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Research Article
Biomass and Flavonoid Production of *Gynura procumbens* (L.). Merr Adventitious Root Culture in Baloon-type Bubble-bioreactor Influenced by Elicitation

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Abstract

Background and Objective: *Gynura procumbens* (Lour.) Merr, an important medicinal plant, contains flavonoids that exhibit various pharmacological effects. Many strategies were used to increase the production of this valuable compound. The addition of biotic and abiotic elicitors is one of the most effective strategies for increasing the production of the bioactive compound. The aim of study is to investigate the effects of biotic (*Saccharomyces cereviceae* extract) and abiotic ($CuSO_4$) elicitors on biomass and flavonoid content of *G. procumbens* adventitious root culture in balloon type bubble bioreactor. **Methods:** Adventitious roots were induced from leaf explants. Two gram adventitious roots were cultivated in 1 L balloon type bubble bioreactor containing 600 mL liquid MS medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose. Then adventitious root growth determined based on fresh and dry weights that were harvested every 7 days up to 35 days of cultivation. Various concentrations of elicitors such as *S. cereviceae* extract (0.025, 0.05 and 0.1%) and $CuSO_4$ (1, 3 and 5 mg L^{-1}) were added on the 28th day to the culture. Adventitious root were harvested 7 day after elicitor treatment the flavonoid content is determined qualitatively by thin layer chromatography (TLC) and quantitatively with HPLC. **Results:** Various concentrations of *S. cereviceae* extract and $CuSO_4$ affected the biomass and flavonoid production of *G. procumbens* adventitious roots. The optimal elicitation strategy was achieved with 0.025% *S. cereviceae* extract and 1 mg L^{-1} $CuSO_4$. The treatment of 0.025% *S. cereviceae* extract resulted the highest biomass production and quercetin content reached 1.9-fold of the control culture. The treatment of 1 mg L^{-1} $CuSO_4$ increased kaempferol content which was 13.3-fold of the control culture. **Conclusion:** Extract of *S. Cereviceae* and $CuSO_4$ at low concentration can enhance production of biomass and flavonoid content of *G. procumbens* adventitious roots. Its condition can be used to develop production in large scale.

Key words: *Gynura procumbens*, flavonoid, biomass, adventitious root culture, saccharomyces cereviceae, copper sulphate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gynura procumbens (Lour.) Merr is an Indonesian medicinal plant belonging to the Asteraceae Family. The plant is commonly found in the tropical areas such as China, Indonesia, Thailand, Malaysia and Vietnam[1]. People use the plant for the treatment of fever, kidney disease, migraine, constipation, hypertension, diabetes mellitus and cancer[2]. It was reported that *G. procumbens* (Lour.) Merr extract had various pharmacological effects such as antioxidant[3-6], anticancer[7-9], anti-inflammatory[10,11], antihyperglycemic and antihyperlipidemic[12,13], antimicrobial[14-16] and organ-protective activities[17,18]. These beneficial effects are related to the presence of bioactive compounds in *G. procumbens* (Lour.) Merr extract. The secondary metabolite compounds found in the plant are flavonoids, saponins, tannins and terpenoids[19].

Flavonoids are the main phenolic compounds of *G. procumbens* (Lour.) Merr. Five flavonoid compounds such as rutin, myricetin, quercetin, apigenin and kaempferol were isolated from *G. procumbens* (Lour.) Merr leaves[4]. The flavonoid compounds of *G. procumbens* (Lour.) Merr showed various health benefits including antioxidant, anticancer, anti-inflammatory, hepatoprotective and antibacterial activities[20-23].

In addition, flavonoids have been used in food processing, cosmetics and pharmaceutical industries. The commercial importance of flavonoids and the need for the renewable resource of the compounds lead an effort to develop their alternative production systems[24]. Chemical synthesis of flavonoids is a difficult and very expensive process. Besides, conventional cultivation is influenced by cultivation periods, low yields and fluctuations in concentrations due to geographical, seasonal and environmental variations[25,26]. Therefore, one of the efforts to increase flavonoid production is using the plant tissue culture. Among the different culture techniques, adventitious root culture is one of the most attractive and efficient systems for producing biomass and secondary metabolite compounds[9]. The successful establishment of adventitious root culture from *G. procumbens* (Lour.) Merr leaf explants for producing biomass and secondary metabolites was reported by Saiman *et al.*[27]. Adventitious root culture has the advantages of being easy and efficient to scale up the production using bioreactor[28]. Various bioreactor designs have been used in adventitious root culture such as stirred tank, bubble column, siphon-mist and modified airlift bioreactor called balloon type bubble bioreactor. Of the bioreactors used in adventitious root culture, balloon type bubble bioreactor and siphon mist bioreactor are the most effective bioreactor for adventitious root[29]. Balloon type bubble bioreactor culture was reported to be suitable for the large-scale production of biomass and secondary metabolite in *Panax ginseng* and *Hypericum perforatum* adventitious root cultures[30,31].

One of the strategies used to increase the production of secondary metabolite in plant cell and tissue culture is elicitation[32-34]. Elicitation can modulate plant defense mechanism stimulating various synthesis pathways and inducing accumulation of secondary metabolite[35]. Many reports showed that various elicitors can trigger accumulation of secondary metabolite of adventitious root cultures in various plants[36-38].

