International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:6, No:10, 2012

Production and Extraction of Quercetin and (+)-Catechin from *Phyllanthus niruri* Callus Culture

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***Abstract*—**Quercetin and (+)-catechin are metabolites present in *Phyllanthus niruri* plant, have potential in medicinal uses as anticancer and antioxidant agents. Studies on production of quercetin and (+)-catechin from *P. niruri* callus culture via *in vitro* technique were carried out and the results were compared to the intact plant. *P. niruri* explants were cultured on Murashige and Skoog (MS) solidified media supplemented with several phytohormone combinations for one month. The metabolites were extracted from *P. niruri* callus and intact plant by using carbon dioxide supercritical fluid extraction (SFE) with ethanol as modifier and solvent extraction techniques. The extracts were analyzed by means of HPLC method. Results showed that *P. niruri* callus culture was successfully established. The highest content of quercetin (1.72%) was found from *P. niruri* callus grown in media supplemented with 0.8mg/L kinetin and 0.2mg/L 2,4-dicholophenoxyacetic acid (2,4-D), which was 1.2 fold higher than intact plant. Meanwhile, the highest amounts of (+)-catechin (0.63%) was found from *P. niruri* callus grown in media with addition of 0.2mg/L 1-naphthalene acetic acid (NAA) and 0.8mg/L 2,4-D. The SFE condition in this study showed better extraction efficiency when higher contents of selected metabolites were found in all SFE extracts compared to the common solvent extracts.

***Keywords*—**Callus culture, *Phyllanthus niruri*, secondary metabolite, supercritical fluid extraction.

I. Introduction

PHYLLANTHUS NIRURI (dukong anak) from family *Euphorbiaceae* is an herb with long history in traditional medicinal uses for treatment of gallstones, jaundice, cancer, diabetes and malaria [1]. The potential of *P. niruri* has been proved scientifically when medicinal compounds such as phenolics, flavonoids, and alkaloids were successfully isolated from the plant. The presence of these metabolites in *P. niruri* has gained interests in plant cell culture research to increase its production via *in vitro* technique. For pharmaceutical based plant products, extraction method has been considered as a critical point since the final products must be free from any toxic components. Conventional extraction methods commonly use organic solvents and sometimes the solvents are difficult to remove after extraction. The modern extraction method called supercritical fluid extraction (SFE) has been chosen because it uses non-toxic solvent (carbon dioxide), which is good for extraction of pharmaceutical based plant products.

In this study, plant cell culture technique has been performed to *P. niruri* plant to enhance the production of quercetin and (+)-catechin. Generally, plant cell culture can be described as the induction of plant cells from intact plant organ on/in artificial medium, which contains all the required nutrients for cell growth within controlled environmental condition. Plant cell culture technique may allow the discovery of potential metabolites produced in a small quantity by the large plant with a mean of a large-scale production for valuable secondary metabolites. This technology is also easier to control compared to common crop, thus, can overcome conventional problems such as unreliable plant sources, destruction of product quality and environmental constraints [2], [3]. Studies on the extraction of *P. niruri* metabolites were also carried out to determine the efficiency of SFE method compared to conventional solvent extraction method. The SFE is one of the separation technologies that use a supercritical fluid as a solvent [4]. Basically, SFE process employed carbon dioxide as the main solvent at supercritical conditions to gain the powerful solvent that has both gas and liquid properties. To get better results, small percentage of polar solvent sometimes was added as a modifier during extraction. Also, by using a different condition of pressure and temperature, SFE can be manipulated to fit in with selective extracts. Compared to conventional method, the main solvent used in SFE is more economic and easy to remove from compounds after extraction [5], allowing a direct applications of metabolites in the food and pharmaceutical industries for the generation of high-value products [6].

