Comparative Study of Peperochromen-A from Sasaladaan [*Peperomia pellucida* (L.) Kunth.] Herbs *In Vivo* and *In Vitro*-Cultered on MS, WPM and DKW Media

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Abstract

Sasaladaan [*Peperomia pellucida* (L.) Kunth.] *has been traditionally used as herbal medicine for decreasing blood sugar. Previous research showed that one of the antidiabetic bioactive compounds from P. pellucida is Peperochromen-A. This plant is wild herb and the propagation is very limited in land cultivation. The objective of this research was to obtain plantlet derived explant from P. pellucida that containing Peperochromen-A. Explant was cultured on different basic medium i.e. Murashige and Skoog (MS), Woody Plant Medium (WPM) and Driver and Kuniyuki (DKW). Plantlet from different basic mediums was extracted by ethanol and compare with Peperochromen-A compound using TLC under UV light 366 nm.*

Result of this research showed that plantlets were obtained from three different basic mediums and the explant has different growth response. TLC analysis showed that plantlet extract from MS, WPM and DKW medium has Peperochromen-A spot. These indicate that Peperochromen-A can be produced from the biosynthetic process in plants resulting from tissue culture, so this tissue culture can be an alternative for propagation of Peperochromen-A compound.

Keywords: *Peperomia pellucida*, Sasaladaan, Tissue Culture, Peperochromen.

Introduction

The world's attention to treat diseases from natural materials has increased. WHO records show that about 65% of the population of developed countries and 85% of the population of developing countries use natural materials. According to the Resolution promoting the role of Traditional Medicine in Health System, in PRC (People's Republic of China) the use of natural ingredients reached 90%. The growth trend of natural medicines in Indonesia in the period 2009-2013 amounted to 82.23%.[16,19] This is because the drugs from natural substances are considered safer and almost have no harmful side effects compared to modern medicine.[7,19]

The genus *Peperomia* is the second largest in the Piperaceae family and comprises more than 600 species widely distributed in Indonesia[16]. *Peperomia pellucida* is a perennial herb that grows wild and clustered in humid places and shaded from the sun. This herb known in Indonesian as "sasaladaan" is one of the plant that has been used as medicinal herbs[9]. Empirical use, in Kalimantan island, water stew from *P. pellucida* herb are used to overcome rheumatism and ulcers[21], fever, wound and skin diseases[9]. Sasaladaan in the Philippines is used to treat headaches, stomachaches and kidney problems[13].

Several studies have shown that sasaladaan has activities as antidiabetics[5,23,24,26], antimicrobials and anticancer[27], antipyretics and depressants[11], analgesics[15], antioxidants[5,17,18], anti-inflammatory and analgesic[2] and antihiperuricemia[25]. This is related to the chemical content possessed by the herbs sasaladaan: flavonoids, tannins, steroids, saponins, triterpenoids, a dimeric ArC_2 compound, monoterpenes and sesquiterpenes[1,3] and chromenes (peperochromens A and B)[24].

The obstacles need to take a long time to produce a medicinal whole; growing in different areas can affect the content of its compounds, growth depends on climate and environmental conditions[6]. Technique of herba sasaladaan development needs to be developed to produce plants on a large scale with a relatively fast time on a limited land and controlled environmental conditions. One alternative method of propagation that can be done is by tissue culture techniques.

Plant tissue culture techniques or *in vitro* cultures can produce secondary metabolites in plant tissue and also in cells that are preserved in aseptically made[3]. In the pharmaceutical field, tissue culture method is advantageous because it can produce a useful secondary metabolite[22].

The advantages of tissue culture in the production of secondary metabolites compared with intact plants include the absence of climate limitations, does not require extensive land and bioactive compounds can be produced

continuously under controlled circumstances. Another advantage of *in vitro* propagation of medicinal plants is being able to produce high quality drugs or medicinal substances[9].