Yeast extract is often used as biotic elicitor in the study of plant-microbe interactions and for the production of secondary metabolite[39]. Yeast extract contains certain components that can induce plant defence responses including chitin, N-acetylglucosamine oligomers, β-glucan, glycopeptides and ergosterol[40]. The component of the cell wall that acts as an elicitor in *Saccharomyces cereviceae* is glucan[41]. The successful application of *S. cerevisiae* yeast extract in elicitation was known to increase the content of rosmarinic acid in *Solenostemon scutellarioides* culture[42] and gymnemic acid in suspension culture of *Gymnema sylvestre*[43]. Happyana *et al.*[44] reported that the addition of *S. cerevisiae* yeast extract in hairy root culture of *Morus macroura* Miq. can increase the production of the secondary metabolite compound.

Copper is a plant micronutrient needed for protein components of several enzymes[45]. Copper in a micronutrient fertilizer is primarily in the form as $CuSO_4$.5H2O and CuO. Copper (II) sulfate ($CuSO_4$.5H2O) becomes the most common Cu source because of its low cost and high solubility in water[46,47]. Copper is one of heavy metals that is essential for the growth of plant but it can also be an abiotic stress that induce the production of secondary metabolite[48]. Many reports have mentioned the successful application of $CuSO_4$ elicitors in increasing the accumulation of secondary metabolite compounds such as grindelloic acid in *Grindella pulchella*[49], lettucenin in *Lectus*[50], anthraquinones in *Rubia tinctorum*[51] and diosgenin in *Dioscorea bulbifera*[52]. The application of 20 μg mL^{-1} $CuSO_4$ can increase the flavonoid content in the *in vitro* cultures of some members of Euphorbiaceae, such as *Baliospermum montanum*, *Codiaeum variegatum* and *Dryptes roxburghi*[53].

To the best to our knowledge, there has been no reports of the effect of *S. cereviceae* extract and $CuSO_4$ elicitors on adventitious root culture of *G. procumbens* (Lour.) Merr. in balloon type bubble bioreactor. Therefore, this study aimed to investigate the effect of biotic elicitor (*S. cereviceae*) and abiotic ($CuSO_4$) on biomass and flavonoid production in adventitious root culture of *G. procumbens* (Lour.) Merr in balloon type bubble bioreactor.

MATERIALS AND METHODS

Plant material: This research is conducted at January-November, 2017 at Plant Tissue Culture Laboratory, Biology Department, Universitas Airlangga, Surabaya, Indonesia. *Gynura procumbens* (Lour.) Merr was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java Indonesia which further was nurtured in polybag with mix of soil and organic fertilizer (50:50%) and incubated at room temperature. Adventitious root was obtained from leaf explants of *Gynura procumbens* (Lour.) Merr.

Adventitious root induction: Leave explants of *G. procumbens* (Lour.) Merr were washed with detergent for 5 min and then rinsed thoroughly with tap water. Explants were sterilized with clorox 10% (v/v) and soaked for 10 min and rinsed 3 times with sterile distilled water. Explants were placed in sterile filter paper in the petri dishes. Explants were cut 1 cm^2 and inoculated in MS solid medium supplemented with 5 mg L^{-1} IBA, 30 g L^{-1} sucrose and 7 g L^{-1} agar. Cultures were maintained in room culture at 25°C in the dark conditions. After 21 days, adventitious roots were harvested for used in the liquid medium.

Adventitious root culture in liquid medium: The objectives of this method were to acclimatize, stabilize, reduce contamination and obtain a large amount of inoculum sources of adventitious root in a short time before being used as inoculum in balloon type bubble bioreactor culture. Twenty one days old adventitious roots were harvested from solid medium and placed in sterile filter paper. Two grams of adventitious root were cultured in Erlenmeyer flask containing 100 mL MS liquid medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose. Cultures were agitated at 100 rpm on shaker incubator in room temperature at 25°C and in the dark conditions. The adventitious root cultures were maintained by routine sub culturing at 21 days intervals. These root cultures were used for further experiments.

Adventitious root culture in balloon type bubble bioreactor: This study used balloon type bubble bioreactor with a capacity of 1 L. Two grams of adventitious roots were inoculated in balloon type bubble bioreactor containing 600 mL MS liquid Medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose. Cultures were maintained for 35 days at room temperature in dark condition with aeration rate of 0.2 vvm. The value of pH medium was measured every 7 days.

Determination of adventitious root growth curve: In order to determine the growth curve of *G. procumbens* (Lour) Merr adventitious root cultures, 2 g of adventitious roots were inoculated in balloon type bubble bioreactor containing 600 mL MS liquid medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose. The growth curve of *G. procumbens* (Lour) Merr adventitious root determined based on fresh and dry weights that were harvested every 7 days up to 35 days of cultivation.

Elicitor treatment: The elicitors of *S. cereviceae* extract were prepared by inoculating the pure culture of *S. cereviceae* from *Potato Dextrose Agar* (PDA) medium into *Potato Dextrose Broth* (PDB) medium. Cultures were incubated in shaker incubator at 100 rpm, 25°C for 48 h (Daihan LabTech Co., Ltd.). Cultures were harvested and centrifuged at 5000 rpm for 10 min (Hettich zentrifugen). After that, the supernatants were removed and the pellets were washed with sterile distilled water 3 times and then dried in oven at 50°C. The dried pellets were crushed into powder. The powders of *S. cereviceae* were then dissolved in distilled water and sterilized (autoclave, 121°C, 1 atm, 15 min).