II. Materials and Methods

A. Callus Culture of P. niruri

P. niruri callus culture was derived from cultivated *P. niruri* plant. Stem explants of *P. niruri* were sterilized in commercial sodium hypochlorite solution (1% v/v) with 2-3 drops of Tween-20 for 40min and rinsed off with sterile distilled water. Explants were cut to 5 – 10mm length in size and cultured horizontally on Murashige and Skoog (MS) media [7], supplemented with 3% (w/v) sucrose, 1% (w/v) agar and phytohormones, which were 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA), kinetin (KIN), and 6-benzylaminopurine (BAP). To

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This study is supported by Malaysia Ministry of Science Technology and Innovation under research grant IRPA 09-02-02-0091-EA234.

study the callus formation, combination of two plant phytohormones (2,4-D with NAA or KIN or BAP) at different ratios (20%, 50% and 80%) were attempted at 1.0, 2.0, and 3.0mg/L of total hormones concentration in MS media. Callus culture of *P. niruri* was incubated at 24 – 26ºC with eight hours daily exposure of fluorescent light. The effect of hormone combination treatments for callus formation was observed visually during two weeks of cultivation.

B. Super Critical Fluid Extraction

Supercritical fluid extraction (SFE) system consisted of CO_2 pump, oven, extraction vessel and collection vial. The condition was referred to the optimized flavonoids SFE extraction for *P. niruri* plant [8]. The main solvent was carbon dioxide and ethanol (HPLC grade) was used during extraction as a modifier. Extraction process was carried out at 60ºC temperature and 200 bars pressure. A total of 3g samples were subjected to static supercritical fluid extraction for 30 minutes followed by dynamic extraction for 3 hours. The extracted analytes were collected every half an hour in a cooled collection vial in order to achieve optimal collection efficiency.

C. Solvent Extraction

Solvent extraction method was conducted as described by Yuan and his colleagues [9]. Five weeks old selected calluses were harvested and dried at 60ºC until constant weight achieved. Dried calluses were extracted with 50% ethanol for 24 hours at 25 - 27ºC. Samples were subjected for 30 minutes sonication prior to filtration of extracts.

D. HPLC Analysis

All *P. niruri* samples were diluted to 10g/L with 50% ethanol prior to analysis. HPLC separation was achieved at 35ºC on a Zorbax RP-C18 column (250 x 4.6mm I.D.) with a particle size of 5μm. The mobile phases were acetonitrile and 0.1% phosphoric acid in deionised water with 1ml/min flow rate. The injection volume of samples was 20μL and chromatogram peaks were detected at 280nm. The external standard used were quercetin (QEN) and (+)-catechin (CT), dissolved and diluted in 50% ethanol at a concentrations range of 0.002 – 0.2g/L. Chromatographic peaks were identified by comparison of the retention time with external standards. The content of metabolite was expressed by unit percentage gram of metabolite per gram of dry sample analyzed.

III. Results and Discussion

A. P. niruri Callus Formation

Studies on phytohormone combination for developing *P. niruri in vitro* culture are still not much reported. In this study, several treatments of phytohormone combination were performed in order to obtain soft and friable *P. niruri* callus, which physically suitable to initiate suspension culture consisted of secondary metabolites. In previous study, 2,4-D hormone was considered as a suitable hormone to induce *P. niruri* callus, but the use of 2,4-D alone was found to be insufficient to achieve target callus [10]. Results showed that combination of NAA and 2,4-D exhibited formation of yellowish and friable callus at ratio of NAA less than 2,4-D. For ratio of NAA same or higher than 2,4-D, roots formation in *P. niruri* callus was observed. This result showed that NAA hormone promoted induction of *P. niruri* roots rather than callus formation. Catapan and his colleagues [11] have also reported similar results when they found roots formation by supplement of NAA hormone alone in medium from other *Phyllanthus* sp.

