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Journal of Chemical and Pharmaceutical Research, 2016, 8(2):808-812

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Production of asiaticoside from hairy roots culture of pegagan (*Centella asiatica* (l.)) urban using chitosan and its derivates as elisitors

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ABSTRACT

Research on production of asiaticoside from hairy root of pegagan, Centellaasiatica (L.), was conducted using chitosan, chito-oligosaccharide (COS), carboksimethyl chitosan (CMC) as elisitors. The objectives of this research were studying the effect of addition of chitosan and its derivates with several concentrations on wet weight of hairy roots of C.asiatica and on asiaticoside content in hairy roots of C.asiatica. The research was conducted using Randomized Design (Nested Design) with 3 replications. Factor A was kind of elicitors, Chitosan, Chito-oligosaccharide (COS), and Carboksimethyl chitosan. Factor B was concentrations of elisitors control, 15, 30, and 45 ppm. The results showed that addition of 30 ppm chito-oligosaccharide resulted in the highest frest weight of hairy roots of C.asiatica (551,68 mg/g) and the highest content of asiaticocide (5,97 mg/g freshweight or 5-folds of control).

Keywords: *Agrobacterium rhizogenes*, *C.asiatica*, hairy root, chitosan, COS, KMK, asiaticocide

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INTRODUCTION

Pegagan (*Centella asiatica* (L.) Urban belongs to family Umbelliferae which is potential as source of medicinal materials for different diseases. This plant contains secondary metabolite i.e. triterpenoids mainly asiaticoside, asiatic acid and madecacid acid. The secondary metabolites are used to prevent osteophorosis old people, increase intelligence of children, improve nerve problem and bood circulation, as anti-aging, cosmetics, as medicine for skin diseases, ovarium anti-cancer, anti-senility and anti-stress.

The need for the source of medicinal materials increases as the need for medicines increases. However, the natural production of secondary metabolite is very limited and the content is influenced by geographical position, climate, and diseases (1).

There have been many efforts done to increase the production of asiaticoside through *in vitro* cultural technique. Kim et al. (2004) found that methyl jasmonate and yeast extract stimulated asiaticoside production in the whole plant cultures of *C. Asiatica* (2). Kiong et al. (2005) reported that triterpenes production increased by supplementing a precursor of squalene to shoot culture of *C. asiatica*. Kim et al. (2007) showed the production of asiaticocide from hairy root cultures of *C. Asiatica* was elicited by methyl jasmonate (3). Loc and Giang (2012) found that asiaticoside content in cell cultures of Centella could be increased about 5 folds by adding 100 µM of 2-hydroxybenzoic acid at day 10 of inoculation .

One of other possibilities to increase production of asiaticoside from hairy roots of *C. Asiatica* was using chitosan as elisitor. Merkli (1997) found that chitosan increased production of diosgeninin suspension culture 3 folds higher compared to plants growing in field. Chitosan as transduction signal could stimulate the production of anthraquinone

in *Rubia tinctorum*culture 3 folds higher than control (4). Elisiting by chitosan 30 mg/50 mL could stimulate production of silymarin in hairy root cultures of *Silybummarianum* 5.26 folds after 96 days with wet weight 0,705 mg/g, and dry weight of hairy roots 0,535 mg/g (5).

Besides increasing the production of secondary metabolites, chitosan could also increase the growth of orchid tissue culture in liquid medium 4 folds faster than control. Besides that, chitosan with concentration 15 mg/l could also increasethe growth of callus culture of oil palm (*Elaeis guineensis* Jacq) (6).

Based on potential of chitosan as elisitor to increase production of varioussecondary metabolites *in vitro* and other effects to increase callus growth, thus a research was done on the production of asiaticocide on hairy roots of *C. Asiatica* with chitosan and its derivates as elisitor.

