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Effect of different drying methods and solvent ratios on biological activities of *Phyllanthus acidus* extracts

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Abstract

The main purpose of this study was to evaluate the antioxidant and α-glucosidase inhibitory activities of *Phyllanthus acidus*. The *P. acidus* fruits were dried using three different methods, namely oven (OD), air (AD) and freeze (FD) dryings and extracted with ethanol at different ratios (50 and 100%). The proximate analysis and total phenolic content (TPC) as well as free radical scavenging and α-glucosidase inhibitory activities were determined. The proximate analysis of *P. acidus* fruit indicated that all the dried samples contained potential nutrient contents. The highest TPC value, α-glucosidase inhibitory and antioxidant activities were observed for 50% ethanolic extract from OD method with TPC value of 28.39 mg GAE/g dried extract, IC_{50} value of 12.394 µg/mL and 64.17% inhibition, respectively. The study revealed that phenolic compounds could be the main contributors to the antioxidant and α-glucosidase inhibitory activities based on the Pearson correlation coefficients with R values of 95.0 and 73.8%, respectively. The study could provide scientific evidence for some folk uses in the treatment of diseases related to the production of reactive oxygen species and oxidative stress.

Introduction

Plant extracts and their bioactive constituents have shown prominent intent in herbal medicine therapies. There is also a strong link between the fruits and vegetable intake and the reduction of numerous chronic diseases (Abas *et al*., 2006; Maisuthisakul *et al*., 2008; Ali *et al*., 2014). In recent years, most researchers have interested in the plant of the genus *Phyllanthus*, regarding their therapeutic potential for the management of many diseases. The extracts and isolated compounds from *Phyllanthus* species were found to exhibit several pharmacological activities such as antiviral, antimalarial, antibacterial, antiplasmodial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, and nephroprotective (Chopade and Sayyad, 2014).

Phyllanthus acidus (L.) Skeels (Euphorbiaceae), commonly known as harfarauri or star gooseberry or Mayom in Thailand, is a widely distributed plant in India and other Asian countries (Leeya *et al.*, 2010; Jain *et al*., 2011). In Malaysia, *P. acidus* is locally known as 'cermai' or kemangul and chermala. The *P. acidus* herbs have been traditionally used in the management of several inflammatory and oxidative stress related disorders, including rheumatism, bronchitis, asthma, respiratory disorder, hepatic disease, and gonorrhea (Chakraborty *et al*., 2012). In Thailand, the leaves of *P. acidus* have been used as anti-hypertensive remedy to relief headache resulting from hypertension (Chongsa *et al.*, 2014). In addition, *P. acidus* can improve eyesight problem, cure cough, and reduce severity of psoriasis, skin disorders and sudorific (Chakraborty *et al*., 2012). The fruits of the plant were used as an astringent while the root and seed are useful as cathartics. The leaves and roots were also used as an antidote to viper venom. However, contradictory bioactivities of this plant were found in several studies. This could be rationalized by the differences in sample's processing which could affect the content of the test samples.

Prior to testing the bioactivities of plant materials, they should be dried and extracted. These factors might affect the phytochemicals and bioactivities of plant materials. Thus, the selection of the valuable drying method and the suitable solvent is necessary. Drying is one of the most important factors that could affect plant efficiency and benefit. It is an old ancient method of food preservation (Korus, 2011; Mediani *et al*., 2013). It is usually applied to inactivate the polyphenol oxidising enzyme and it can be either performed by conventional or industrial techniques (Lim and Murtijaya, 2007). Moreover, drying aids to minimise the water activity that ultimately retards the

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microbial growth which in turn helps to conserve the desirable qualities and reduces the storage volume (Gupta *et al*., 2011). It was reported that some drying techniques have physically or chemically negative effects on plant materials, which affect their qualities and bioactivities to treat various diseases. Most of the diseases are caused by free radicals, which need to be managed in order to control the diseases. The research on the effect of drying and extraction solvent on antioxidant and other bioactivities related to free radicals of *P. acidus* is lacking. Thus, there is a need to optimise the valuable processing parameters that provide the maximum benefit and efficiency of *P. acidus*.

In view of the fact that most of the current treatments of various diseases acquired some toxic effects and caused other serious ailments, chemical and biological studies on medicinal and edible plants have been increased in order to discover safer bioactive compounds from natural sources. Therefore, the aims of this study were to evaluate the proximate composition of the dried and fresh *P. acidus* fruit and to determine the antioxidant and α-glucosidase inhibitory activities of dried *P. acidus* extracted with 50 and 100% ethanol.