The elicitors of $CuSO_4$ were prepared by dissolving $CuSO_4$.5H2O in distilled water and then sterilized before they were added in the culture. Various concentrations of *S. cereviceae* extract (0.025, 0.05 and 0.1%) and $CuSO_4$ (1, 3 and 5 mg L^{-1}) were added into the adventitious root culture in balloon type bubble bioreactor on the 28th day of cultivation (in the exponential phase of adventitious root growth). Adventitious roots were then harvested after 7 days of elicitor addition.

Extraction and analysis of flavonoid content: A 0.5 g dried biomass powders of each adventitious root treatments were soaked with 10 mL of methanol at room temperature for 24 h. This process was done twice. The extracts were filtered and then concentrated to 6 mL at room temperature. The methanol extracts were partitioned with n-hexane with a ratio

of 1:1 to remove non-polar compounds. Subsequently, the methanol extracts were partitioned with ethyl acetate with a ratio of 1:1[54]. The extracts of ethyl acetate were then analyzed the flavonoid content. The flavonoid content was analyzed qualitatively by thin layer chromatography (TLC) and quantitatively with HPLC. The kaempferol and quercetin compounds are used as standard.

A qualitative analysis using thin layer chromatography (TLC) was performed by preparing 2 mL of ethyl acetate extract of each adventitious root treatments, then concentrated to 1 mL, subsequently the extracts were spotted on silica gel GF254 and eluted using chloroform: Methanol (9:1). Spots were visualized using UV at 312 nm wavelength.

The quantitative analysis of flavonoids in ethyl acetate extract of adventitious roots was performed with HPLC Agilent 1100 series with PDA detector and C8 column (4.8×160 mm, 5 µm, Zorbax Eclipse XDB C8). The eluen used was acetonitrile: trifluoroacetic acid (82:18), sample volume 5 µL, flow rate 1 mL min^{-1}, column temperature 30°C and detected with 370.8 nm wavelength. Identifications of compounds were done by comparing the value of RT (Retention Time) samples with standard compounds. The concentration of the compounds was determined from the integration of the peak area of sample and the corresponding standard.

Statistical analysis: All experiments were carried out in a completely randomized design and performed twice in replications. The data of adventitious roots biomass were descriptively analyzed and presented as means±standard deviation.

RESULTS

Growth curve of *G. procumbens* adventitious root in balloon type bubble bioreactor: In the present study, in order to determine the culture period and the optimal time for adding elicitor to produce biomass and flavonoid of adventitious root, the growth phase was observed through the growth curve of adventitious root. The growth curve was obtained from the correlation between fresh and dry weight with cultivation time. Based on fresh and dry weight, the growth curve of adventitious root for 35 days indicated a linear pattern. The growth curve exhibited lag phase and exponential phase but there was no stationary phase observed yet. As shown in Fig. 1, the growth of adventitious root showed the lag phase on the early to 10th day culture and the exponential phase started from the 10th day to the end of culture.

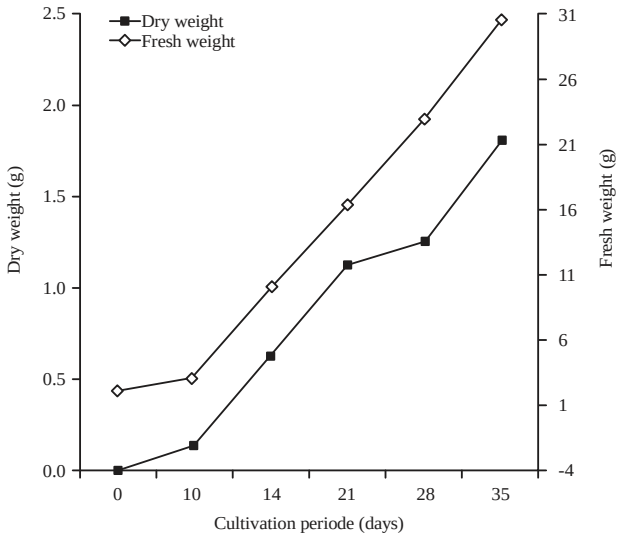

Fig. 1: Growth curve of *G. procumbens* adventitious root in balloon type bubble bioreactor based on fresh and dry weight

***S. cereviceae* extract and $CuSO_4$ effect on *G. procumbens* adventitious roots biomass:** Adventitious roots treated with *S. cereviceae* extracts and $CuSO_4$ had similar, normal and healthy in morphology (Fig. 2). The effect of elicitors of *S. cereviceae* extract and $CuSO_4$ on fresh and dry weight of *G. procumbens* adventitious root in balloon type bubble bioreactor can be seen in Fig. 3.

The addition of *S. cereviceae* extracts and $CuSO_4$ in *G. procumbens* adventitious root culture affected the growth of adventitious roots. The lower concentration of *S. cereviceae* extracts promoted the growth of root while the higher concentration inhibited the growth of root as compared to the control culture. The biomass of adventitious root treated with *S. cereviceae* extract yielded the highest fresh and dry weight at 0.025% (47.27±1.52 g) and the lowest at 0.1% (19.77±3.36 g).

The fresh weights of adventitious root in $CuSO_4$ treatments were lower than control. The lowest fresh weight was found in the treatment of 1 mg L^{-1} $CuSO_4$. While at concentrations of 1 and 3 mg L^{-1} produced higher dry weights than control culture. The $CuSO_4$ treatments produced the highest dry weights at a concentration of 1 mg L^{-1} (3.07±0.90 g) and the lowest at concentration of 5 mg L^{-1} (1.05±0.73 g).