EXPERIMENTAL SECTION

Nested design was used with two factors, A (kind of elisitor): A1) chitosan; A2) chito-oligosaccaride (COS); A3) carboximethyl chitosan (CMC), and B (elisitors concentrations): B1) control B2) 15 ppm; B3) 30 ppm; B4) 45 ppm. Hairy roots of pegagan were induced using *Agrobacterium rhizogenes* strains R1000 (7). Explant of leaves used to induce hairy roots derived from one week old cultured explants *in vitro*. Hairy roots of *C.asiatica*were propagated using compact MS medium. Propagated roots were cut into ± 2 cm then were treated with elisitorsby adding them to liquid MS medium through mili pore membrane (0,22 µm) acceptically. Then the roots were incubated for 1 week.Wet weight of hairy roots was measured at age of 1, 2, 3 and 4 months using analytical balance.

Assesment of asiaticocide content in hairy roots culture of *C.asiatica* was done using HPLC by taking 50 mg hairy roots from each treatment. Hairy roots were ground finely. Then 0,1 M extract was added with 1 mL MeOH hidrocloric acid (1:1) and was vortexed, Then the exstract was added with 1mL MeOH: 0,1 M hidrocloric acid (1:1) and was vortexed, then centrifuged for 15 minutes at 14.000 rpm. Supernatant was lioptioned and resuspended with 1 mL HPLC eluen (solution containing 6 g KH_2PO_4, 1.95 g hexilamine; 50 mL H_3PO_4 1 M and 60 mL asetonitrilin 1 L solution with pH 2,8). Then it was screened with milleks 0,45 µm and injected to HPLC, Shimadzu type C- R4A with the following condition: wave length 206 nm, velocity 1 mL/minute, injection speed volume 20 µL/100 mL, column µ Bondapak C.18.

Data on fresh weight of hairy roots and asiaticocide were analized using nested design and DNMRT at 5% confidence level

RESULTS AND DISCUSSION

Fresh weight of hairy roots

Observation on growth of hairy roots of *C.asiatica* after given elisitor for 4 months showed the increase on fresh weight of hairy roots at all given concentrations of chitosan, COS, and CMC (as shown in Figure 1). Fresh weight of hairy roots was the same at age of 1 and 2 months. After 3 months, treatment with COS 30 ppm showed the highest fresh weight and increased until the last observation, 4 months. Adding elisitor chitosan, COS, CMC after 1 month, wet weight of each treatment was almost the same at concentration 15, 30, 45 ppm.

Treatment with chitosan, COS, CMC 30 ppm increased fresh weight at age 3 months, while at concentration 45 ppm and age 4 months the fresh weight increase was higher. During 4 months observation the fresh weight showed the same pattern from three elisitors at 15, 30 and 45 ppm. The lowest fresh weight was on control followed by 15 and 45 ppm, and the highest was on COS 30 ppm as shown in Figure 1.

In general giving elisitor COS showed the highest fresh weight of hairy roots (as shown in Table 1). Fresh weight of hairy roots of C.asiatica given chitosan and its derivates at age of 4 months was different among the treatments. Given chitosan elisitor 0, 15, 30 and 45 ppm showed different fresh weight. Treatment with chito-oligosaccaride (COS) 30 ppm showed the highest fresh weight (551.68 mg/g fresh weight) and control showed the lowest fresh weight (180.02 mg/g fresh weight). Average fresh weight of hairy roots of C.asiatica given COS 0 ppm, 15 ppm, 30 ppm and 45 ppm was different among treatments.

