High throughput analysis on selected polyphenol production and Principal Component Analysis (PCA) in *Phyllanthus watsonii* grown under different environmental conditions

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Abstract
Polyphenol production in *Phyllanthus watsonii* (species of Dukung anak) planted in different environmental conditions was investigated. The plants were grown in two different environments: A – in the net house under semi-controlled environmental condition, and B – in an open environment. Environmental parameters such as light intensity (kLux), soil moisture content (%), relative humidity (% RH) and surrounding temperature (°C) were taken on daily basis for comparison. Water extract of the samples showed that *P. watsonii* planted in the open environment yielded higher amounts of polyphenols compared to that planted in semi-controlled environment. However, there was significant inconsistency (*p* < 0.05) in the polyphenol yield among the triplicates of *P. watsonii* planted in the open environment compared to that of the semi-controlled environment. Statistical results from principal component analysis (PCA) and quantifications of four known phenolic acids (geraniin, corilagin, rutin and quercetin glucoside) from LCMS/MS verified these findings. The findings revealed that polyphenol production in *P. watsonii* can be significantly influenced under different environmental conditions.

Keywords: *Phyllanthus watsonii*, polyphenols, environmental factors, LCMS/MS, Principal Component Analysis

Introduction
Malaysia is well known for its diversified and well evolved rainforest in the world. Rich in more than 1,500 species of floras, Malaysia is a place where tropical medicinal plants can be easily grown and cultivated. These plants, which are used traditionally to treat common occurring diseases such as jaundice, gastrointestinal problems and diarrhoea, are now stealing the limelight as a potential alternative medicine in pharmacological studies for the treatment of degenerative diseases such as cancer, cardiovascular, Alzheimer’s and many more. Recently, there is a surge in the research of medicinal plants for their medicinal properties in Malaysia. Findings have shown that they are highly rich in polyphenols; mainly comprising flavonoid, phenolic acids, tannins, stilbenes or lignans (D’Archivio et al. 2007; Ignat et al. 2011). However, studies on the effects of environmental conditions on preharvest stage in medicinal plants are still lacking (Jorge et al. 2006). Bauer and Tittel (1996) and Werner (2000) in their reports stated that the phytochemical composition in plants varies a lot due to many factors during...
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the preharvest and postharvest processes. Environmental factors such as light intensity, temperature, nutrients and water availability were identified to contribute to the variations in the production of phytochemicals in plants during the preharvest stage.

The genus *Phyllanthus* (Euphorbiaceae) or locally known as ‘Dukung anak’ is widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat many diseases such as intestinal infections, diabetes, jaundice, as an astringent etc. (Basharat 1998; Calixto et al. 1998). Research on the water and methanolic extracts of various types of subspecies belonging to this genus has shown to have antimicrobial (Tan et al. 2011), anticancer (Yin et al. 2010), anti-inflammatory (Perianayagam et al. 2004) and many health beneficial properties. Our previous studies on the extracts of *Phyllanthus watsonii* have shown to possess anticancer (Yin et al. 2010), antiviral (Lee et al. 2013) and anticholesterol (unpublished data) properties.

The planting material was, however, harvested from the net house (semi-controlled environment) where the plants were grown in pots to minimise the fluctuations of the environmental conditions on the production of polyphenols. For commercialisation purposes, it is economical that *P. watsonii* to be grown in the field. But to date, there has been no reports on the effect of different environmental conditions on the production of important polyphenols in *P. watsonii*. Therefore, this study investigated how *P. watsonii* grown in different environmental conditions affects the production of polyphenols.

**Materials and methods**

Standard (rutin) and sodium diethyldithiocarbamic acid ACS reagent were purchased from Acros Organics (NJ, USA). Other standards such as quercetin glucoside and corilagin were purchased from Fluka-Sigma Aldrich (Germany) and Toronto Research Chemicals Inc. (Canada) respectively. Acetonitrile LC grade and formic acid 98 – 100% were purchased from Merck (Germany). HPLC grade methanol was obtained from Fisher (Canada).

