita andreana*. Cancer Lett., 189: 11-16.

[11] Devendra, N. K., Vijaykumar B. M., Subhash, B., and Y. N. Seetharam (2010). Phytochemical Profile and Antibacterial Properties of the Fruit and Leaf of the *Momordica dioica* (Roxb.) Willd. *Pharmacologyonline* 3: 207-211.

[12] Devendra N. K., Raghunandan Deshpande and Y. N. Seetharam (2010). Anti-inflammatory activity of *Trichosanthes cucumerina* L. var. *cucumerina* seed. *Pharmacologyonline*. 2: 172-176.

[13] Peters, R., T. Saleh, M. Lora, C. Patry, A. de Brum-Fernandes, M. Farias and R. Ribeiro-do-Valle, (1999). Anti-inflammatory effects of the products from *Wilbrandia ebracteata* on carrageenan-induced pleurisy in mice. Life. Sci., 64: 2429-2437.

[14] Abbas, D., G. Simon, R. Ali, N. Hossein, M. Masoud, N. Lutfun and D. Satyajtt, (2006). Flavone Cglycoside and cucurbitacin glycoside from Citrullus colocynthis. DARA, 14(3): 109-114.

[15] Jing. N. and D. J. Tweardy. (2005). Targeting Stat3 in cancer therapy. Anticancer Drugs, 16.601-607.

[16] Tannin-Spitz T, S. Grossman, S. Dovrat, H. E. Gottlieb, and M. Bergman. (2007). Growth inhibitory activity of cucurbitacin glucosides isolated from *Citrullus colocynthis* on human breast cancer cells. Biochem Pharmacol. 73:56- 67.

[17] Jiazhi, S. Michelle A.B., Richard J, Sandra K.L., Domenico C and Sa ïd M.S. (2005). Cucurbitacin Q: a selective STAT3 ac-tivation inhibitor with potent antitumor activity. Oncogene., 24: 3236–3245

[18] Blaskovich, M., J. Sun, A. Cantor, J. Turkson, R. Jove and S. Sebti, (2003). Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res., 63: 1270- 1279.

[19] Sun, J., M. Blaskovich, R. Jove, S. Livingston, D. Coppola and S. Sebti, (2005). Cucurbitacin Q: a selective STAT3 activation inhibitor with potent antitumor activity. Oncogene, 24: 3236-3245.

[20] Prabuseenivasan, S., M. Jayakumar, N. Raja and S. Ignacimuthu, (2004). Effect of bitter apple, *Ctrullus colocynthis* (L.) Schrad seed extracts against pulse beetle, *Callosobruchus maculatus* Fab. (Coleoptera: Bruchidae). Entomol., 29: 81-84.

[21] Torkey, H.M., Abou-Yousef, H.M., Abdel A, zeiz, A.Z. and Hoda, E.A. (2009) Farid Insecticidal Effect of Cucurbitacin E Glycoside Isolated from *Citrullus colocynthis* Against *Aphis craccivora* Australian Journal of Basic and Applied Sciences, 3(4): 4060-4066,).

[22] Duke, 2006. Dr. Duke's Phytochemical and Ethnobotanical Databases, Ethnobotanical uses of *Citrullus colocynthis* (Cucurbitaceae). Available on-line at: http://www.ars-grin.gov/cgi-bin/duke/ethnobot.pl

[23] N. K. Devendra, E. G. Attard, D. Raghunandan, Y. N. Seetharam (2011) Study on Seasonal Variation on the Content of Cucurbitacin of Various Vegetative Parts of *Trichosanthes cucumerina* L. var. *cucumerina* International Journal of Plant Research. 1(1): 25-28 DOI: 10. 5923/j.plant.20110101.04

[24] Murashige T, F. Skoog. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.

[25] Yang P. S, Z. Liu, W. Cao, and C. T. Che. (1991). Cucurbitacin contents in *Hemsleya dolichocarpa*. Am. J. Chin. Med, XIXX: 51-56.

[26] Everaldo G. A. and S. Anthony. (2001). *Ecballium elaterium:* an in vitro source of cucurbitacins. Fitoterapia, 72:46.

[27] Sakuta M, A. Komamine, F. Constabel, I. K. Vasil. (1987). Cell Culture and somatic genetics of plants, Vol. 4, Cell culture in phytochemistry. U.S.A.: Academic Press, 97-114.

[28] Halaweish, F. T. and D. W. Tallamy. (1998). Production of cucurbitacins by cucurbit cell cultures. Plant Science, 131, 209-218.

[29] Yuan, X., Z. Wang, J. Liu, and J. She. 2009. Development of plant regeneration system from seed-derived calluses of centipede grass *Eremochloa ophiuroides* (Munro.)Hack. Scientia Horticulturae, 120:96-100.