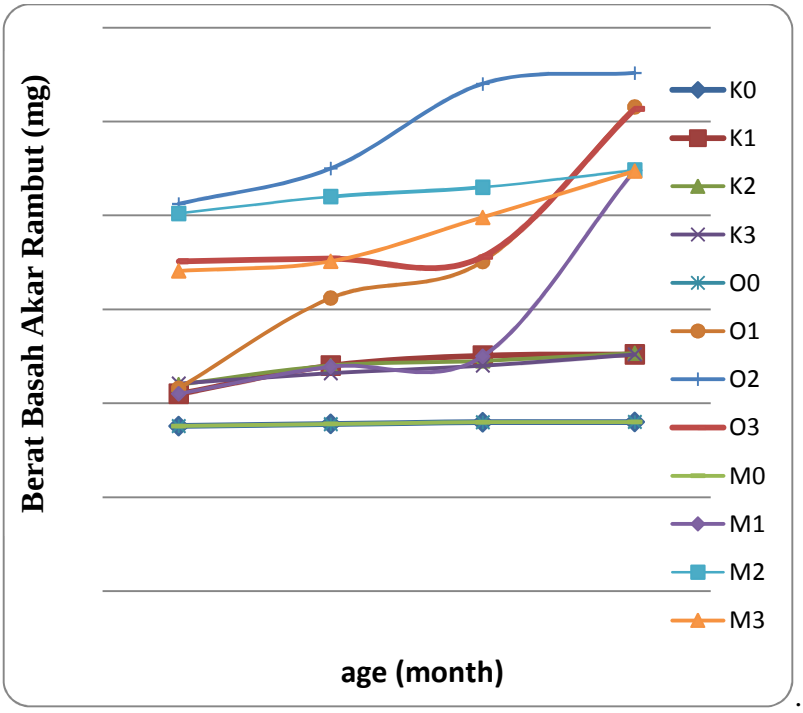

Figure 1. Fresh weight of hairy roots culture of pegagan from 1-4 months at each treatments: Control, chitosan 15 ppm (C1), chitosan 30 ppm (C2) chitosan 45 ppm (C3), COS 15 ppm (O1), COS 30 ppm (O2), COS 45 ppm (O3), CMC 15 ppm (M1), CMC 30 ppm (M2), CMC 45 ppm (M3)

(a) **(b)** **(c)**

Figure 2. Hairy roots culture of *C.asiatica* with elisitor. (a) COS 30 ppm (b) CMC 30 ppm (c) Chitosan 30 ppm

Table 1. Fresh weight of hairy roots of pegagan after addition of elisitors (mg/g)

Elisitor	Concentrations (ppm)			
	0	15	30	45
Chitosan	1180.03 g	252.03f	252.35ef	252.01f
COS	180.02g	515.67b	551.68a	513.35 c
CMC	180.01g	448.02d	448.23d	447.27d

Numbers followed by the same letters in the same rows were not significantly different at 5% confidence level

Fresh weight of hairy root of *C.asiatica* given CMC 15, 30, and 45 ppm was not significantly different among them. Hairy roots culture of *C. asiatica* added by COS 30 ppm showed the highest average fresh weight (551.68 mg/g fresh weight), this was caused by a lot of roots grew (as shown in Figure 1). Wet weight given COS 15 ppm and COS 45 ppm showed average fresh weight 515.67 mg/g and 448.01 mg/g. This indicated that given elisitor chitosan, COS, and CMC could influence fresh weight of hairy roots of *C. asiatica*. Keng, Wei and Bhatt (2010) showed that elisitor chitosan also increased biomass cell on roots of *Eurycoma longifolia* culture to produce alkaloid which was on chitosan 100 ppm with fresh weight 2,83 g while on control 1,47 g. Giving lower (10 ppm, 25 ppm) and higher (150 ppm) concentration of chitosan decreased fresh weight of cell biomass, which were 0.56 g; 0.88 g; and 1.07 g respectively.

Asiaticoside Content

Asiaticoside content in hairy roots of pegagan with COS treatment was higher compared to those of chitosan and CMC (as shown in Table 2). The highest asiaticocide content was obtained in treatment at 30 ppm COS, followed by 15 and 30 ppm COS, i.e. 5.97, 5.23 and 5.20 mg/g fresh weight respectively. Asiaticoside content in CMC treatment was higher than in chitosan. Asiaticoside content with treatment CMC at concentration 15, 30, and 45 ppm were 5.23, 5.97 and 5.20 mg/g fresh weight. Although treatment with chitosan produced lower content of asiaticoside compared to COS and CMC, but it was higher than control.