Materials and Methods

Plant materials

Phyllanthus acidus fruit was obtained from Malaysian Agricultural Research and Development Institute, MARDI (Serdang, Selangor). The sample was identified by Mohd Hafizi as an in-house botanist at the herbarium of Institute of Bioscience, Universiti Putra Malaysia under the voucher specimen (SK 2990/16). The fruits were cleaned and the seeds were separated before subjected to air (AD), oven (OD), and freeze (FD) dryings.

Chemical reagents

Concentrated sulphuric acids, mix catalyst (96% sodium sulphate anhydrous + 3,5 cuprum sulphate + 0.5% selenium dioxide, sodium hydroxide, indicator solution, petroleum ether, absolute ethanol, dimethyl sulfoxide (DMSO), sodium carbonate, and phosphate buffer were supplied by Merck (Darmstadt, Germany). The other chemicals include α-glucosidase enzyme, glycine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, quercetin, and p-nitrophenyl-α-D-glucopyranose (PNPG) were purchased from Sigma (Aldrich, Germany).

Proximate analysis

The nutrient and chemical compositions of fresh and dried *P. acidus* fruits were determined using AOAC 2005 methods (AOAC, 2005).

Drying methods

The FD samples were prepared by placing in the deep freezer at -80°C. Then, the sample was put in a Scan VacCoolSafe freeze drier functioning at the temperature of -96°C and vacuum pressure of 0.765 hectopascal until the samples were dried completely to constant weight. For OD, the fresh samples were dried in a forced-air convection oven at the temperature 45°C until the samples were completely dried. For AD, drying was performed at ambient temperature until the complete drying was reached.

Sample extraction

The samples were then ground to a fine powder using laboratory grinder. Then, 10 g of each sample was sonicated after mixing with 200 mL of different ethanol ratios (50 and 100%). Each extract was filtered through a Whatman filter paper no 1, dried using a rotary evaporator, and lyophilized to ensure no water remained in the crude extract. All of the extracts were kept at 4˚C before further analysis.

Determination of total phenolic content (TPC)

The Folin-Ciocalteu test was conducted to evaluate the TPC of *P. acidus* fruits by previously described method (Lee *et al.*, 2014). The standard compound used was gallic acid. A volume of 20 µL of sample (50 and 100% ethanol extracts at 5000 µg/mL concentration) was mixed with 100 µL of Folin-Ciocalteu reagent in a 96-well plate. Then, 80 µL of sodium carbonate solution (75%) was added after 5 min of incubation. The plate was covered and stored in the dark area for 30 min. The absorbance was recorded at 765 nm by using an SPECTRAmax PLUS micro-plate reader. Same procedure was repeated by replacing samples with gallic acid at 8 serial-diluted concentrations (7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0, and 1000.0 µg/mL) for standard graph plotting. The results were expressed in milligram of gallic acid equivalents (GAE) per gram of dried extract (mg GAE/g dried extract).

Determination of DPPH assay

The diphenyl-1-picrylhydrazyl (DPPH) assay was performed according to the procedures of reported method (Mediani *et al*., 2014). The assay was conducted in 96-well plate using 5000 µg/mL of stocks samples. A volume of 50 µL from each sample was transferred to the plate. Then, each well was loaded with 100 µL of DPPH (5.9 mg/100 mL). The plate was covered and stored in the dark for

30 min. The absorbance was recorded at 515 nm by using SPECTRAmax PLUS micro-plate reader (Molecular Devices, LLC, California, US). The scavenging capacity (SC) was calculated as followed the equation below:

$$\%SC = [(a_0 - a_s) / a_0] \times 100\%$$

Where a_0 is a difference of absorbance of the reagent blank and as is a difference of absorbance for sample or standard.

