Diversity of bioactive secondary metabolites produced by medicinal plants of *Physalis angulata* L. (Ciplukan)

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Abstract. Plants are a main source of various types of secondary compounds. Plant secondary compounds play a greater role in interacting with the environment than contributing to the growth and development. Therefore, different environmental conditions besides influencing growth can also affect the profile and concentration of secondary compounds. Ciplukan (*Physalis angulata* L.) has been known as medicinal plants. The medicinal properties are derived from bioactive secondary compounds especially withanolide and physalin. This study aims to identify the diversity of secondary metabolites found in *in vitro* callus and plants tissue of ciplukan. Withanolide and physalin profiles of callus tissues, cotyledonary shoot-derived plantlet and germinated seeds-derived plant, were evaluated by LC-MS analysis. The LC-MS analysis of methanol extract showed the diversity in the amount and type of withanolide and physalins. This study confirmed that in undifferentiated callus cultures and *in vitro* induced plantlet of *P. angulata* the biosynthesis activity was not altered and the accumulation sites of withanolides were not missing. However *in vitro* regenerated plant produced withanolides and physalins in higher number. Therefore, modification of plant cell culture system to improve withanolide including physalin production of *P. angulata* is a good future prospect.

1. Introduction

Secondary metabolites are natural products which are mostly in the form of small organic molecules. Plants synthesize a variety of secondary metabolites, with complex chemical compositions which are not essential for the growth, development and reproduction but are very important to interact with the environment for the survival of the organism itself and to fulfill important physiological tasks. They play a role in resistance to pests and diseases, attractiveness of pollinators, and interactions with symbiotic microorganisms [1]. In general, secondary metabolites are produced by specialized cells or tissues and their production may be limited to families, genera and even certain species. In addition, they are unique, diverse and adaptive [2]. In addition to varies in the presence, quantity and quality in individual plants, between individuals and groups the plant secondary metabolites also variable among species populations, individuals, and even in different plant parts. Diversity also found in the specificity of biochemical interaction between plants and their enemies. In addition small structural variations can also have different effects [3].
Withanolides are group of compounds with a basic structure withaferin. This compound has more than 650 member. Withanolides has biological activity as anti-inflammatory, antitumor, cytotoxic, immunomodulating and cancer chemopreventive as well as antibacterial and antifungal properties. In addition, withanolides also acts as insecticidal activities, mostly as feedant deterrents, and selective phytotoxicity. Withanolides has been shown to be a powerful anti-feeding and immunosuppressant in insects [4]. Most of the known withanolides included in this group are subdivided into 13 subgroups, including the parent skeleton of withaferin A (1), withaphysalins, and physalins [5]. In Solanaceae withanolides only limited to subfamily Solanoide including genus Physalis. Physalis is one of the four main contributing genera of the withanolides structure in addition to the genus Jaborosa Juss., Datura L., and Withania.

Various types of withanolides have been isolated from the genus Physalis. Ten types of withanolide have been isolated from the aerial part of P. peruviana [6], two new types of withanolide were isolated from all parts of the plant [7]. Eight types of withanolides have been isolated from P. coztomatl [8]. In the aerial part of P. hispida, 9 new types of withanolide were also isolated along with 9 identified species [9]. In addition to P. pubescens [10] cytotoxic withanolides has also been isolated from P. angulata [11;12]. Withanolide isolated from P. angulata [13] and P. angulata var. Villosa [14] was detected to have anti-inflammatory and antiproliferative activity.

Besides withanolides Physalis spp also contains other steroidal constituents namely Physalins [15]. Ethanolic extract of the whole plant of Physalis angulata L. contained physalin F which showed cytotoxicity in vitro on five human and three animal cancer cell lines [16]. The extracts and physalin D from Physalis alkekengi were potential as antibacterial, especially against Gram-positive bacteria [17]. P. angulata also synthesised physalin B and Physalin F which showed antiplasmodial activity [18]. Combined knowledge about chemical composition and biological activity serves as the basis for the sustainable use of biodiversity in the development of new products including pharmaceuticals and medicines.

Secondary metabolites often present in a small amount in cell. In other site, the utilization of in vitro techniques has been proved able to increase secondary compounds in a sustainable manner and in greater quantities than the results of synthesis in the parent plant. Since the chemical composition of secondary metabolites profiles was affected by change of environmental condition [19]. The production of specific secondary metabolites has been frequently treated by environmental micro-stress. This study aims to identify the diversity of secondary compounds, especially withanolide in P. angulata plants that grow in nature as well as the results of induction in vitro.