For combination of KIN and 2,4-D treatment, results showed a successful formation of yellowish, soft and friable callus, which is very useful for developing a liquid culture system. The induction of callus from *Phyllanthus* sp. by using KIN hormone alone has been reported previously but lack of physical description of the callus [11]. However, treatments with the lower ratio of KIN as compared to 2,4-D (at 1.0 and 2.0mg/L of total hormone concentrations) had resulted in formation of *P. niruri* callus with roots. The different result was found at combination hormone of BAP and 2,4-D when most of the treatments produced white, hard, and compact callus. This finding is in parallel to another study on *Phyllanthus* sp. where they found induction of similar type of callus by addition of BAP alone in culture media [12]. Nevertheless, supplement of BAP ratio less than 2,4-D at low concentration of total hormone in MS media productively induced soft and friable *P. niruri* callus.

Among all treatments, four hormone combinations were selected based on the physical properties of callus that is friable and soft to be extracted for metabolites analysis (see Fig. 1). The treatments were A-callus (NAA:2,4-D = 20:80, 2mg/L), B-callus (KIN:2,4-D = 80:20, 1mg/L), C-callus (KIN:2,4-D = 50:50, 3mg/L) and D-callus (BAP:2,4-D = 20:80, 1mg/L).

Fig. 1 Photo of *P. niruri* callus

B. Metabolites Analysis

Quercetin and (+)-catechin are flavonoid compounds, which can be found in green vegetables, fruits, and tea and contributed to wide pharmacological properties such as antioxidant, anticancer, antibacterial and anti-inflammatory activities. These flavonoids were commonly extracted from various types of plant by using solvent extraction method [13]. In this study, extraction of quercetin and (+)-catechin from *P. niruri* callus was studied by using supercritical fluid extraction (SFE) method and compared with solvent extraction method. Contents of selected metabolites from the intact plant (stem part) were also determined to be the control. Fig. 2 displays

HPLC chromatogram and chemical structures of the reference standards.

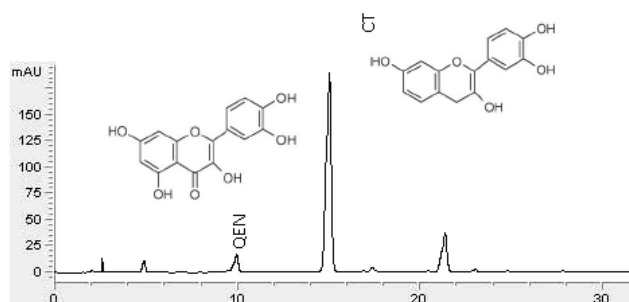

Fig. 2 HPLC chromatogram of quercetin and (+)-catechin standard mixture

By using SFE method, *P. niruri* extracts were collected every half an hour during extraction time sequentially to study the separation of quercetin and (+)-catechin from the samples. Fig. 3 shows content of the metabolites from *P. niruri* extracts. In general, large amounts of quercetin and (+)-catechin were extracted at 1.0 and 1.5 hours extraction time. A total of three hours extraction was found to be adequate to gain 3g of samples due to decreasing amount of both metabolites during final collection.

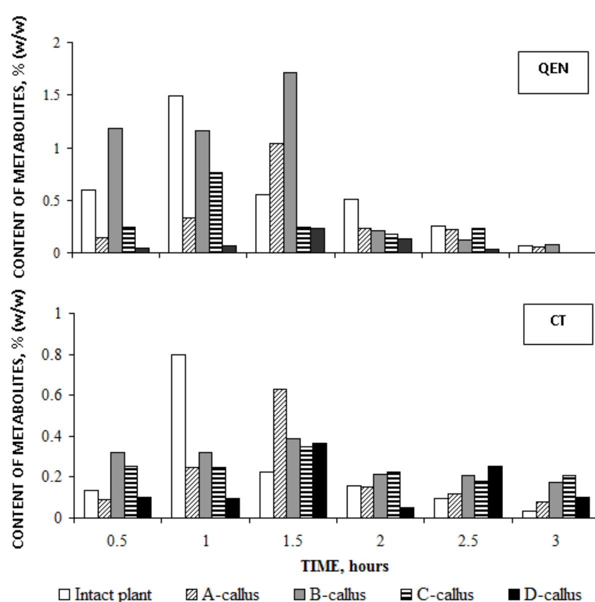

Fig. 3 Contents of quercetin (QEN) and (+)-catechin (CT) from *P. niruri* intact plant and callus extracted by using SFE method

