Comparison of Organosulfur Bioactive Compounds in Bulb, Callus and Cells Suspension of Single Garlic (*Allium sativum* L.)

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Abstract. Single garlic (*Allium sativum*. L) is one of herbs widely used for healing various kinds of health problems such as diabetes, hypertension, cholesterol, atherosclerosis, and cancer. Benefits of single garlic as a medicinal plant was caused by organosulfur bioactive compounds which are widely used in the field of health including allin, allicin, allyl sulphide, ajoene and dithiin. This study aimed to identify and compare a content of organosulfur bioactive compounds in bulb, callus and cells suspension. Callus induction was carried out by culturing the buds explants on MS basal medium with 2.4 D and kinetin. The cells suspension were conducted by culturing callus in liquid medium with 2.4 D and kinetin. HPLC technique was used to analyse organosulfur bioactive compound in samples. The HPLC chromatograms confirmed the same presence of 30 organosulfur bioactives compounds. Among the 30 detected allin, allicin, allyl trisulphide, E1 propenyl allyl sulfdide, 2 propenyl 1 propenyl disulfide, 2 vinyl 4H 1,3 dithiin, 3 vinyl 4H 1,2 dithin and ajoene were found as the major compounds in samples. The ratio of bioactive compounds in the bulb, callus and cells suspension is 3.5 : 1.4 : 1. Although produced lower yields but callus and cell culture have a potency to produce organosulfur bioactive compounds. Yields can improved by addition elicitor, precursor or implementation of bioreactor cultur system, so that it can be used to support the use of herbal medicines.

Keywords : single garlic, organosulfur compounds, bulb, callus, cells suspension

1. INTRODUCTION

Secondary metabolites in plants is chemical metabolic compounds that does not play a direct role for their growth, but these secondary metabolites have various biological activities that can be used by humans. One of the plants producing secondary metabolites that have various biological activities is single garlic (*Allium sativum*. L.). Single garlic is a garlic produced from seed which does not develop
well because of unsupportive environment conditions so it produce one clove only [1]. Currently single garlic is widely used as an herbal for cure various kinds of health problems, such as diabetes, hypertension, cholesterol, atherosclerosis, and cancer [2]. These benefits are due to the presence of volatile oil and other active compounds [3]. Bioactive compounds in garlic as a whole there are reported to be more than 100 kinds of compounds that are biologically very useful [4]. Approximately 33 of these compounds are organosulfur [5]. Organosulfur compounds are compounds which are rich in sulfur content, causing garlic to have distinctive taste and odor characteristics [6] as well as the properties that play a role in its pharmacological functions [2]. Of the several organosulfur bioactive compounds contained in a single garlic, five main groups of organosulfur bioactive compounds that have a role in the health field are alliin, allicin, allyl sulfide, ajoene and dithiin. Currently in vitro culture techniques are needed, among others to produce disease-free plants, rapid multiplication of plants that have rare genotypes, transformation of plant genomes and production of secondary metabolites that have commercial value [7], [8]. In addition, factors such as seasonal changes, environmental conditions, plant age, difficulties in spreading certain species causing limited availability of bioactive compounds can be overcome by using plant cell culture [9], [10]. Several types of cell cultures that can be used to produce plant bioactive compounds include callus culture and cell suspension culture. Several studies have been carried out to produce bioactive compounds through cell culture, for instance; accumulation of alkaloid compounds in callus culture Carantus roseus [11], accumulation of sulfur compounds in callus culture of Allium hirtifolium Boiss [12], accumulation of flavonoid compounds in Centella asiatica L. cell suspension culture [13], increased accumulation of scopoletin in Spilanthes acmella Murr cell culture . [14]. This study aims to identify and compare the content of a single garlic organosulfur bioactive compound in bulb, callus and cell suspension. as an effort to support the development of herbal medicines.

2. MATERIALS AND METHODS
The tools used in this study include Laminar Air Flow (LAF), autoclaves, inoculation equipment (dissecting kit), analytical balance, culture shelves, and glassware which include glass cups, measuring cups, measuring flasks, glass mixers, storage bottles of solution stock. To culturing buds explants and callus used solid and liquid MS medium with the addition of 2.4 D and kinetin growth regulator. The sample used in this study was a single garlic of Tawangmangu Baru variety obtained from Magelang-Central Java.

2.1. Callus Induction
Prior to inoculation, a single garlic bulb was sterilized using 5.25% sodium hypochlorite (NaOCl) then rinsed with sterile aquades,. Bud explants of the basal meristem section were cultured on the Murasighe & Skoog (MS) solid medium with the addition of 0.3 ppm 2,4-D and 0.5 ppm kinetin [15]. The culture was incubated with a light intensity of 600 lux, lighting for 16 hours and a temperature of 25-26°C for 8 weeks. Multiplication is done by doing sub-culture every 4 weeks with the same solid medium composition.