The aim of this research is to know the growth response of plantlet *P. pellucida* on MS medium (Murashige and Skoog) Woody Plant Medium (WPM) and Driver-Kuniyuki Walnut medium (DKW) and to know whether tissue culture method can produce the same secondary metabolite with comparator of peperochromen-A compound obtained from isolation of wild sasaladaan herb extract.

Material and Methods

Materials: The tools used for tissue culture include the BC-11E S039213 autoclave, hot plate, magnetic stirrer, Laminar Air Flow (LAF), petridish, dissection equipment (large tweezers, small tweezers, scalpel blades), UV lamps λ 254 nm and λ 366 nm and common glass tools used in the Tissue Culture Laboratory. Research materials used for tissue culture include alcohol, aquadest, 1% swallow, chlorox, bactericidal (Streptomycin Sulfate), MS medium (Murashige and Skoog) Woody Plant Medium (WPM), Driver-Kuniyuki Walnut medium (DKW), fungicide (mixture carbendazim and mankozeb) 1%, medium, HCl, $HgCl_2$, NaOH, spirtus, sucrose. TLC plates coated with silica gel GF_{254} (Merck 0.25 mm). *P. pellucida* is obtained from the collection of tissue culture laboratory, Faculty of Agriculture, Padjadjaran University.

Tissue Culture Technique

Tools sterillization: Equipments are washed with washing soap, rinsed, then dried. The dry tools are wrapped in paper (except for the culture bottle). All the tools are sterilized with autoclave at 121^0C and pressure of 1.5 Psi (kg / cm^2) for 45 minutes.

Medium Preparation (MS, WPM, DKW) and Medium Sterilization: Medium is prepared by taking stock solution of 500 mL, then mio-inositol and sucrose are added. The solution was homogenized using a magnetic stirer and conditioned at pH 5.8 for MS medium, pH 5.6 WPM medium and 5.5 DKW medium by adding NaOH when pH is too low and when pH is too high HCl was added. Then agar was added. The solution is stirred and boiled. After boiling, the solution is poured into ± 10ml bottle culture per bottle. The bottle is covered with 0.3 mm PP plastic and fastened with rubber. Media is sterilized with autoclave at 121^0C, pressure 1.5 kg / cm^3 for 45 minutes. After that, the bottles are placed on the culture shelves.

Explant Sterilization: Explant usualy comes from the top of the sasaladaan herb (*P. pellucida*). Explant was cleaned first using a brush then washed with running water, then soaked with 1% detergent solution, rinsed again with running water and aquades. After that, explant was soaked with 0,5% $HgCl_2$ solution, 1% fungicide solution and 1% bactericidal solution soaked and rinsed again with water. Further sterilization was done with 0.5% sodium hypochlorite solution in LAF room.

Explant culture: Explant that had used for this experiment was part of *P. pellucida* shoot. Explant was placed on Petridish, part of *P. pellucida* shoots ready to cut using a scalpel. The mouth of the bottle was preheated with a bunsen lamp to prevent contamination. Then explant was planted on each medium with sterile tweezers. To maintain sterilization of the appliance, the scalpel and tweezers were always heated before use. Before closing, the bottle mouth is reheated. After that, the bottle was covered with aluminum foil and tied with rubber. Bottles were labeled according to treatment and date of planting.

Maintenance: Maintenance of the explant culture was done by putting them on the shelves, sprayed with 70% alcohol and kept at the room temperature between 21-25^0C and provided by irradiation of 20 watts fluorescent light.

Data Analysis: Qualitative analysis by way of visual data was analyzed by using descriptive method. Quantitative analysis was done by weighing the fresh weight of plantlets or shoots that grow using the analytical balance.

Secondary Metabolite Examination with Thin Layer Chromatography: Secondary metabolite monitoring was done by TLC. The resulting tissue culture from three different media (MS , WPM and DKW) was removed from the culture bottle and cleaned from the rest of the planting medium. The plantlet and shoots from each medium were grinded in mortar to become small pieces and then soaked in 96% ethanol that has been redestilated for 2 x 3 hours, extracts were filtered and collected in bottles. The extraction process was done until the extract was clear. The ethanol extract was evaporated by vacuum to become a concentrated extract.