The physical condition of the medium was observed at the beginning of cultivation. The pH values at each treatment were changed during cultivation. Changes in pH showed a decrease but not significant (Fig. 4a and b). Root cultures that had an average initial pH of 5.3-5.1 were decreased weekly but

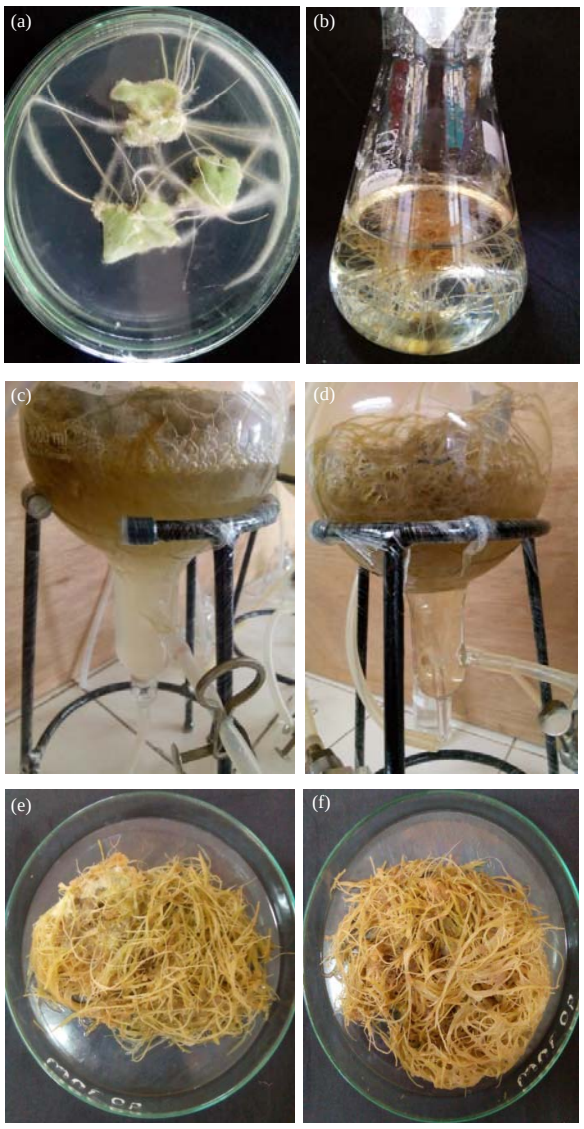

Fig. 2(a-f): *G. procumbens* adventitious root culture in balloon type bubble bioreactor, (a) Induction of adventitious roots from leaves explants, (b) Adventitious roots culture in liquid medium before move to bioreactor, (c) The highest biomass after addition of 0.025% *S. cereviceae* extract in bioreactor, (d) The highest biomass after addition of 3 mg L^{-1} $CuSO_4$ and (e-f) Morphology of adventitious root after elicitation with 0.025% *S. cereviceae* extract and 3 mg L^{-1} $CuSO_4$

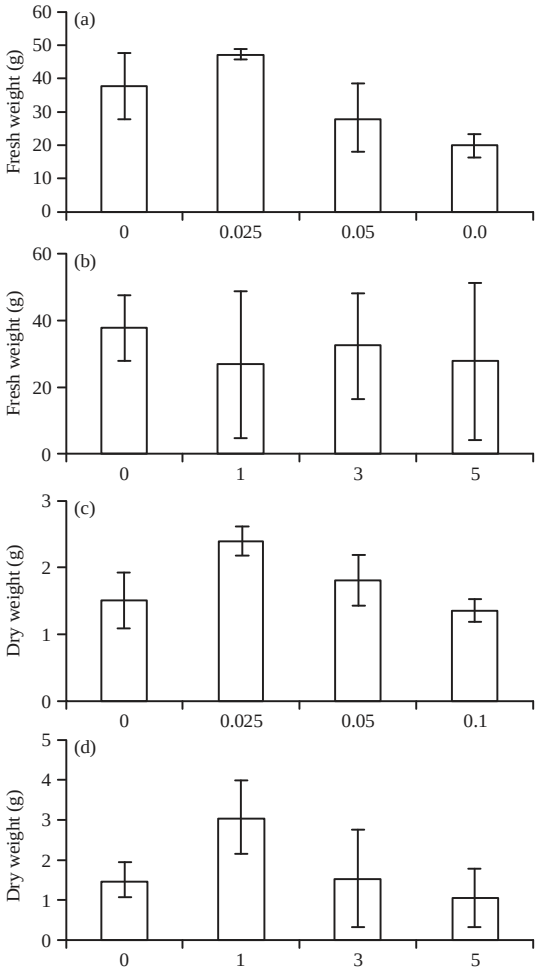

Fig. 3: Effect of *S. cereviceae* extract and $CuSO_4$ on biomass of *G. procumbens* adventitious root (fresh and dry weights). Each value represents Mean±SD of two replicates

not drastically and even pH values remained unchanged or remained the same as the previous week. Until the 21st day of culture, the pH value was at 5 but on the 28th day, the pH value was below 5.