Two treatments were applied in this study and labelled as treatment A: plant samples grown inside the net house (or semi-controlled environment) and treatment B: plant samples grown outside the net house (or open environment). Both treatments were conducted at the first quarter of the year.

In treatment A, six pots (75 cm x 20 cm) with two pots per replicate were chosen and for treatment B, three plots of land outside the net house with the area of 3 m² (2 m x 1.5 m) each was outlined. The outlined plots were later dug for about 15 cm deep prior to the addition of new soil. Mixture of organic soil and sand (3:1) was prepared, filled in both pots and plots and used to germinate *P. watsonii* seedlings. Both treatments were irrigated with the same amount of water (twice a day), and fertilised (40 g NPK Blue diluted in 10 litres of water) once a week.

Reading of the soil moisture content (%) (Aquaterr M-300, Aquaterr Instruments, USA), surrounding temperature (°C) (Traceable® Dew-Point/Wet-Bulb/RH/Temp Meter, Fisher Scientific, USA), relative humidity (% RH) (Traceable® Dew-Point/Wet-Bulb/RH/Temp Meter, Fisher Scientific, USA) and sunlight intensity (LUX) (EA30 Wide Range Light Meter, Extech Instruments, USA) were taken on daily basis in both planting conditions. After 31 days of germination, *P. watsonii* were harvested, washed, ground, freeze dried (FreeZone 6plus, Labconco, USA) and stored in –20 °C (Fisher & Paykel, USA) for further usage.

The extraction procedures were carried out according to Tan et al. (2011) with a minor modification. Freeze dried samples (0.5 g) were weighed into 50 ml falcon tubes. A volume of 20 ml of water extraction buffer (WEB) (20 mM of sodium diethyldithiocarbamic acid
in 1% formic acid) was added into the samples and homogenized for 1 min. Samples were then vortexed (Multi Reax, Heidolph Instruments, Germany) for 30 min before being centrifuged (2-16K Sigma, Germany) at 8,900 rpm at 4 °C for 5 min. All supernatants were filtered (Whatman No. 40, UK) and the extraction procedure was repeated three times. A volume of 2 ml from the final volume of water extracted samples was aliquoted into micro-centrifuge tubes and concentrated (Concentrator 5301, Eppendorf, Germany) until dry. Dried extracts were reconstituted with 30% methanol with a final concentration of 20 mg/ml before analysis. Each sample was run in triplicates with injection volume of 20 µl.

Separation, quantification and identification of polyphenols were done using a LCMS/MS system comprising HPLC (1200 series, Agilent Technologies, Germany) and mass spectrometer (3200 QTrap LCMS/MS, AB Sciei, USA). Separation of polyphenols was achieved with the usage of a reverse phase C18 Hypersil Gold column (5 µm particle size, 150 x 4.6 mm, Thermo Scientific, USA) preceded by a guard column (5 µm particle size, 10 x 4.6 mm, Thermo Scientific, USA).

The temperature and flow rate were set to be at room temperature (not controlled) and 1 ml/min respectively. Two mobile phases were chosen for polyphenol separation: solvent A – 0.1% formic acid, and solvent B – 0.1% formic acid in acetonitrile. The solvent gradient was set as follows: 5 – 40% B in 50 min increased to 90% B for 15 min, and at 65 min the gradient was reduced to 5% for 4 min to equilibrate the column. Wavelength at 280 nm was used to detect the polyphenols from the extracts. Electron Spray Ionisation (ESI) at temperature 500 °C was used to ionise the polyphenols in negative mode. Enhance mode (trap function) was chosen to obtain the MS/MS fragmentations.

For quantification purpose, four polyphenols (marker compound), which are commercially available, found in Phyllanthus were selected and their standard curves prepared (Liu et al. 2002; Xiaoli et al. 2008; Yin et al. 2010; Elrashid et al. 2011; Jay et al. 2011). Standards of quercetin glucoside, rutin and corilagin were weighed accurately and diluted in methanol before being subjected into LCMS/MS separately. Quantification of geraniin was done using the standard curve obtained from corilagin since unavailability of geraniin standard in the local market. Besides, corilagin is also a suitable substitute to quantify geraniin as it is the closest related compound to geraniin in terms of its chemical structure (Wu et al. 2011).