The next step was monitoring secondary metabolites with TLC. The plantlet extracts, wild sasaladaan extract and Peperochromen-A were stained on a silica gel plate GF_{254}. The silica plate is inserted in a TLC chamber saturated with *n*-hexane: ethyl acetate (6:4) for MS medium, *n*-hexane: ethyl acetate (7:3) for WPM medium and chloroform: ethyl acetate (7:4) for DKW medium until the eluent reaches the boundary marker. The TLC results are seen under UV light 254 nm and 366 nm.

Results and Discussion

Explant observation on MS, WPM and DKW Media: Table 1 showed that the average days of root emergence were 7 days after the explant were planted on MS medium. The explants planted on the MS medium was part of the wild sasaladaan shoot. The planlet roots formation was affected by sulfur concentration in medium. Sulfur has an important role to promote plantlet's root growth, endurance and plant body protection[4]. The average days of leaf emergence were 10 days after the explant was planted on

MS medium. This can be affected by nitrogen in MS medium.

The nitrate elements intake by explants require a low pH, on the other hand ammonium intake causes the liberation of H^+ so that the medium becomes acidic. Medium Murashige and Skoog (MS) provide nitrogen in the form of NH_4NO_3 salt, this is a good strategy and has a double advantage, because in addition to its complete source of Nitrogen, the nitrogen in the salt form can mantain the pH of the medium[4].

The average days of the plantlet's shoot appearing on MS medium are 13 days after the explant was planted on MS medium. This is influenced by the presence of cytokinin or auxin which is endogenous growth regulator (ZPT) contained by explant. The role of ZPT is to stimulate the division and enlargement of cells present in plant shoots and cause the growth of new buds[4].

Total weight of fresh plantlet obtained on MS media is as much as 48.85g. Pierik[20] claimed that the growth of plantlets in one plant species may differ depending on factors such as the original explant position in plants and growth conditions.

The *P. pellucida* explants planted on WPM medium bottle number 1-5 were from wild *P. pellucida* shoot while bottle number 6-11 was from subculture. The days of roots, leaves and shoots appearence on *P. pellucida* roots explant after planting on WPM medium were shown on the table 2. There are the differences between the root growth of the wild *P. pellucida* explant and the subculture *P. pellucida* explant. The explant from wild *P. pellucida* has longer days of roots growth response as it apperared on the 3rd bottle 10 days and the 2nd bottle 17 days after the expalnt was planted on the WPM medium, compared to explant that had planted previously on the same medium (subculture) on the 6th bottle and the 11th on 7 days. That's because the is more nutrients absorbed by explant from subculture so that the regeneration power increases, because it has grown on the same medium before.

The average days when the shoots appear on explant derived from wild plants were 20 days, whereas in explant derived from the subculture were at 10 days after the explant was planted on WPM medium. Shoot formation could be a sign of of regeneration explant inoculated on tissue culture medium. Nutrients in medium were absorbed by explants such as nitrogen (N), potassium (K), sulfur (S), iron (Fe), potassium (K), phosphorus (P), zinc (Zn) and thiamine are required for forming a new shoot and it can stimulate cell division, so thats can increase the activity of metabolism in explant tissue cause plant cells[26].

Leaf growth response on WPM growth media is relatively slower compared to root and shoot growth. The average leaf that appears on explant derived from wild plants is 24 after the explant was planted on WPM medium.

Table 3 shows that the explant of sasaladaan subculture has faster root growth respon than the wild sasaladaan explant. Jumroh et al[10] mentioned that subculture has more sterile condition and it is able to absorb more nutrients in new planting media compared to previous media. The subculture results can develop depending on the growing rate of explants.

The average appearance of shoots on DKW media is 15 days after the explant was plante on DKW medium. The type of shoot that is formed is called axillary buds, that is the buds which are formed directly without going through the callus phase. The appearance of shoots, can also be influenced by micronutrients such as copper (Cu). Cu deficiency can cause the plant shoots will furl and inhibit plant growth. The formation of shoots shows the success of plant regeneration. The average leaves appear on DKW media, in 17 days. Sufficient quantities of Nitrogen in a DKW medium will increase leaf growth of explant. Micronutrient elements such as Manganese or vitamin can help the formation of leaves, Cl administered in Thiamine HCl and Pyridoxine HCl[25].