Table 2. Asiaticocide content in hairy roots culture of *C.asiatica* (mg/g/ fresh weight) after addition ofelisitors

Elisitor	Concentration (ppm)			
	0	15	30	45
Chitosan	1,33 h	3,17 e	3,10 f	3,07 g
COS	1,32 h	5,23 b	5,97 a	5,20 c
CMC	1,33 h	4,15 c	4,07 c	3,94 d

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The higher effectiveness of treatment with COS as elisitor in increasing production of asiaticocide compared to those of CMC and chitosan might relate to characters of COS which dilute more in water so that it could influence osmotic pressure of medium. This was in accordance with higher fresh weight of hairy roots in treatment with COS. According to Lin (2009)(8), COS has low molecule weight so that it dilutes easier in water. Tyler *et al*.(1988) stated that kind and dosage of elisitor used determined effectiveness in inducing secondary metabolites. Shibuya (2001) reported that chito-oligosaccharide was an active elisitor for biosynthesis of enzyme in paddy cell suspension culture.

According to Andrea *et al.* (2004), chitosan as transinduction signal could stimulate secondary metabolite production in *Rubia tinctorum* culture (4). With the treatment anthraquinone increased 3 folds compared to control. Some research results showed that the increase production of secondary metabolites with chitosan as elisitor was better than others for examples in hairy roots culture of *Hyoscyamus muticus* (9) and callus culture of oil palm (6).

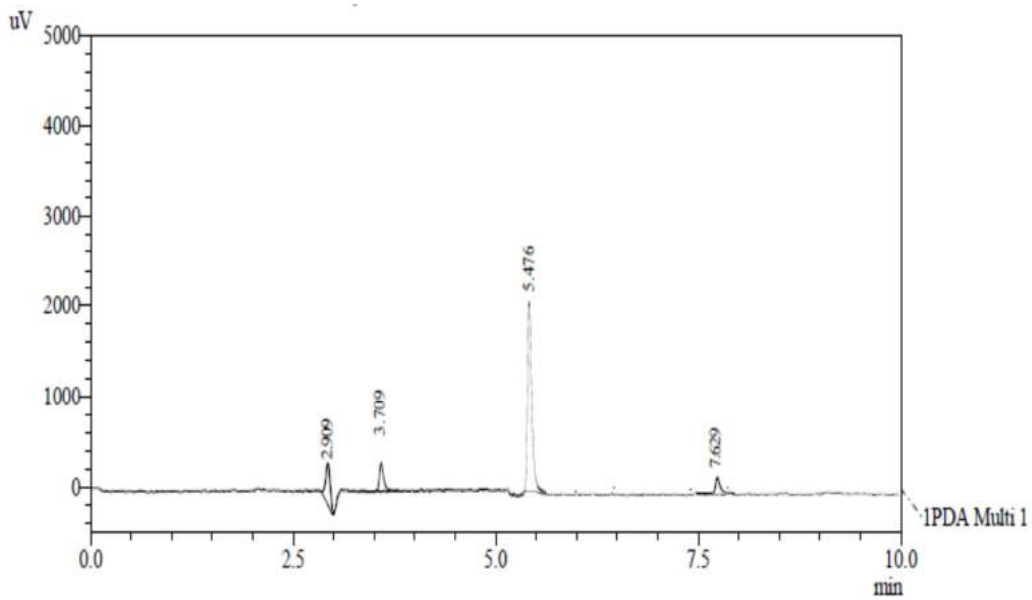

Figure 3. Chromatogram of asiaticocide standard

Hasanloo *et al* (2013) found that chitosan elisitor could increase production of silymarin as secondary metabolite in hairy roots culture of *Silybummarianum* 2,9 folds higher than control (5). Prasad *et al* (2011) also report that chitosan elisitor could also increase camalexin by genes inducing mechanism which were transcripted and translated to become enzyme involved in biosynthesis path of the secondary metabolite (10). Chitosan and its derivates are first signal of transduction gene associated with G protein reseptor. Further more, induction process could be trough adenilatesiklase pathorphosporilase as a second messenger to activate gen stimulating enzymes involved in metabolism of triterpenoid (11).