***S. cereviceae* extract and $CuSO_4$ effect on *G. procumbens* adventitious root flavonoid production:** TLC results indicated the presence of flavonoid compounds in the extracts on the various elicitor treatments of *S. cereviceae* extract and $CuSO_4$ (Fig. 5). The presence of flavonoid compounds was shown by the spots that have the same Rf value as the standard Rf value. The elicitor treatments of *S. cereviceae* extract and $CuSO_4$ had the same Rf value with Rf value of kaempferol (0.63) and quercetin (0.34).

The flavonoid contents of adventitious roots were quantitatively analyzed using HPLC. Figure 6 shows HPLC chromatograms of the extracts from adventitious roots (a-c) and roots of mother plants (d).

The flavonoid content determined using linear equation of the calibration curve of standard compound and the peak areas of adventitious roots extracts. Our result indicated that

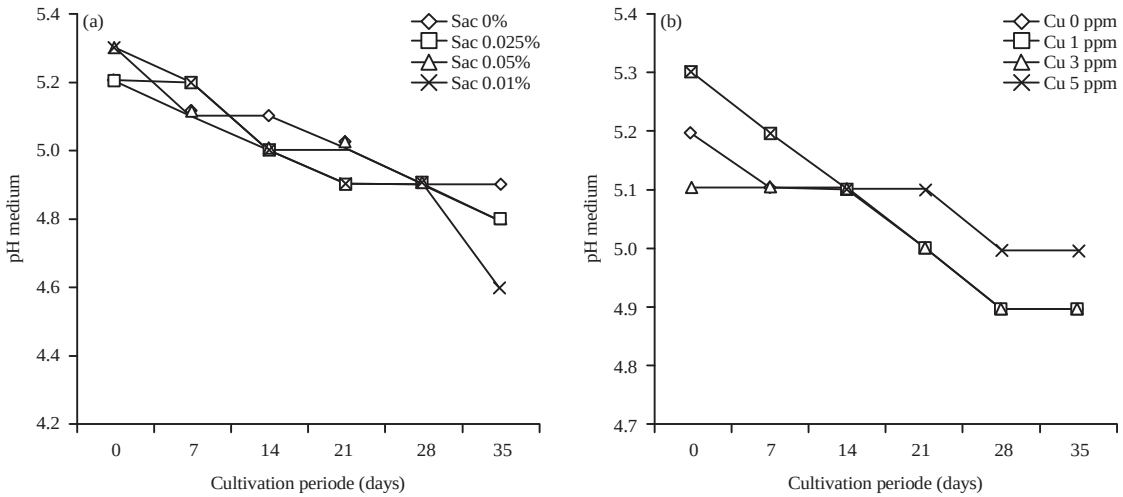

Fig. 4: Physical condition of medium during 35 days (a) The change of pH value in *S. cereviceae* extract treatment and (b) The change of pH value in $CuSO_4$ treatment

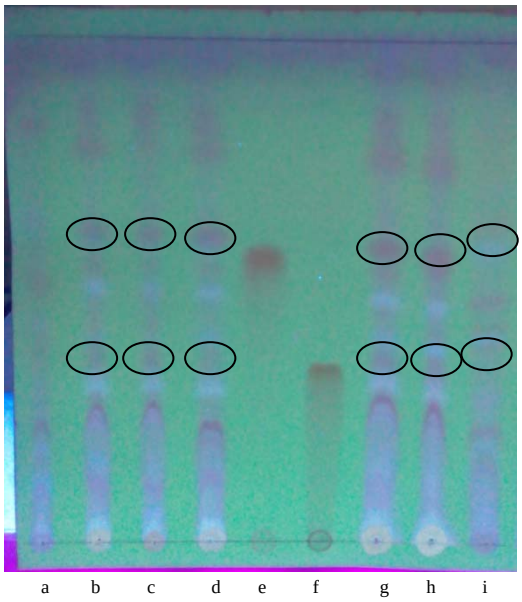

Fig. 5: TLC chromatogram under UV light λ_{312} sequentially from left to right (a) Control, (b) 0.025% *S. cereviceae*, (c) 0.05% *S. cereviceae*, (d) 0.01% *S. cereviceae*, (e) Kaempferol, (f) Quercetin, (g) 1 mg L^{-1} $CuSO_4$, (h) 3 mg L^{-1} $CuSO_4$ and (i) 5 mg L^{-1} $CuSO_4$

the addition of *S. cereviceae* extract and $CuSO_4$ can increase the production of adventitious roots as compared to the control (Table 1). The addition of 0.25% *S. cereviceae* extract led to an increase of quercetin compound about 25.7725 mg L^{-1}. Quercetin content in the treatment of 0.025% *S. cereviceae* extract increased 1.9 times compared to the control. While the treatment of *S. cereviceae* extract at concentration 0.05 and 0.1% have the content of kaempferol compound 1.4013 mg L^{-1} and 1,2507 mg L^{-1} respectively. The content of kaempferol compounds in this treatment increased 3.7 times and 3.3 times, respectively, compared to the controls. The treatment of high concentration of *S. cereviceae* extract decreased the flavonoid content compared with the low concentration of *S. cereviceae* extract. These results indicate that addition of *S. cereviceae* extract with lower concentrations was more effective in increasing the flavonoid content in adventitious root culture.

