THE INFLUENCE OF COPPER AND COBALT IONS ADDITION IN CELL SUSPENSION CULTURE OF *Impatiens balsamina* L. TOWARD COUMARINS YIELD

Zainab[1] dan Soegihardjo C.J.[2]
Faculty of Pharmacy Ahmad Dahlan University

Abstract

Background. *Coumarins display various bioactivities in vitro such as anticoagulant, estrogenic, dermal photosensitizing, antimicrobial, vasodilator, molluscicidal, anthelmintic, sedative and hypnotic, analgesic, antimalarial, antitumor, anti-HIV, antiviral, Ca antagonistic, cytostatic, inhibition of 5-lipoxygenase, inhibition of monoamine oxidase which are very potent for medicine. The study on cell cultures of Impatiens balsamina L. have been done before and the cell cultures were capable of producing coumarins derivate, scopoletin and isofraxidin. The aim of this research was to study the effect of addition copper and cobalt metal ions into cell culture of I. balsamina on the yield of coumarins.*

Method. *Increasing the yield of coumarins in cell cultures of I. balsamina, was achieved by addition of elicitor Cu and Co metal ions in the liquid MS media supplemented with 1.0 mg/L kinetin and 0.1 mg/L 2,4-dichlorophenoxyacetic acid (1K; 0.1D) which initiated from callus grown on solid MS media (1K; 0.1D). Concentrations of Cu and Co metal ions which used in this study were 10, 20, and 30 fold of normal MS media (250, 500, 750 µg/L). The cell culture on treatment media were incubated at 22-28 ^{0}C with continuous dark periods on rotary shaker for 30 days. The quantitative analysis of coumarins in cell suspension cultures was measured by TLC Scanner.*

Result. *The addition of Cu metal ion could only produce one coumarin at Rf 0.72 and the highest coumarin production detected was 0.148%. While the addition of Co metal ion could produce complete coumarins as mother plant at Rf 0.23, 0.32 and 0.72 with highest coumarin production was 0.260% (ion Co 750 µg/l).*

Conclusion. *In short the addition of Cu and Co metal ions had influence both qualitative and quantitative of coumarins content in cell suspension culture of I. balsamina L.*

Keyword : *Impatiens balsamina L., cell suspension cultures, elicitor, Cu and Co ions, coumarins.*

[2]Faculty of Pharmacy Gadjah Mada University

ISBN : 978-979-18458-5-4

INTRODUCTION

Impatiens balsamina L. has been used as traditional medicine and had content of naphthoquinone, coumarin derivates, flavonoids, and saponins. Previous study has been reported that cell suspension culture of *I. balsamina* were capable of producing coumarin derivatives, scopoletin and isofraxidin (Panichayupakaranant, 2002). The coumarin derivatives have various of biological activities such as: anticoagulant, estrogenic, dermal photosensitizing, antimicrobial, vasodilator, molluscicidal, anthelmintic, antimalarial, antitumor, anti-HIV, antivirus, Ca antagonist, cytostatic, inhibitor of 5-lipookxigenase, inhibitor of monoamine oxidase that potentially develop into phytomedicine (Ojala, 2001; De Simone, *et al.*, 2001; Prince, *et al.*, 2006).

The development of plant tissue culture which produces high value of chemical compound is driven by the possibility to control the culture conditions that can lead to secondary metabolic pathways and the ability of cells to grow in bioreactor. Plant secondary metabolites are the result of plant interaction with the environment and can be induced and enhanced by the addition of biotic or abiotic elicitors. The addition of abiotic elicitor has several advantages including availability, relatively low price, and also available in pure form (Sudha & Ravishankar, 2002; Threlfall & Whitehead, 1988).

Plant cells react to the increasing concentration of metal ions from the environment by various mechanisms, including formation of metallothionein, phytochelatin, amino acids (cysteine, hystidine), organic acids (citric, malic) or secondary metabolites. As abiotic elicitors, Co^{2+} and Cu^{2+} ions, are more frequently used and get more attention because they give a positive effect on secondary metabolite production. Metal Mg, Co, and Ni are potential elicitors on the production of coumarins (Siatka, *et al*., 1995; Tapia, *et al*., 2001).

This study is aimed to determine the effect of Cu and Co ions addition in the MS medium of coumarins content in cell suspension cultures of *I. balsamina*.