2. Material and Methods

Plant materials

Variation of secondary metabolites were evaluated in two types of in vivo plant and two types of in vitro tissues. In vivo wild P. angulata L. (Ciplukan) with the height approximately 10 cm were derived from corn field area at South District, Malang, East Java. The two types of plants in vivo have different leaf edge morphologies, namely serrated (figure 1A) and entire leaf (figure 1B). Secondary compound profiles in in vivo plants will be compared with those profiles in in vitro plantlet and callus tissue of P. angulata. In vitro plantlets (figure 1C) were initiated from in vitro cotyledonal node explants which cultured on MS + 2 mg/L BAP + 0.05 mg/L IAA for shoot induction [20]. Subsequently, the multiplied shoots were subcultured into MS free hormone medium to promote growth of individual plantlet. Plantlets with approximately 3 cm height were used to analyze withanolide profile. Meanwhile, callus tissues (figure 1D) were induced from cotyledonal node explants in medium MS + 2.0 mg/L BAP + 4.0 mg/L 2,4-D [unpublished].
**Figure 1.** *In vivo* and *in vitro* source of Physalis tissues. A) *In vivo* Physalis plant with serrated leaf, B) *in vivo* Physalis plant with entire leaf, C) *in vitro* Physalis plantlet two weeks after subculture, D) *in vitro* callus derived from cotyledonary node explants of Physalis three weeks after subculture.

**LC-MS analysis of secondary compounds withanolides**

*In vivo* plant samples were washed with water to remove soil media. While *in vivo* callus samples and *in vitro* plantlets were rinsed to separate from agar media. The sample was dried and grounded with mortar and pestle. The mashed samples were weighed approximately 2 g, dissolved with 95% methanol with a ratio of 1:5 (material: methanol), homogenized and allowed to stand for 24 hours at cold temperatures. Then the solution was filtered with a vacuum filter Erlenmeyer to obtain filtrate and pellets. The pellet was homogenized and refined 3 times then the collected filtrate was evaporated to separate the solvent and methanol until a final volume of half of the initial volume was obtained. Subsequently the extract was dissolved with methanol to a concentration below 100 ppm then the supernatant was purified with a solid phase extraction (SPE). The solution to be analyzed was filtered by cellulose acetate filter with 0.45 μm pore size then degassed and put in a dry and contaminant free bottle and ready for LC-MS analysis.

**3. Result and Discussion**

All sample extracts of *P. angulata* revealed the presences of bioflavonoids, sterols & triterpenoids, glycosides, acidic compounds, and reducing sugars. The results of LCMS analysis on all Physalis tissue samples detected compounds with a retention time range of 1.212 – 44.064 min (figure 1). The total compounds detected were 75 but the number of compounds detected in each sample was different, ranging from 60 - 73 (Table 1). *In vitro* tissues had fewer type of compounds than *in vivo* Physalis plants. Callus *in vitro* tissue contains the least number of compounds.
Among all 75 compounds the 47 withanolides compounds were identified (Table 1). Most types of withanolide were detected in all samples except physoperuvine (1), peruvianoxide (3) and withanolide E (10) which were not detected in in vivo plants with serrated leaf. In vitro plantlet produced similar types of secondary metabolites to in vivo plants with entire leaf. This profile showed that in in vitro plantlet of P. angulata the biosynthesis activity of withanolides was not altered and the accumulation sites were not missing. Meanwhile, one type of whitanolide, Physalolactone B 3 O β glucopyranoside, was not detected in callus cultures. It is suspected that in undifferentiated callus the site for biosynthesis of secondary compounds has not been fully formed yet.

Secondary compounds produced by plants to interact with the environment both biotic and abiotic. Great diversity of secondary metabolites occur in many spheres and are determined by many factors. Variations can occur in numbers, presence or absence, quantitative or qualitative, as well as within or between individuals and groups [3]. Variations in concentration can be influenced by age and size of plants as a consequence of changes in resource requirements for growth such as nutrients, light and water [21]. The concentration of secondary compounds in tissues can also be related to specific regulations in their biosynthesis which are strongly influenced by environmental conditions [22]. In this study the differences in leaf edge shape in in vivo P. angulata plants are thought to reflect differences in plant physiological age. Differences in the types of withanolides secondary compounds found in high concentrations between the two indicate a difference in the need for strategies to interact with the environment.