Secondary Metabolites Examination Result with Thin Layer Chromatography: Plantlets produced from MS, WPM and DKW media were extracted three times with ethanol 96% for 3 hours. Plantlets extract from MS, WPM, DKW and Peperochromen-A media were analyzed by Thin Layer Chromatography on silica gel plate GF_{254} using eluent *n*-hexane: ethyl acetate (6: 4) for plantlets from MS medium and (7: 3) for plantlets from WPM media and for plantlets from DKW media using chloroform: ethyl acetate (7: 3). The TLC results were observed in 366 nm UV light, compared with compounds of Peperochromen-A. Chromatogram results can be seen in table 4.

Table 4 shows TLC results of *P. pellucida* plantlet extract from MS, WPM and DKW. All plantlets extract produced Peperochromen A. The compound was characterized by yellow spot under UV 366 nm with Rf value of 0.24; 0.125; 0.816 in various eluent. TLC chromatograms of the *P. pellucida* wild herb extract, *P. pellucida* plantlet extract and Peperochromen-A from MS, WPM and DKW medium can be seen in figure 1.

In the TLC chromatogram, the spot from *P. pellucida* planlet extract has the same yellow color intensity as Peperochromen-A spot, while on the results of chromatogram wild *P. pellucida* herb extract was seen as a reddish yellow patch covered by the presence of chlorophyll and the concentration of the compound was too small. Heriyanto and Limantara stated that the amount of chlorophyll from a plant depends on the growing place and environmental conditions[8]. Yellow color spots on the extract of tissue culture results are more visible than wild

P. pellucida extract, because the peperochromen-A concentration of tissue culture is influenced by the nutritional content of elements of macronutrients, micronutrients and vitamins. Masoumian et al[14] stated that amino acids in media such as glutamine as one of the precursors can increase the production of flavonoids.

There is a difference between secondary metabolite concentration resulting from sasaladaan plant tissue plant extract more than sasaladaan wild extract. This is because the formation of secondary metabolites by cultured plants is influenced by various factors such as controlled environmental conditions, light intensity and stable temperature, medium pH and nutritional composition of the media used. Kumar et al stated that the effect of constant temperature on tissue culture can increase secondary metabolite content, based on temperature incubation 20, 25, 30 and 32^{o}C.

The highest flavonoid content (1.67 + 0.04 mg / g) was yielded at temperature 25^{o}C[12]. In plantlet (*in vitro* growth) light lasts continuously for 24 hours, while in wild plants it lasts according to fluctuation of light and termperatur in nature. The appearence of first roots, leaves and shoots on the plantlets can be seen in figure 2.

Table 1
Explant growth response to MS Medium

MS medium Bottle Number	Days of roots emergence	Days of shoots emergence	Days of leaves emergence	Plantlet weight (g)
1	8	14	11	8.97
2	6	13	10	1.60
3	8	13	12	3.14
4	7	15	11	1.98
5	7	11	9	10.16
6	7	11	9	1.96
7	8	14	11	7.93
8	7	15	12	1.36
9	6	14	11	1.81
10	7	12	9	0.33
11	6	14	11	8.70
12	7	12	9	0.92
Average	7	13	10	Total= 48.85