METHODS

Callus growth

The seeds of *I. balsamina* were surface sterilized with 20 %v/v of commercial sodium hypochloride solution (Bayclin) for 12 min. After three times washing with sterile distilled water, the seeds were placed in glass pot containing double wet filter paper. After a week, all germinated seeds become sprout. The hypocotyl part from each sprout was cut ± 5 mm in length then they were used as explants. The 2 or 3 explants were placed in a glass pot containing 10 ml of MS basal medium supplemented with 1.0 mg/L kinetin, 0.1 mg/L 2,4 D, 3% (w/v) sucrose and 0.8% (w/v) Difto Bacto agar (callus induction medium). Four week after initiation, callus was transferred to the same medium for subculture. The callus cultures were maintained at 22-28^{0}C under 16 h light, using fluorescent lamps (Phillips TL 40 W) 50 cm distance.

Initiation cell suspension cultures of *I. balsamina*.

The cell cultures of *I. balsamina* were performed by using callus obtained from MS medium supplemented with 1.0 mg/L kinetin, 0.1 mg/L 2,4 D. About 1 g callus was cut into small pieces with sterilized scalpel and placed into 250 Erlenmeyer flask containing 50 ml of liquid medium supplemented with 1.0 mg/L kinetin, 0.1 mg/L 2,4 D. The flasks were incubated on rotary shaker at ± 100 rpm, 22-28^{0}C, 16 h light. The cell culture were maintained under these conditions and sub cultured every 2 week until we got sufficiently number of cell suspension cultures for further treatment.

Treatment of Cu and Co ions addition in cell suspension cultures.

In order to study the influence of copper and cobalt ions on the coumarin content in cell suspension cultures of *I. balsamina*, ca. 4-5 g of cell aggregates were transferred to a series of treatment media (see Table 1) and incubated on rotary shaker at ± 100 rpm, 22-28^{0}C, continuous dark period (black plastic wrap) for 30 days. After 30 days incubation, biomass of cell suspension cultures were separated from liquid media using paper filter by vaccum Buchner. The biomass were washed three times with distilled water to ensure that they free of liquid medium before being dried in oven at 40^{0}C until constant weight.

Tabel I. The composition of Cu and Co ions in liquid MS medium treatment

	Concentration of Cu and Co ions in liquid MS (μg/L)							
	LC	**DC**	**10 Cu**	**20 Cu**	**30 Cu**	**10 Co**	**20 Co**	**30 Co**
Ion Cu	25	25	250	500	750	25	25	25
Ion Co	25	25	25	25	25	250	500	750

Keterangan :
LC : Control of Continuous light
DC : Control of continuous dark

Qualitative and quantitative analysis of coumarins content

Thin Layer Chromatography (TLC) was used as method for detection coumarin accumulated in the cell cultures. Silica gel 60 F_{254} was used as stationary phase and chloroform: methanol (4:1) as mobile phase. The identitification of compounds were confirmed by the obtained Rf, spot characteristics under UV_{254}, UV_{365} irradiation before and after sprayed with 5% KOH in ethanol. For quantitative assay, the 5 μL of 10 % w/v from each sample in methanol extracted by reflux for 30 min were applied as bands on TLC plate of silica gel 60 F_{254}. Then TLC plate was eluted by solvent of chloroform: methanol (4:1) to run for a distance of 8.5 cm. The conditions of the TLC scanner instrument were set to a maximum wavelength using D_2 lamp; slit dimension 10.0 x 0.2 mm; monochromator bandwidth 20 nm. The measurement was set to the mode of absorption with scanning speed of 20 mm/s. The area under the peaks of coumarin were integrated and converted to concentration using its calibration curve. The calibration curve was established from standard coumarin in the range of 0.1-2.0 μg (0.1 ; 0.25; 0.5; 1.0; 2.0 μg), with linear equation Y = 1.2533 X + 0.3937 (R = 0.9919).

RESULTS AND DISCUSSION

Qualitative analysis of coumarins compounds were done by thin layer chromatography with silica gel F_{254} as stationary phase and chloroform: methanol (4:1) v/v as mobile phase. All spot of coumarin compounds on the chromatogram indicated the distinctive of fluorescence quenching under the UV_{254} light irradiation and gave the intense blue or blue green fluorescence (simple coumarins), yellow, brown, blue or blue green fluorescence (furano and pyranocoumarins) under UV_{365} light irradiation. The non-substituted coumarin flouresces yellow-green in UV_{365} only after treatment with KOH reagent or vapour ammonia (Wagner & Bladt, 1996). Coumarin spots on chromatograms were detected at Rf 0.23, Rf 0.32 and Rf 0.72, as shown in Table II.