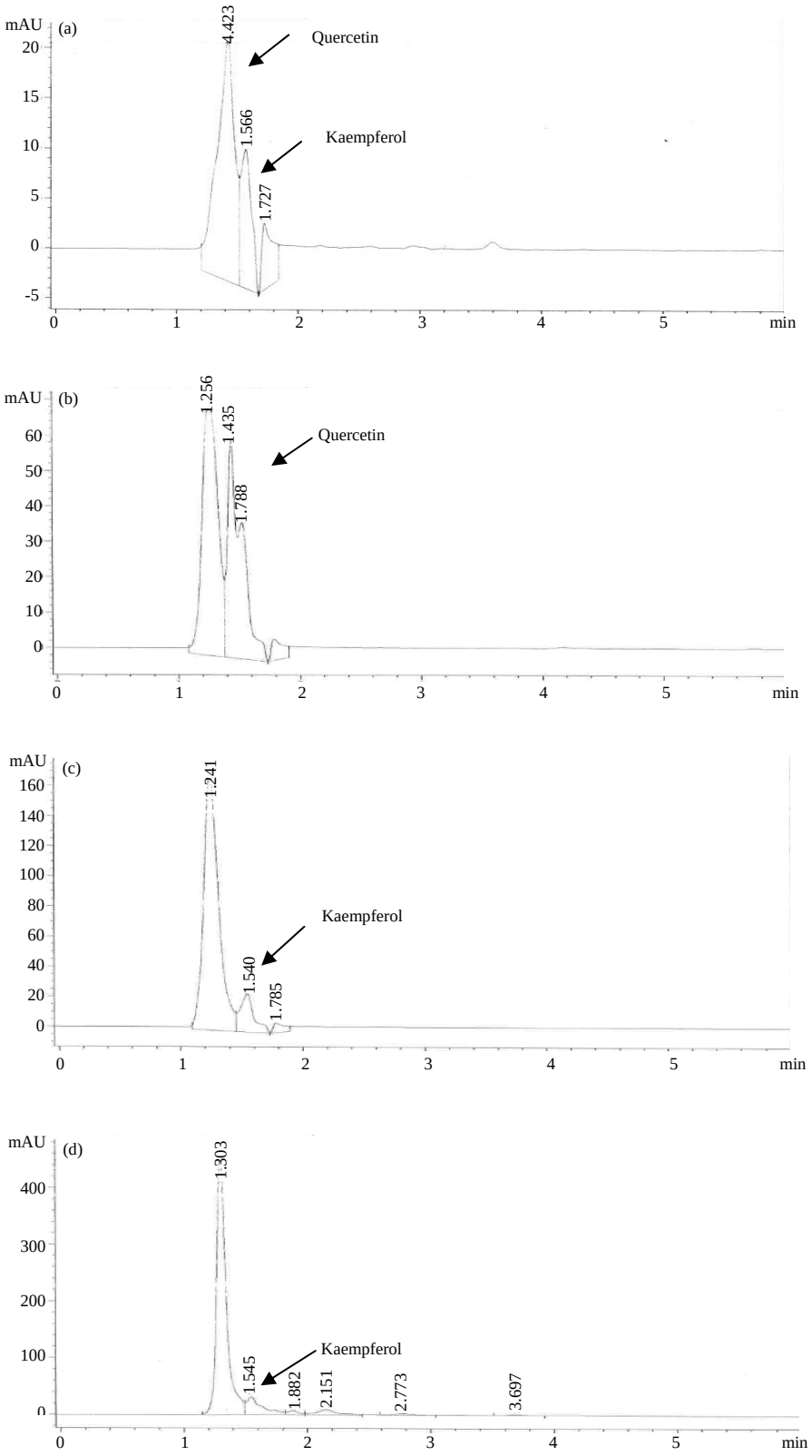

Fig. 6(a-d): HPLC profiles of kaempferol and quercetin in *G. procumbens* adventitious roots and roots of wild plants, (a) Control culture, (b) Culture treated with 0.025% *S. cereviceae* extract, (c) Culture treated with 1 mg L^{-1} $CuSO_4$ and (d) Root extract of mother plants

Table 1: Effect of *S. cereviceae* extract and $CuSO_4$ on Flavonoid Production of *G. procumbens* Adventitious root

Treatments	Concentration	Flavonoid content (mg L^{-1})	
		Kaempferol	Quercetin
Control (untreated root)		0.3763	13.7972
S. cereviceae extract	0.025%	-	25.7725
	0.050%	1.4013	-
	0.100%	1.2507	-
$CuSO_4$	1 mg L^{-1}	4.9906	-
	3 mg L^{-1}	0.7068	-
	5 mg L^{-1}	3.9469	-
Root of mother plants		8.4002	-

The treatment of various concentrations of $CuSO_4$ had higher kaempferol content than the control culture. $CuSO_4$ treatment at concentrations of 1 and 5 mg L^{-1} had kaempferol content about 13.3-fold (4.9906 mg L^{-1}) and 10.5-fold (3.9469 mg L^{-1}), respectively, than the control. The treatment of $CuSO_4$ with concentration of 3 mg L^{-1} had kaempferol content of 0.7068 mg L^{-1}. The content of kaempferol compound in the treatment of $CuSO_4$ with a concentration of 3 mg L^{-1} had higher content of kaempferol (1.9-fold) than control but lower than the treatment of $CuSO_4$ with concentrations of 1 and 5 mg L^{-1}.