The B-callus treatment resulted in the highest quercetin content among other samples (4.47% w/w) after 90 minutes collection and 1.2 fold higher (1.72% w/w) as compared to intact plant. Meanwhile, the highest (+)-catechin content (0.63% w/w) was derived from A-callus treatment collected after 90 minutes extraction, comparable to (+)-catechin derived from *P. niruri* intact plant. However, B-callus treatment gave the highest total content of (+)-catechin (1.62% w/w) when compared to other callus samples and intact plant after complete extraction. This finding showed that B-callus treatment resulted in the best performance on quercetin and (+)-catechin production *in vitro* as compared to other treatments.

C. Comparison of Extraction Methods

SFE and solvent extraction methods were applied to understand the efficiency of each method in extraction of studied metabolites. Table I summarizes the extracted amounts of quercetin and (+)-catechin in *P. niruri* samples while Fig. 4 presents chromatograms of *P. niruri* intact plant extracts by means of different extraction methods. The HPLC chromatograms demonstrated the effectiveness of using SFE for both metabolites by increasing peaks of quercetin and (+)-catechin. Furthermore, some other natural compounds in *P. niruri* plant were also found to be isolated by this method.

TABLE I
EXTRACTED AMOUNTS OF QUERCETIN AND (+)-CATECHIN

P. niruri samples	SFE modified ethanol*		Solvent extraction[a]	
	Quercetin (w/w)	(+)-Catechin (w/w)	Quercetin (w/w)	(+)-Catechin (w/w)
Intact plant	1.50%	0.80%	1.17%	0.16%
A-callus	1.04%	0.63%	0.98%	0.36%
B-callus	1.72%	0.39%	1.10%	0.22%
C-callus	0.76%	0.35%	0.36%	0.03%
D-callus	0.24%	0.37%	0.18%	0.05%

* Data taken from the highest content from each run
[a] Average of three replicates

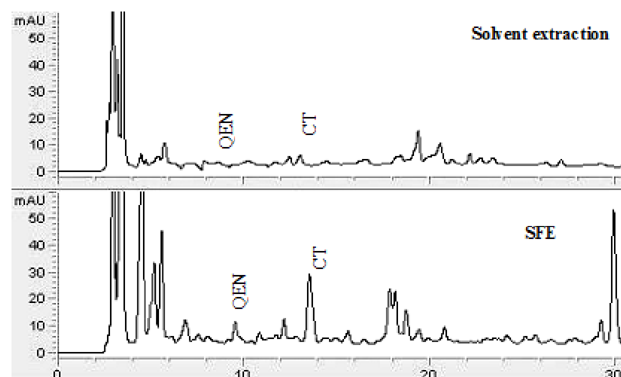

Fig. 4 Chromatogram of *P. niruri* plant extracted by solvent extraction and SFE methods

The content of quercetin from B-callus and C-callus were respectively 1.6 and 2.1 fold higher with SFE than solvent extraction methods. This finding was contradict to previous study [5], where they found quercetin quantities to be 2 to 5-fold higher with common ethanol extraction than with modified SFE using ethanol. Additionally, (+)-catechin content from C-callus and D-callus were found to be 11.7 and 7.4-fold higher in SFE as compared to the conventional method. The addition of modifier ethanol to SFE system have improved results for extracting catechins and this finding was in conjunction with previous studies on tea extraction [14].

IV. Conclusion

This study has investigated the production of quercetin and (+)-catechin from *P. niruri* callus culture via *in vitro* technique. Results showed that friable and high-yielding *P. niruri* callus has been successfully obtained with a potential to be developed for a higher scale production. At the same time, studies on different extraction methods demonstrated better efficiency of quercetin and (+)-catechin production using modified SFE as compared to common solvent extraction.

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