Generally, all standards were serially diluted ranging from 500 µg/ml to 31.25 µg/ml and quantified based on area under the graph (counts) against the concentration at 280 nm absorbance. Area under the graph of each polyphenol from P. watsonii was calculated in triplicates and subsequently quantified based on the standard curve. All experimental results were expressed as mean ± standard deviation.

Statical analysis
A thorough statistical method was utilised to compare the results obtained in this study. The values of the mass/charge (m/z) ratio obtained were used for PCA using MarkerView™ 1.1 software (Applied Biosystems/MDS Sciei) which supports the data from the LCMS/MS. Pareto scaling was chosen as the default scaling method to generate the PCA results.

Results and discussion
In this study, two common planting conditions for herbs among farmers and agriculturists were selected and how these conditions may influence the production of polyphenols in P. watsonii were studied. Treatment A which involves germination of herbal seeds in pots and grown in the net house under a semi-controlled environment has been utilised since 1990 by Westerners and currently been widely adapted in the Southeast Asian countries (Padaranee et al.
2011). Planting of crops in the net house minimised yield losses due to environmental stresses and pest and disease infections. On the contrary, treatment B, which has been used worldwide for large scale productions and growing of *Phyllanthus* sp. in the open field, was reported by Wan Zaki and Musa (2007) previously. Regardless of the type of planting condition used, the success rate of growing herbs depends solely on both biotic and abiotic factors (James et al. 2000).

Four common environmental factors (light intensity, soil moisture content, relative humidity and surrounding temperature) were chosen in this study and their readings were taken twice a day (9 am and 3 pm) for 31 days prior to harvest. The results were plotted as average for the 31 days and shown in *Figure 1*. Studies related to various environmental factors in the production of phytochemicals in certain plants have shown that environmental factors do affect the production of phytochemicals (Daniels 1971; Shamir et al. 2001; Levine and Pare 2009; Pollastrini et al. 2010).

In this study, a distinct relationship was observed between the four environmental factors which act synergistically with each other. The shaded net inside the net house provided a more consistent light exposure based from the readings plotted in *Figure 1* compared to the outside of the net house. Higher light intensity readings were registered outside the net house during the evening, whereas consistent readings were registered inside the net house for both period of time (morning and evening). The same pattern was observed as well for the

![Figure 1](image)

*Figure 1. Readings of light intensity, surrounding temperature, relative humidity and soil moisture plotted for Phyllanthus watsonii planted inside (treatment A) and outside (treatment B) the net house for an average of 31 days (p <0.05). Readings were taken twice per day (9 am and 3 pm)*
soil moisture content. Relative humidity for both inside and outside of the net house seemed to be related with the surrounding temperature. Relative humidity decreased as the temperature increased at late evening (3 pm).

**Analysis of *P. watsonii* extracts via LCMS/MS**

The analysis was carried out in two steps, namely the identification of four phenolic acids in *P. watsonii* and quantification of these phenolic acids. The data obtained hereafter were used to compare the fluctuation in the synthesis of polyphenols, if any in *P. watsonii* planted under different planting conditions. Identification and confirmation of geraniin, corilagin, rutin and quercetin glucoside was done by comparing their retention time ($R_t$) and mass fragmentation with the standards and as well data reported from previous studies (Table 1).

Figure 2 shows the chromatogram obtained from liquid chromatography using diode array detector (DAD) at 280 nm. Qualitative assessment based from the DAD absorbance indicates an increment of two folds in rutin and quercetin glucoside in *P. watsonii* grown inside the net house compared to the one planted in open environment. Geraniin had an absorbance at 320 mAU, two folds higher in *P. watsonii* planted in treatment B compared to treatment A. No significant difference was measured in corilagin absorbance in both treatments.