2.2. Initiation and multiplication of cell suspensions
The initiation was carried out on callus that had been obtained by culturing 1 gram of callus into 25 ml MS liquid medium with the addition of 0.3 ppm 2,4-D and 0.5 ppm kinetin in a 100 ml erlenmeyer. Incubation was done by shaking using a shaker at 100 rpm and a temperature of 20°C. Multiplication is done by transferring 5 ml of cell suspension into 20 ml of a fresh MS liquid medium on a 100 ml erlenmeyer. Multiplication is done by doing sub-culture every 2 weeks with the same liquid medium composition.
2.3. Analysis of organosulfur bioactive compounds
Extraction was carried out on bulb, callus and cell suspension. The extraction begins by crushing the buds, callus and filtering the cell suspension. Blended buds, callus and cell pellets were dissolved in 95% methanol (1:5). The solution is stirred until homogeneous and let stand for 24 hours at cold temperatures and in a closed bottle, then the solution is filtered to obtain the filtrate. Addition of solvents and filtering is carried out again to the pellet up to 3 times with a ratio of the same amount of solvent as before. The collected filtrate was then centrifuged at a speed of 5000 rpm for 20 minutes to be taken supernatant.
Content or level determination of organosulfur bioactive compounds in the supernatant was carried out using HPLC with Shim-pack VP ODS 5 µm 150 x 4.6 mm a stationary phase, of 10 mM Potassium dihydrogen phosphate: Acetonitrile (1:1) (v/v) a mobile phase, flow rate 1 ml/min, UV detector 210 nm.

3. RESULT AND DISCUSSION
Organosulfur bioactive compounds in the sample were separated and identified using the HPLC method. The results of HPLC analysis were obtained in the form of signal chromatograms in which there are peaks which describe the number of organosulfur bioactive compounds in the sample. Chromatograms from HPLC analysis results from bulb, callus and cell suspension extract are shown in Figure 1. The peaks on the chromatogram are formed according to the retention time (RT) of each compound. The peak height is the absorption response of the chromatogram expressed in millis Absorption Units (mAU).
Figure 1. HPLC chromatogram of organosulfur bioactive compounds in (a) bulb. (b) callus. (c) cell suspension

Table 1. Content of organosulfur bioactive compounds based on HPLC chromatograms

| Peak Number | Compounds                          | Retention Time (RT) | Content of organosulfur compounds (µg/g) in Bulb, Callus, Cell suspension |
|-------------|------------------------------------|---------------------|--------------------------------------------------------------------------------|
| 1           | 1,2 Epithiopropane                 | 1,155              | 3648.98, 874.59, 1033.53                                                        |
| 2           | Allyl mercaptan                     | 1,158              | 9331.61, 2472.70, 2730.32                                                       |
| 3           | Dimethyl disulfide                  | 1,203              | 2089.91, 435.94, 657.62                                                          |
| 4           | 2,5 Dimethylthiophene               | 1,318              | 2588.95, 585.25, 782.72                                                          |
| 5           | 3 Methyl 2 cyclopentene 1 thione    | 1,322              | 1557.87, 384.87, 643.64                                                          |
| 6           | Isobutyl isothiocyanate             | 1,341              | 2262.03, 583.04, 788.03                                                          |
| 7           | 2,5 Dimethyltetrahydrothiophene     | 1,348              | 1903.54, 530.79, 587.90                                                          |
| 8           | Propyl disulfide                    | 1,357              | 2422.05, 579.11, 788.97                                                          |
| 9           | 1,3 Dithiane                        | 1,383              | 3814.25, 874.80, 1147.91                                                         |
| 10          | Allyl methyl disulfide              | 1,391              | 3648.66, 923.53, 1266.12                                                         |
| 11          | 2,3,4 Trithiapentane                | 1,461              | 1904.41, 437.40, 654.31                                                          |
| 12          | 1,2 Dimercaptocyclopentane          | 1,479              | 2077.63, 481.25, 672.72                                                          |
| 13          | 2 Vinyl 4H 1,3 dithiin              | 1,517              | 2088.42, 484.39, 633.40                                                          |
| 14          | 3 Vinyl 4H 1,2 dithiin              | 1,519              | 2238.29, 576.40, 756.56                                                          |
| 15          | E1 Propenyl allyl disulfide         | 1,544              | 2602.09, 628.40, 890.96                                                          |
| 16          | 2 Propenyl 1 propenyl disulfide     | 1,547              | 1909.21, 433.75, 568.93                                                          |
| 17          | Allyl propyl disulfide              | 1,587              | 3290.64, 920.69, 1026.87                                                         |
|   | Compound                             |   |   |   |   |
|---|--------------------------------------|---|---|---|---|
| 18| Allyl methyl trisulfide              | 1.618 | 3117.69 | 969.64 | 996.09 |
| 19| Deoxyalliin                          | 1.665 | 7130.87 | 2079.76 | 2486.00 |
| 20| Allyl 2 propenethiosulfinate         | 1.666 | 5693.95 | 1693.22 | 1848.74 |
| 21| Trans 1 propenyl allyl thiosulfinate| 1.667 | 4524.03 | 1316.32 | 1528.23 |
| 22| 1 propenyl allyl thiosulfinate       | 1.669 | 4137.07 | 1255.63 | 1438.13 |
| 23| 2 propene 1 sulfinothioic acid 1 propenyl ester | 1.671 | 2780.98 | 826.41 | 926.74 |
| 24| Allicin                               | 1.673 | 15883.77 | 4558.18 | 4732.77 |
| 25| Alliin                                | 3.208 | 31272.83 | 9761.96 | 10604.27 |
| 26| Cycloalliin                           | 3.211 | 8354.22 | 2392.79 | 2759.36 |
| 27| Allyl trisulfide                     | 3.505 | 2436.08 | 728.87 | 824.40 |
| 28| 3,5 Diethyl 1,2,4 trithiolane        | 4.733 | 2595.50 | 773.95 | 812.06 |
| 29| Ajoene                                | 7.935 | 13570.85 | 3614.27 | 4048.50 |
| 30| Diallyl heptasulfide                 | 11.508 | 4335.0 | 1267.57 | 1329.13 |