Of the various treatments of *S. cereviceae* extract and $CuSO_4$, the *S. cereviceae* extract at 0.025% exhibited maximum quercetin content and the maximum kaempferol content was found in the addition of 1 mg L^{-1} $CuSO_4$. So, the addition of 0.025% *S. cereviceae* extract was the most effective treatment to increase the production of quercetin compound meanwhile the addition of 1 mg L^{-1} $CuSO_4$ was the most effective treatment to increase the production kaempferol compounds in *G. procumbens* adventitious root culture. Although the addition of *S. cereviceae* extract and $CuSO_4$ increased the production of flavonoid compounds, the kaempferol content of adventitious roots were still lower than in the root of mother plant.

DISCUSSION

The growth curve of adventitious root for 35 days culture periods exhibited lag phase and exponential phase but there was no stationary phase observed yet. The lag phase is a period of energy production. The lag phase is characterized by accumulation of weight, without cell division. The exponential phase is the maximum period of cell division and biosynthetic stage[55,56].

To produce large-scale secondary metabolites effectively, it is important to understand when in the growth phase a specific product is formed. The information can be used to develop an effective strategy of secondary metabolite production. Production of secondary metabolites in vitro can occur at almost any stage of culture growth[57]. The hairy root culture of *Catharanthus roseus* in dark conditions produced maximal tabersonine and ajmalicine in the exponential phase but serpentine (ajmalicine derivative) was produced maximally in the stationary phase[58]. Tan *et al.*[59] reported that callus cultures of *Centella asiatica* L. urban produced the highest flavonoid content in the exponential phase.

The duration and addition time of elicitor is a key factor in increasing secondary metabolite production. The addition of elicitors in the exponential phase were reported to be able to increase ginsenoside production in cell culture of *Panax ginseng*[60] and flavonoids in cell culture of *Hypericum perforatum*[61]. Torkamani *et al.*[62] reported that 7 days exposure of elicitor was the most effective duration of elicitation in increasing valerenic acid significantly in hairy root culture of *Valeriana officinalis* L. The elicitor additions for 7 days in root culture were also reported to be effective in increasing the production ginsenoside[63] and tanshinone[64]. Therefore, in this present study, the elicitors were added in the exponential phase, on the 28th day and exposed for 7 days to obtain optimal results in increasing flavonoid content of *G. procumbens* adventitious root culture in balloon type bubble bioreactor.

This study showed that there was an increase of biomass production in the elicitor treatment of *S. cereviceae* extract at 0.025% concentration and then decreased with increasing of *S. cereviceae* extract concentration. Increased biomass production in elicitor of *S. cereviceae* extract was also reported by Prakash and Dayaram[65] where the addition of *S. cereviceae* extract increased the production of shoot biomass in *Bacopa monniera* (L.) Pennell culture. Increases in biomass production may have been due to the *S. cereviceae* extract acting as a nutrient that can increase root growth.

Yeast extract is usually used as a supplement in order to induce plant growth because of its high amino acids and it is also used as a growth nutrient in callus cultures. However, variations in yeast concentration will have different responses to the plant growth, where addition of yeast extract at higher concentration will inhibit the growth whereas lower concentration of yeast extract was found promoting the plant growth effectively[66,67]. So in this study, the addition of *S. cereviceae* extracts at higher concentrations can lead to decreased adventitious root biomass. Decreased adventitious root biomass probably due to stress conditions in root culture[68]. *S. cereviceae* may act as an allelochemical that causes stress conditions[65]. The decreases in adventitious root biomass is also probably due to the addition of concentration of *S. cereviceae* extract is too high causing the media becomes turbid and concentrated resulting in hypertonic conditions in the media. Hypertonic conditions in the media cause higher osmotic pressure outside the cell so that the water is drawn out of the cell. Cells that lose more water will become shrink (crenate) resulting plasmolysis and eventually inhibit cell growth[69]. This stunted cell growth will eventually lead to a decrease on adventitious roots biomass (Fig. 3a). Elicitor treatment of yeast extract resulting in decreased biomass was also reported by Marsik *et al.*[70] on adventitious root culture of *P. ginseng*.

The highest concentrations of $CuSO_4$ (5 mg L^{-1}) resulted in a decrease in fresh and dry weight adventitious roots when compared to control. The decreased biomass most likely due to stress conditions in root culture caused by Cu accumulation. Cu is the plant micronutrient needed for protein components of several enzymes[45]. However, high concentrations of $CuSO_4$ have toxic effects on plant cells[71]. The addition of $CuSO_4$ in high concentrations was also reported inhibiting plant regeneration in *Dioscorea bulbifera* L. cultures[52]. Meanwhile the addition of $CuSO_4$ at concentrations of 1 and 3 mg L^{-1} increased the dry weight of the adventitious roots. Similar result indicating an increasing biomass on Cu treatment was reported by Rhee *et al.*[72], where the addition of Cu^{2+} ions in the medium increased biomass in root culture of *Angelica gigas.* This is related to the role of Cu in plant metabolism. Cu acts as a catalyst in the plant metabolism and component of enzymes or proteins that play a role in oxidation and respiration processes such as cytochrome oxidase i.e., respiratory enzymes mitochondria and plastonin, a protein chloroplast[73].

The pH values in the treatment of various concentrations of *S. cereviceae* extracts and $CuSO_4$ decreased during the cultivation period from 0-35th day. But, the decrease of pH value was not drastic and significant. Therefore, it didn't inhibit the absorption of sucrose, macronutrient and micronutrient, so the growth of adventitious root can increase optimally. The decrease of pH value in adventitious root culture medium was also reported by Lulu *et al.*[74] and Manuhara *et al.*[75]. The decrease of pH in culture medium is caused by MS medium containing ammonium. The source of ammonium in MS medium is ammonium nitrate that is important as a buffer and a source of nitrogen. When a cell requires nitrogen, the cell takes ammonium and releases H^+ into the medium then causes acidic conditions on the medium[75].