Assessment on the chromatogram showed a clear fluctuation in the yield of these phenolic acids. Standard curves of these selected phenolic acids were then plotted and their respective equations and regression were obtained as follows: corilagin ($y = 9009.3x + 385.16$, $R^2 = 0.999$), rutin ($y = 14217x + 120.18$, $R^2 = 0.999$), and quercetin glucoside ($y = 14161x + 15.128$, $R^2 = 0.999$). The quantification data obtained were proven to be in line with the above qualitative assessment. Rutin and quercetin glucoside were detected two to three folds higher in *P. watsonii* cultivated in treatment A while geraniin content was almost nine folds higher in *P. watsonii* planted in treatment B. Yield of corilagin showed no significant difference between the two treatments.

Generally, both chromatograms showed significant variations in the synthesis of polyphenols in *P. watsonii* planted under different environmental conditions. Studies have shown that environmental factors such as sunlight intensity, temperature and amount of water can exert environmental stress on plants, and as a response to these stresses plants may trigger a series of enzymes in the biosynthesis of various phenolic compounds which act as antioxidants (Diallinas and Kanellis).

<table>
<thead>
<tr>
<th>Peak</th>
<th>$R_t$ (min) (approx)</th>
<th>Mass/Charge (m/z)</th>
<th>Product ions (EPI MS/MS)</th>
<th>Identified compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>20.5</td>
<td>633 [M-H]-</td>
<td>301, 275, 245, 169</td>
<td>Corilagin</td>
<td>Liu et al. (2002); Xiaoli et al. (2008); Yin et al. (2010)</td>
</tr>
<tr>
<td>b</td>
<td>21.0</td>
<td>951 [M-H]-</td>
<td>933, 765, 463, 301</td>
<td>Geraniin</td>
<td>Xiaoli et al. (2008); Yin et al. (2010), Wei et al. (2010); Yin et al. (2010)</td>
</tr>
<tr>
<td>c</td>
<td>26.0</td>
<td>609 [M-H]-</td>
<td>301, 271, 255, 179</td>
<td>Rutin</td>
<td>Wei et al. (2010); Yin et al. (2010)</td>
</tr>
<tr>
<td>d</td>
<td>26.7</td>
<td>463 [M-H]-</td>
<td>301, 271, 255, 243</td>
<td>Quercetin glucoside</td>
<td>Wei et al. (2010); Yin et al. (2010)</td>
</tr>
</tbody>
</table>

Table 1. HPLC-ESI-MS of four polyphenols obtained from *Phyllanthus watsonii* corresponding to the DAD chromatogram in Figure 2.
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1994; Reymond et al. 2000; Liu et al. 2006). This might suggest the significant increment in the production of geraniin from *P. watsonii* planted in treatment B was due to the environmental stresses. On the other hand, for a consistent production of polyphenols, a stable environmental condition as depicted inside the net house may provide a more suitable condition for the yield of various polyphenols as seen in Figure 2.

Principal Component Analysis (PCA) from LCMS/MS results

PCA was chosen as the suitable statistical tool to help in identifying and distinguishing the variations in all phytochemicals produced in *P. watsonii* planted in different environments, thus showing any fluctuations in the yield of polyphenols (*MarkerView*™ 2005). Figure 3 (a) shows the score plot of the variation among samples planted in treatment A (denoted with solid circles) versus treatment B (denoted with dotted circles). Samples planted in both treatments were significantly grouped into two separate quadrants, mainly at the positive and negative quadrants in principal component 1 (PC1 Score or X-axis) with 46.5% variation. On contrary, principal component 2 (PC2 score or Y-axis) showed a significant variation in the distribution of triplicates among *P. watsonii* planted in treatment B compared to treatment A. The distributions of planted samples as shown in the score plot were in line with the distribution of their detected phytochemicals from the loading plot as in Figure 3 (b). The inconsistency in the production of these phytochemicals among *P. watsonii* planted outside the net house may due to several environmental factors.

The sunlight intensity data collected inside the net house for both period of time (9 am and 3 pm) showed minimal fluctuations throughout the growing phase despite higher readings were observed at the outside of the net house during the evening.
than in the morning. The minimal fluctuation in the sunlight exposure may suggest the consistency of total polyphenol yield among the triplicates of *P. watsonii* planted in treatment A (*Figure 3a*).