Figure 2. Comparison of major organosulfur bioactive compounds in (a) bulb, (b) callus, (c) cell suspension

As shown in Figure 1, the peaks formed indicate the number of bioactive compounds found in the sample. In the bulb, callus and cell suspension samples, there were organosulfur bioactive compounds with the same amount of 30 compounds. The time required for the formation of peaks in each type of compound is also the same for all three samples. The difference in the three samples is the absorption response on the chromatogram which means that there are differences in the levels of organosulfur bioactive compounds.

In Table 1 it is shown that among the three samples, the highest levels for all types of organosulfur bioactive compounds are contained in the bulb. Compare with callus, levels of organosulfur bioactive compounds in cell suspensions are slightly higher compared to levels of organosulfur bioactive compounds produced by callus. Figure 2 shows the comparison of organosulfur bioactive compounds in bulb, callus and cell suspension against five main organosulfur compounds, namely alliin, allicin, ajoene, allyl sulfide (1 propenyl allyl disulfide, 2 propenyl 1 propenyl disulfide, allyl trisulfide, allyl methyl disulfide, allyl sulfide (1 propenyl allyl disulfide, 2 propenyl 1 propenyl disulfide, allyl
trisulfide, allyl methyl disulfide, allyl sulfide propyl disulfide, allyl methyl trisulfide, diallyl heptasulfide) and dithiin (2 Vinyl 4H 1,3 dithiin, 3 Vinyl 4H 1,2 dithiin).

The graph shows the level of organosulfur compounds from high to low of the three samples are alliin, alicin, ajoene, allyl sulfide group and dithiin group. Ratio of levels of organosulfur bioactive compounds in bulb, cell suspension and callus is 3,5: 1,4: 1. The content of organosulfur bioactive compounds in cell suspension and callus are smaller than in bulb. The low production of organosulfur compounds in cell culture compared to the content of the bulb is partly due to the synthesis of secondary metabolites in cells that have not been differentiated in some cell cultures or plant tissue is usually much lower than in intact plants [16].

Cell culture methods that have been carried out have the potential to be able to produce organosulfur bioactive compounds, even though the levels of organosulfur bioactive compounds in callus and cell suspensions are lower than organosulfur bioactive compounds in bulbs. Manipulation of cell culture growth conditions can stimulate an increase in secondary metabolites [16]. Alternatives that can be done to increase the accumulation of bioactive compounds including the addition of elicitors and precursors [17]. The use of elicitors or so-called elicitation is one of the effective strategies in increasing the productivity of bioactive compounds [18], [19].

CONCLUSION

Based on this research, it can be concluded that callus culture and cell suspension culture of single garlic have the potential to produce organosulfur bioactive compounds. Accumulation of organosulfur bioactive compounds have a possibility to enhance by addition of elicitors, precursors or implementation of bioreactor cultur system.

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REFERENCES

[1] Untari I. 2010. Bawang Putih Sebagai Obat paling Mujarab Bagi Kesehatan. GASTER. Vol 7. No 1.