Table 2
Explant growth response to WPM Medium

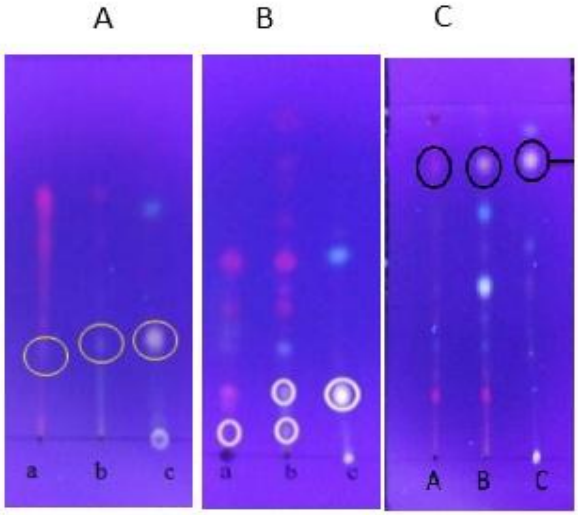
MS medium Bottle Number	Days of roots emergence	Days of shoot emergence	Days of leaf emergence	Plantlet weight (g)
1	15	28	38	1.91
2	17	27	31	1.70
3	10	20	33	2.33
4	14	21	29	4.63
5	13	22	28	4.21
6	7	10	17	8.22
7	7	10	17	6.82
8	7	10	18	9.33
9	7	10	18	5.02
10	7	10	17	1.32
11	7	12	18	1.30
Average	10	16	24	Total= 46.79

Table 3
Explant growth response to DKW Medium

MS medium Bottle Number	Days of roots emergence	Days of shoots emergence	Days of leaves emergence	Plantlet weight (g)
1	9	11	15	1.94
2	9	11	15	1.66
3	9	11	20	1.96
4	15	15	18	1.38
5	13	15	17	1.91
6	13	15	18	2.03
7	13	16	16	1.48
8	13	14	16	1.07
9	13	14	17	1.94
Average	11	13	16	Total= 48.85

Table 4
TLC Results of Ethanol Extract *P. pellucida*

Medium	Eluent	R*f* from wild *P. pellucida* extract	R*f* from plantlets extract	R*f* Peperochromen-A
MS	*n*-hexane: ethyl acetate (6: 4)	0.23	0.24	0.25
WPM	*n*-hexane: ethyl acetate (7: 3)	0.1	0.125	0.138
DKW	chloroform: ethyl acetate (7: 3)	0.8	0.816	0.83



Detected by UV 366 nm
A= MS medium
B= WPM medium
C= DKW medium
a = Wild *P.pellucida* extract
b = Plantlet *P.pellucida* extract
c = Peperochromen-A (yellow spot)

Figure 1: TLC Chromatograms of *P. pellucida* plantlet from MS, WPM and DKW mediums

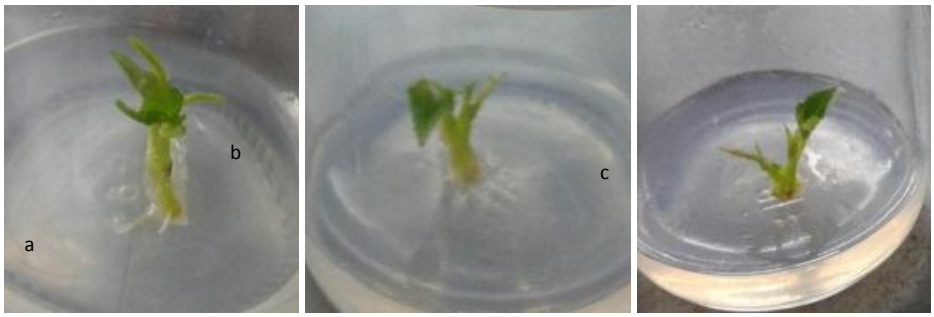


Figure 2: The *P. pellucida* plantlet's first roots (a), leaves (b), dan shoots(c)

Conclusion

The results of this study indicate that sasaladahan plantlet has been obtained from tissue culture on MS, WPM and DKW media. The response of shoot bud growth was different in MS, WPM and DKW basic media. TLC results of *P. pellucida* planlet extracts from the three tissue culture mediums can produce Peperochromen-A characterized by a yellow spot detected with UV light 366 nm with a R*f* value of 0.24; 0.125; 0.816 respectivelly in *n*-hexane: ethyl acetate (6: 4), *n*-hexane: ethyl acetate (7: 3) and chloroform: ethyl acetate (7: 3) mobile phase.

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