The highest asiaticoside (5.97 mg/g fresh weight) with treatment 30 ppm COS when compared to control (1.32 µg/mg wet weight), it was 5 folds increase. The production was much more higher compared to other elisators used by former researchers.

Kim *et al* (2004) reported that production of asiaticocide *C.asiatica*by hairy roots culture from transformation *A.rhizogenes* R 1000 could increase production of triterpenoid 2 folds compared to field conditioning 0.514 mg/g dry weight to become 2.695 mg/g dry weight(2). This research showed that three elisitors (chitosan and its derivates) could increase production of triterpenoid in hairy roots culture of *C.asiatica* (as shown in Table 2). Azis *et al* (2004) found triterpenoid content in *C.asiatica* tissue culture was 0,79 mg/dry weight(12). Kim *et al* (2007) reported triterpenoid content in hairy roots culture of *C.asiatica* was 1.21 µg/dry weight(3).

CONCLUSION

The highest wet weight (551.68 mg/g fresh weight) and asiaticocide content (5.97 mg/g fresh weight or 5 folds) in hairy roots culture of *C.asiatica* was obtained by adding Chito-oligosaccharide 30 ppm.

Acknowledgement
1. Dr. Pipih Suptijah, Faculty of Fishery and Maritime Science, Bogor Agricultural Institute, Bogor, Indonesia
2. Dr. Ermayanti, Indonesia Science Institute, Cibinong Bogor.

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by Zahanis Zahanis

Submission date: 22-Sep-2020 01:20PM (UTC+0700)
Submission ID: 1393682599
File name: oduction_of_asiaticoside_from_hairy_roots_culture_of_pegagan.pdf (2.09M)
Word count: 2492
Character count: 13015

Available online www.jocpr.com

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RESULTS AND DISCUSSION

Fresh weight of hairy roots

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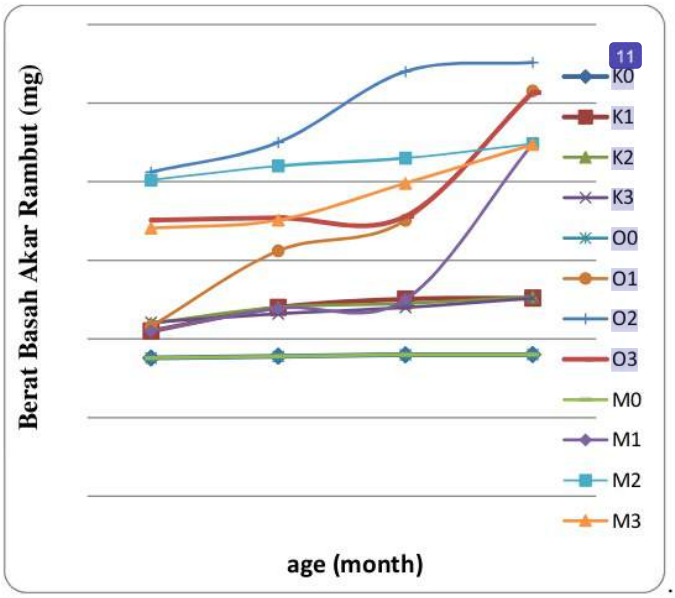