Table 1 Various compounds in in vivo and in vitro tissues samples detected by LC-MS method
Note: sample type I: in vivo plant with serrated leaf; II: in vivo plant with entire leaf; III: in vitro plantlet; in vitro callus, number in the ( ) showed withanolide and sterol related compounds, score 1 in each sample profile shows the presence of withanolides compound. * indicates withanolide compounds (no. 1, 2, 10 and 47) which were not detected in certain sample.

The detected withanolide compounds also showed varied content between samples (Table 2). In vivo plant samples which have different leaf edges showed different variations in the profile of secondary metabolites. In vivo plant with entire leaf had 47 withanolides compounds which was similar to in vitro plantlet. While in vivo plant with serrated leaf only had 44 withanolide compounds without three types of withanolides compounds (no. 1, 2, and 10) the callus culture did not have only one type of withanolide compound (no. 47) (Table 1).
Table 2 Variation in composition and concentration of withanolides compounds in four different tissue samples of *P. angulata*

| Sample Type | Composition | Concentration (mg/g) | In vitro | Concentration (mg/g) | In vitro Callus | Concentration (mg/g) |
|-------------|-------------|----------------------|----------|----------------------|-----------------|----------------------|
| In vivo Plant with serrated leaf | Physalin H | 33.34 | Physalin H | 31.41 | Physalin H | 27.32 | Physalin H | 31.44 |
| | Withagulatin G | 22.72 | Withagulatin G | 20.14 | Withagulatin G | 21.16 | Withagulatin G | 22.72 |
| | Withagulatin C | 18.24 | Withagulatin C | 16.20 | Withagulatin C | 17.50 | Withagulatin C | 18.24 |
| | Witherolactone D | 21.18 | Witherolactone D | 19.65 | Witherolactone D | 19.30 | Witherolactone D | 21.18 |
| | Witherolactone E | 23.81 | Witherolactone E | 22.35 | Witherolactone E | 22.00 | Witherolactone E | 23.81 |
| | Witherolactone F | 23.97 | Witherolactone F | 22.94 | Witherolactone F | 22.96 | Witherolactone F | 23.97 |
| | Witherolactone G | 24.71 | Witherolactone G | 23.11 | Witherolactone G | 22.88 | Witherolactone G | 24.71 |
| | Witherolactone H | 24.71 | Witherolactone H | 23.11 | Witherolactone H | 22.88 | Witherolactone H | 24.71 |
| | Witherolactone J | 29.21 | Witherolactone J | 27.25 | Witherolactone J | 26.80 | Witherolactone J | 29.21 |
| | Witherolactone K | 30.91 | Witherolactone K | 28.50 | Witherolactone K | 28.00 | Witherolactone K | 30.91 |
| | Witherolactone L | 31.09 | Witherolactone L | 29.11 | Witherolactone L | 28.50 | Witherolactone L | 31.09 |
| | Witherolactone M | 31.09 | Witherolactone M | 29.11 | Witherolactone M | 28.50 | Witherolactone M | 31.09 |
| | Witherolactone N | 31.09 | Witherolactone N | 29.11 | Witherolactone N | 28.50 | Witherolactone N | 31.09 |

Almost all types of withanolides detected in in vivo plant with serrated leaf have a lower content than in vivo plant with entire leaf, except two compounds namely 4 Deoxyphysalolactone and withangulatin G. Both of them were present in much higher amounts than in the in vivo plant with entire leaf which reach 148.64 µg/g and 185.70 µg/g, respectively. On the contrary, in other three samples concentration of 4 Deoxyphysalolactone and withangulatin G was only less than 30.0 µg/g. The secondary metabolites profile of in vitro regenerated plantlets and callus were more closely resembled to *P. angulata* plants with entire leaf than serrated leaf. Almost all the withanolide contents produced by *in vitro* plants increased compared to those produced by plants *in vivo*. However *in vitro* callus tissue produced withanolides in lower concentration than *in vivo* and *in vitro* plants.

Types of secondary metabolites detected in four samples of *P. angulata* tissues showed that the diversity of withanolides groups almost similar between *in vivo* and *in vitro* plants. The production of secondary metabolites have been frequently undertaken via tissue culture techniques in both callus and cell suspension cultures. However the result of this study showed that the appropriate culture condition should be improves to obtain the optimum concentration of withanolide compounds from callus culture.

**Conclusion**

This study shows that the diversity of withanolides compounds in *P. angulata* is influenced by the physiological age of plants as indicated by variations in leaf morphology, tissue types and growing environment. *In vitro* techniques have the potential to be applied to increase the accumulation of withanolides.
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