[2] Hernawan, U. E., Setyawan, A. D. 2003. Senyawa Organosulfur Bawang Putih (Allium Sativum L) dan Aktivitas Biologinya. Biofarmasi, 1(2):65-76.

[3] Bharat, P. 2014. Comparative Analytical study of Single Bulb and Multi Bulb Garlic (Allium sativum Linn.). International Journal Of Ayurveda & Alternatif Medicine, 2(4):86-91.

[4] Challem, J. 1995. The Wonder of Garlic, (Online) (www.drpasswater.com/nutrition_library/wonder_garlic.html), diakses 12 September 2017.

[5] Palani, S., Joseph, N.M., Nisha, M.J., Tegene, Y., Zacharia, A. 2014. Medicinal Properties of Garlic-A Concise Review. Current Research in Pharmaceutical Sciences, 4(4):92-98

[6] Crozier, A., Clifford, M.N., Ashihara, H. Plant Secondary Metabolites. Oxford: Blackwell Publishing Ltd.

[7] Debnarh, M., Malik, C.P., Baisen, P.S. 2006 Micropropagation : a tool for the production
of high quality plant based medicines. Curr Pharm Biotechnol 7:33–49

[8] Altpeter, F., Springer, N.M., Bartley, L.E., Blech, A.E., Brutnell, T.P., Citovsky, V., Conrad, L., Gelvin, S.B., Jackson, D., Kausch, A.P., Lemaux, P.G., Medford, J.I., Orozco-Cardenas, M., Tricoli, D., VanEck, J., Voytas, D.F., Walbot, V., Wang, K., Zhang, Z.J., Stewart, C.N. 2016. Advancing crop transformation in the era of genome editing. Plant Cell 28:1510–1520.

[9] Cherdshewasart, W., Subtang, S., Dahlan, W. 2007 Major Isoflavonoid Contens of the Phytoestrogen Rich-herb Pueraria Mirifica in Comparison with Pueraria lobata. Journal of Pharmaceutical and Biomedical Analysis, 43(2):428-434.

[10] Castro, F., Braga, Q., Sousa, M., Coimbra, C., Chagas, R. 2016. Callus Induction and Bioactive Phenolic Compounds Production from Byrsonima Verbascifolia (L.) DC. (Malpighiaceae). Revista Ciência Agronômica, 47(1):143-151.

[11] Verma, K.A., Singh, R.R., Singh, S. 2013. Mutation Breeding in Catharanthus roseus (L.) G. Don: An Overview. Journal of Pharmacognosy and Phytochemistry, 2(1):334-337.

[12] Hoseinpoor, E.M., Mortazaeinezhad, F. 2016. Recognition of Sulfur Compounds in Tissue Culture Different Organs of Persian Shallot (Allium hirtifolium Boiss) by GC/MS. Journal of Herbal Drugs, 6(4): 219-225.

[13] Tan, S.H., Mahmood, M., Ariff, A. 2013. Synergism Affect Between Inoculum Size and Aggregate Size on Flavonoid Production in Centella asiatica (L) Urban (pegaga) Cell Suspension Culture. International Journal of Research in Engineering and Technology, 2(8):244-253.

(a) [14] Abyari, M., Nasr, N., Soorni, J., Sadhu, D..2016. Enhanced Accumulation of Scopoletin in Cell Suspension Culture of Spilanthes acmella Murr. Using Precursor Feeding. Brazilian Archive of Biology and Technology, 59: e1615053.

[15] Fauziah, A., Widoretno, W. 2015. Regenerasi Tanaman dari Eksplan Kalus Bawang Putih (Allium sativum L) secara In Vitro, Jurnal Biotropika, 3(1):32-35

[16] Collin, H.A. 2001, Secondary Product Formation In Plant Tissue Culture, Plant Growth Regulation, 34: 119-134

[17] Rao, S., Usha, K., Arjun. 2015. Production of Secondary Metabolites from Callus Cultures of Centella asiatica (L.) Urban, Annal of Phytomedicine 4(1):74-78.

[18] Sharma, M., Sharma, A., Kumar, A., Basu, K.S. 2011. Enhancement of Secondary Metabolite in Cultured Plant Cells Through Stress Stimulus. American Journal of Plant Physiology, 6(2):50-71.

[19] Giri, C., C., Zaheer, M. 2016. Chemical Elicitors Versus Secondary Metabolite Production In Vitro Using Plant cell, Tissue and Organ Cultures: Recent Trends and A Sky Eye View Appraisal, Plant Cell, Tissue and Organ Culture, DOI: 10.1007/s11240-016-0985-6