Our result showed that flavonoid content in *G. procumbens* adventitious root can be increased by both biotic (*S. cereviceae* extract) and abiotic ($CuSO_4$) elicitors. Plants produce a various secondary metabolite compounds that are useful for interacting with the environment and for developing defense systems against stressful conditions and pathogen attacks. Biotic and abiotic elicitors are external stimuli that can trigger the changes in plant cells that will ultimately result in the accumulation of secondary metabolites that help plants deal with stressful conditions. The stimulus is received by the receptor, which then generates secondary messenger activation that transmits signals to the cells through signal transduction pathways leading to gene expression and biochemical changes resulting in secondary metabolite production[76].

The *S. cereviceae* extract treatment resulted in increasing kaempferol and quercetin production in *G. procumbens* adventitious root. The yeast extract is a biotic elicitor reported to increase effectively the production of secondary metabolites in plant cultures. Park *et al.*[77] reported that the addition of yeast extract to *Pueraria lobata* cell culture can stimulate the accumulation of isoflavone and daidzein. The yeast extract contains several components that can induce plant defense responses, including chitin, N-acetylglucosamine oligomers, β-glucan, glycopeptide and ergosterol[40]. In *S. cereviceae* extract contains glucan that can induce the production of phytoalexin[68]. Phytoalexin is a plant defense compound whose production is triggered by infection or predation and it usually has antifungal or antibacterial activity[78]. Phytoalexins include terpenoid, stibene, polyacetylene and flavonoids[79,80]. The increase of secondary metabolite content in plant culture treated with *S. cereviceae* extract was also reported by Sahu *et al.*[42] where elicitation with polysaccharide fraction of yeast extracted isolated from *S. cerevisiae* was found to increase significantly the accumulation rosmarinic acid (1,5-fold) in *Solenostemon scutellarioides* culture.

$CuSO_4$ increased the kaempferol production significantly in *G. procumbens* adventitious culture especially at the

lowest concentration of 1 mg L^{-1} (13.3-fold). Copper (Cu) is one of the heavy metals essential for plant growth but can also be an abiotic stress that induces the production of reactive oxygen species (ROS)[48]. The production of ROS in plants will cause the plant to activate a plant defense mechanism, such as by producing flavonoids as antioxidants that counteract ROS as well as chelating the ROS-producing metal[81,82]. Ali *et al.*[83] reported that Cu stimulated the accumulation of super oxide anions (O^{2-}), hydrogen peroxide (H_2O_2) and saponins in *P. ginseng* suspension culture. The addition of the abiotic elicitor of $CuSO_4$ has also been reported to increase anthocyanin content in *Vaccinium phalae* cell culture[84], betacyanin in *Portulaca* cell suspension cutlture[85] flavonoids in *Ginko biloba* cell culture[71] and *digitalis lanata* cell culture[86]. In the study of Kim *et al.*[71], $CuSO_4$ showed a significant effect on the accumulation of quercetin which reached 0.526 m L^{-1}. Quercetin concentration increased up to 12 times in the $CuSO_4$ treatment compared to control. However, high concentrations of $CuSO_4$ decreased secondary metabolite production which is probably caused by toxic effects of Cu on growth and accumulation of secondary metabolites[83].

The addition of *S. cereviceae* extract and $CuSO_4$ at high concentration decreased the biomass and flavonoid production compared to the low concentration. It probably depended on the relative tolerance of plant cell under stress condition. This result showed that a low concentration of elicitors was more effective on both biomass and flavonoid production. At 0.025% *S. cereviceae* extract treatment, the quercetin content increased 1.9-fold (25.7725 mg L^{-1}) of the control culture whereas it wasn't detected in the root of mother plant. The addition of 1 mg L^{-1} $CuSO_4$ can increased kaempferol content 13.3-fold (4,9906 mg L^{-1}) of the control culture but kaempferol content of adventitious roots were still lower than in the root of mother plant. So we suggest the need of further investigation on the addition time and duration of elicitor in order to know the optimum production of flavonoid in *G. procumbens* adventitious root culture.

CONCLUSION

The present study demonstrated that *S. cereviceae* extract and $CuSO_4$ had different effect on biomass and flavonoid production in *G. procumbens* adventitious root culture in ballon type bubble bioreactor. Quercetin content reached 25.7725, which was 1.9-fold of the control in the treatment 0.025% *S. cereviceae* extract. The treatment of 1 mg L^{-1} $CuSO_4$ increased kaempferol content reached 4.9906 mg L^{-1}, which was 13.3-fold of the control culture. So, The addition of 0.025% *S. cereviceae* extract was the most effective treatment for increasing biomass and quercetin production while kaempferol was more increased significantly in the treatment of 1 mg L^{-1} $CuSO_4$.

SIGNIFICANCE STATEMENT

The study discovered the *S. cereviceae* extract and $CuSO_4$ on the biomass and flavonoid production in *G. procumbens* adventitious root culture and found effective in increasing the content even at the lowest concentrations which is not explored by the previous studies. The results of study could help the researchers in understanding the mechanism for increasing the bioactive components in medicinal plant. Thus best theory on it may be arrived at.

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