ISBN : 978-979-18458-5-4

Tabel II. Coumarins detection on mother plant and biomass of *I. balsamina* cell suspension cultures in Cu and Co ions addition for 30 days

No	Rf	Treatment											Coumarin
		Leaf	Stem	C	LC	DC	10Cu	20Cu	30Cu	10Co	20Co	30Co	
1	0.07	+	+	-	-	-	-	-	-	-	-	-	-
2	0,23	-	-	-	+	+	-	-	-	+	+	+	+
3	0,26	+	+	-	-	-	-	-	-	-	-	-	+
4	0,32	-	-	+	+	+	-	-	-	+	+	+	+
5	0,61	-	-	+	+	+	+	+	+	+	+	+	-
6	0,72	-	+	+	+	+	+	+	+	+	+	+	+
7	0,78	+	-	-	-	-	-	-	-	-	-	-	-
8	0,84	+	+	-	-	-	-	-	-	-	-	-	-
9	0,94	+	+	-	-	-	-	-	-	-	-	-	-
P	0,82	-	-	-	-	-	-	-	-	-	-	-	+

Keterangan:

P : Coumarin standart 0,1 % w/v in methanol + : spot

C : Callus - : no spot

DC : Control of continuous dark

LC : Control of continuous light

All concentration of Cu ion addition treatment showed that only one spot of coumarin was produced (Rf 0.72), while spots of coumarins Rf 0.23 and 0.32 were not produced. The highest coumarin concentration was achieved 0.148% in 10 fold addition of Cu ion (250 µg/l). The all concentration of Co ion addition treatment were able to produce 3 coumarins at Rf 0.23, Rf 0.32 and Rf 0.72 same as mother plant. There are qualitatively and quantitatively differences among mother plant, treatment of Cu and Co ion addition toward coumarin content. The coumarins content at Rf 0.23 was found in plant origin, control of cell suspension and treatment of Co ion addition with the highest concentration 0,047%. While in the callus and Cu treatment produced no coumarin at Rf 0.23 because the callus consist of undifferentiated cells so they could not synthesize these coumarins.

The coumarin with Rf 0.32 was detected in mother plant, callus, control of cell suspension, and cell suspension with Co ion treatment, whereas the Cu ion treatment was not produced. This is caused by the addition of Cu ion which lead cells toxicity so the cells are unable to synthesize the coumarin compounds. In the treatment of Cu ions, the highest coumarin content was 0.148 % on 10 fold addition of Cu ion (250 ug/L) higher than that of mother plant (0.08 %).

The coumarin compounds with Rf 0.72 was detected in mother plant, callus, control of cell suspension and cell suspension in all treatments with both Cu and Co ions. The highest concentration of coumarin content was 0.260 % on 30 fold addition of Co ion

(750 µg/L) higher than that mother plant (0.08 %). The quantitative assay of coumarins content was performed by TLC scanner, the result can be seen in Table III and Figure 1.

ion is more suitable to be used than copper ion as an elicitor in *I. balsamina* suspension cultures to stimulate the secondary metabolites production specialy for coumarins.

Tabel III. Coumarins content in mother plant, callus and biomass of *I. balsamina* cell suspension cultures

Treatment	Coumarin (%)		
	Rf 0,23	Rf 0,32	Rf 0,72
Callus	0	0.026 + 0.004	0.186 + 0.049
LC	0.014 + 0.008	0.072 + 0.003	0.196+ 0.038
DC	0.022 ± 0.009	0.121 ± 0.004	0.169 ± 0.032
10 Cu	0	0	0.148 ± 0.027
20 Cu	0	0	0.115 ± 0.040
30 Cu	0	0	0.131 ± 0.034
10 Co	0.034 ± 0.004	0.118 ± 0.009	0.189 ± 0.057
20 Co	0.042 ± 0.011	0.125 ± 0.012	0.232 ± 0.058
30 Co	0.046 ± 0.006	0.075 ± 0.008	0.260 ± 0.050
Stem	0.074 ± 0.007	0	0.010 ± 0.004
Leaf	0.037 ± 0.001	0.082 ± 0.008	0.077 ± 0.022