Figure 1. Fresh weight of hairy roots culture of pegagan from 1-4 months at each treatments: Control, chitosan 15 ppm (C1), chitosan 30 ppm (C2) chitosan 45 ppm (C3), COS 15 ppm (O1), COS 30 ppm (O2), COS 45 ppm (O3), CMC 15 ppm (M1), CMC 30 ppm (M2), CMC 45 ppm (M3)

(a) (b) (c)

Figure 2. Hairy roots culture of *C.asiatica* with elisitor. (a) COS 30 ppm (b) CMC 30 ppm (c) Chitosan 30 ppm

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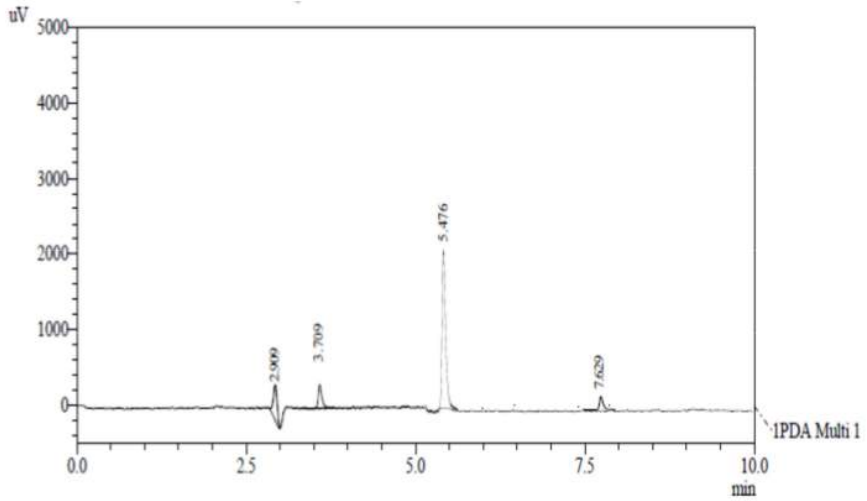

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Hasanloo *et al* (2013) found that chitosan elisitor could increase production of silymarin as secondary metabolite in hairy roots culture of *Silybummarianum* 2,9 folds higher than control (5). Prasad *et al* (2011) also report that chitosan elisitor could also increase camalexin by genes inducing mechanism which were transcripted and translated to become enzyme involved in biosynthesis path of the secondary metabolite (10). Chitosan and its derivates are first signal of transduction gene associated with G protein reseptor. Further more, induction process could be trough adenilatesiklase pathorphosporilase as a second messenger to activate gen stimulating enzymes involved in metabolism of triterpenoid (11).

The highest asiaticoside (5.97 mg/g fresh weight) with treatment 30 ppm COS when compared to control (1.32 µg/mg wet weight), it was 5 folds increase. The production was much more higher compared to other elisators used by former researchers.

Kim *et al* (2004) reported that production of asiaticocide *C.asiatica*by hairy roots culture from transformation *A.rhizogenes* R 1000 could increase production of triterpenoid 2 folds compared to field conditioning 0.514 mg/g dry weight to become 2.695 mg/g dry weight(2). This research showed that three elisitors (chitosan and its derivates) could increase production of triterpenoid in hairy roots culture of *C.asiatica* (as shown in Table 2). Azis *et al* (2004) found triterpenoid content in *C.asiatica* tissue culture was 0,79 mg/dry weight(12). Kim *et al* (2007) reported triterpenoid content in hairy roots culture of *C.asiatica* was 1.21 µg/dry weight(3).

CONCLUSION

The highest wet weight (551.68 mg/g fresh weight) and asiaticocide content (5.97 mg/g fresh weight or 5 folds) in hairy roots culture of *C.asiatica* was obtained by adding Chito-oligosaccharide 30 ppm.

Acknowledgement
1. Dr. Pipih Suptijah, Faculty of Fishery and Maritime Science, Bogor Agricultural Institute, Bogor, Indonesia
2. Dr. Ermayanti, Indonesia Science Institute, Cibinong Bogor.

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