Our data is in agreement with the findings of other researchers; for an example Rosa and Rodrigues (2001) and Schreiner (2005) that have also showed that broccoli grown outdoor at different seasons influences the phytochemical and ascorbic
acid biosynthesis pathways. Thiele et al. (1996) in their study found that plant sample exposed to high visible light radiation may increase the production of xanthophylls cycle and triggers the productions of various secondary metabolites. Although *P. watsonii* planted in treatment B yielded higher amount of total phenolic acids (*Figure 4*), its consistency in terms of the total yield should be taken into consideration.

A case study done by Smith and Burford (1992) on parthenolide concentration in feverfew tablets and capsules found low parthenolide concentration than that mentioned in the product labels. They suggested that this inconsistency may due to certain preharvest and postharvest factors that lead to unexpected decrease in its quality.

The growth of roots in *P. watsonii* could be another lead factor which contributes to the fluctuation of total polyphenol yield in treatment B. The fibrous roots of *P. watsonii* serve as an important organ for a better absorption of water and mineral salts. Planting under the net house involves using pots with drainage holes at the bottom and this allows a better chance of water and mineral salts to be drained out compared to those samples planted in the open field. Angela et al. (2009) in their study showed that although planting crops in the open field is economical, excess water which leads to waterlogging in the soil can reduce the oxygen intake in the roots. This will eventually reduce the ability of root cells for a maximum growth and impair the absorption of water and mineral salts.

Angela et al. (2009) finding is in line with the soil moisture content data collected in our study. Higher percentage of soil moisture content (approximately 60 – 70%, *Figure 1*) was calculated for *P. watsonii* planted outside the net house which may associate with the excess water retention due to rain water. The collected data was further supported by the findings from Aparna and Germund (2000). They concluded that intermediate soil moisture content (60 – 70%) will dilute the amount of important nutrients in the soil for roots’ absorption, thus retarding the growth of the shoots in fibrous roots type plants. This may indicate the fluctuation of total polyphenols yield in *P. watsonii* planted in treatment B.

**Conclusion**

*Phyllanthus watsonii* planted in the open environment produced higher amounts of polyphenol when compared to that planted in the net house under semi-controlled environmental conditions. However,
environmental variations in the open field resulted in significant fluctuation of polyphenol yield in \textit{P. watsonii}. A more consistent yield of polyphenol was observed in \textit{P. watsonii} grown in semi-controlled environment. For commercialisation purpose, it is important that herbs and herbal products comply with regulatory and standardisation procedures which demands a consistent yield of bioactive compounds. Therefore to ensure consistent and standardised supply of herbs, it is recommended that herbs such as \textit{Phyllanthus} be planted under a semi-controlled environment such as in the net house.

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Abstrak
Penghasilan polifenol dalam *Phyllanthus watsonii* (spesies Dukung anak) yang ditanam dalam keadaan persekitaran yang berbeza telah dikaji. Tanaman ini ditanam di dua keadaan persekitaran berbeza: A – di dalam rumah jaring di persekitaraan separa terkawal, B – di luar rumah jaring. Sepanjang kajian, parameter persekitaraan seperti keamatan cahaya (kLux), lembapan tanah (%), kelembapan relatif (% RH) dan suhu persekitaraan (°C) telah direkod setiap hari untuk tujuan perbandingan. Ekstrak air daripada *P. watsonii* menunjukkan sampel yang ditanam di persekitaran luar menghasilkan polifenol yang banyak berbanding dengan sampel yang ditanam di persekitaran separa terkawal. Walau bagaimanapun, perbezaan bererti (*p* < 0.05) dalam penghasilan polifenol antara triplikasi *P. watsonii* yang ditanam di persekitaran luar dapat dilihat berbanding dengan yang ditanam di persekitaran separa terkawal. Keputusan statistik daripada analisis komponen utama (PCA) dan kuantifikasi empat asid fenolik (geraniin, corilagin, rutin dan quercetin glucosida) daripada LCMS/MS turut menyokong keputusan tersebut. Keputusan ini menunjukkan penghasilan polifenol dalam *P. watsonii* dipengaruhi secara signifikan apabila ditanam dalam keadaan persekitaran yang berbeza.