N : 3 replicates

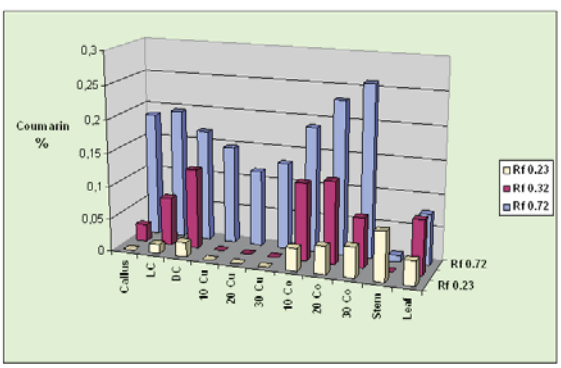

Figure 1. The histogram of coumarin content at Rf 0,23, Rf 0,32 and Rf 0,72 in the samples (laef, stem, and cell suspension cultures of ***I. balsamina***)

CONCLUSIONS

In general the addition of Cu and Co metal ions influenced both qualitative and quantitative of coumarins content in cell suspension culture of *I. balsamina* L. Our finding suggested cobalt

ACKNOWLEDGEMENT

The author wishes to thank Faculty Pharmacy of Ahmad Dahlan University and DIKTI for the support in this research.

REFERENCES

De Simone, F., Aquino, R., De Tommasi, N., Mahmood, N., Piacente, S., and Pizza, C., 2001, Anti-HIV Aromatic Compounds from Higher Plants in: *Bioactive Compounds from Natural Sources*, Corrado Tringgali (Ed), Taylor & Francis Inc, New York, 324, 325.

Ojala, T., 2001, Biological Screening of Plant Coumarins, *Academic Disertation*, Faculty of Science University of Helsinki, 21-30.

Panichayupakaranant, P., Tewtrakul, S., 2002, Plumbagin Production by Root Cultures

of *Plumbago rosea*, *Elect. J. Biotech.* 5: 228-232.

Panichayupakaranant, P., 2001, Naphthoquinone Formation in *Impatiens balsamina* Cell Cultures, *Pharm. Biol.* Vol 39. No. 1

Prince, M., Campbell, C.T., A. Robertson, T.A., Wells, A.J., and Kleiner, H.E, 2006, Naturally occurring coumarins inhibit 7,12-dimethylbenz[*a*]anthracene DNA adduct formation in mouse mammary gland, *J. Carcin.*, http://carcin.oxfordjournals.org/misc/terms.shtml 27 (6): 1204-1213.

Siatka, T., Kasparova, M., Sklenarova, H., and Solich, P., 1995, *Effect of Manganese (II), Cobalt (II), and Nickel (II) Ions on the Growth and Production of Coumarins in The Suspension Culture of Angelica archangelica* L., www.pubmed.gov diakses tanggal 01-03-2007.

Stokigt, J., Obitz, P., Falkenhagen, H., Lutterbach, R., and Endre, S., 1995, Natural Product and Enzymes from Plant Cell Cultures, *Plant Cell Tiss. Org. Cult.* 43: 97-109.

Sudha, G., and Ravishankar, G.A., 2002, Involvement and Interaction of Various Signaling Compounds on The Plant Metabolic Events During Defense Response, Resistance to Stress Factors, Formation of Secondary Metabolites and Their Molecular Aspects, *Plant Cell Tiss. Org. Cult.* 71: 181-212.

Tapia, G.T., Aparicio, A.J., Monroy, M.R., Sanchez, A.D., and Lopez, G.G., 2001, Influence of Cobalt and Other Microelements on the Production of Betalains and the Growth of Suspension Cultures of Beta vulgaris, *Plant Cell Tiss. Org. Cult.* 67: 19-23.

Threlfall, D.R., and Whitehead, I.M., 1988, The Use of Metal ions to Induce the Formation of Secondary Product in Tissue Culture, in Robins, R.J. & Rhodes, M.J.C. (eds), *Manipulating Secondary Metabolism in Culture*, Cambridge University Press, Cambridge, New York, New Rochelle, Melbourne, Sydney, pp.51-56.

Wagner, H., and Bladt, S., 1996, *Plant Drug Analysis: A Thin Layer Chromatography Atlas*, 162, 196, 275, 306, Spinger-Verlag, Berlin