α- Glucosidase inhibition assay

The assay of α-glucosidase inhibitory activity was performed as described in previous method (Lee *et al*., 2014). The ρ-nitrophenyl-β-D-glucopyranosidase (PNPG) in 50 mM phosphate buffer was used as the substrate and the experiments were carried out at pH 6.5, which is comparable to the condition of the intestinal fluid. The samples extracts were prepared at 5000 µg/mL and 6 serial dilutions were performed. Then, 10 µL of samples extracts were mixed with 130 µL of 30 mM phosphate buffer in a 96-well microplate followed by the addition of 10 µL of enzyme. Negative control was prepared by replacing sample with solvent. While, the blank solvent and the blank sample were prepared by 140 µL of 30 mM phosphate buffer with 10 µL of sample and 140 µL of 30 mM phosphate buffer with 10 µL of solvent, respectively. Then, the plates were incubated at room temperature for 5 min. Next, 50 µL of PNPG was added to each well of the sample, blank solvent, and negative control, while the rest were loaded with 50 µL of 30 mM phosphate buffer. The mixtures were incubated for another 15 min at room temperature. The reaction was stopped by adding 50 µL of 2 M glycine (pH 10). Then, the absorbance was measured using spectrophotometer at a wavelength of 405 nm. The α-glucosidase inhibitory activity of the test sample was expressed as percentage (%) of inhibition, which was calculated as $[(a_n-a_s)/a_n] \times 100\%$, where an is the difference in absorbance of the negative control and all the blanks and as is the difference in absorbance of the sample and all the blanks. In this assay, the results are represented as the IC_{50} (µg/mL), which is the concentration of the *P. acidus* extract required to inhibit the enzyme's activity by 50%.

Statistical analysis

Experimental results were expressed as the mean ± standard deviation of three replicates. Analysis of variance (ANOVA) was used to show the significant difference in the results. Pearson correlation test was also performed using Minitab software (Version 16. Minitab Inc, State Collage, PA, USA). For Pearson correlation, the IC_{50} was converted to $1/IC_{50}$ to invert the relation between absorbance and the activity. The GraphPad Prism (Version 5. San Diego, CA, USA) was used to execute the analysis of significant difference with 95% as confident level.

Results and Discussion

Proximate analysis

The proximate composition of *P. acidus* fruits is shown in Table 1. As shown in the data, the highest composition of fresh fruit was water with the value of 90.61%, while the lowest composition was crude fat with the value of 0.58%. In the case of dried fruits, AD sample appeared to contain the highest water content among all drying methods with the composition percentage of 14.76. However, no significant differences were found among different drying methods as p value obtained was greater than 0.05. Therefore, the hypothesis of the variance of moisture contribution on the difference of bioactivities and the tested analysis is excluded. In addition, AD *P. acidus* fruit also showed to contain the highest fat, protein and fibre contents than others, with the composition values of 7.7, 1.08 and 9.53%, respectively. Meanwhile, OD samples exhibited highest carbohydrate content with 68.07% as well as ash content with 5.35%. However, most of the proximate composition of the *P. acidus* fruits was not significantly ($p > 0.05$) different, suggesting that the three different dried *P. acidus* fruit exhibited similar nutritional composition.

Yield of extraction

Extraction yield (Table 2) was chosen as the first evaluation of the effects of different drying methods and ethanol ratios. From the results, there was significant ($p < 0.05$) difference in the extracts as a function of the drying methods and ethanol ratios. The yield of extraction of 50% ethanol extracts of FD, AD and OD fruits were 59.09, 40.09, and 65.75%, respectively. While the yield of extraction for absolute ethanol extracts were 45.64, 19.11, and 28.52% for FD, AD and OD, respectively. For the solvent ratio, 50% ethanol provided higher yield of extraction as compared to absolute ethanol. This observation was in agreement with the previous study, in which the higher the water ratio, the higher the total amount of solids obtained (Spigno *et al.*, 2007). On the other hand, results obtained also shown that drying methods have much influence on the extraction yield of both 50 and 100% ethanol extracts. This was similar to previous findings, in which the extraction yield could

Table 1. Proximate composition of *Phyllantus acidus* fruits

Samples	Moisture	Ash	Protein	Fat	Crude fibre	Carbohydrate
Fresh	90.61 ± 0.33	0.72 ± 0.07	1.50 ± 1.13	0.58 ± 0.38	2.06 ± 0.14	4.55 ± 1.40
Freeze dried	13.61^{a} ± 0.58	4.16^{a} ± 1.71	6.23^{a} ± 0.40	0.01^{a} ± 0.01	9.01^{a} ± 3.37	67.35^{a} ± 4.83
Air dried	14.76^{b} ± 0.25	4.80^{a} ± 0.40	7.70^{b} ± 0.39	1.08^{a} ± 0.44	9.53^{a} ± 0.55	61.8^{a} ± 1.11
Oven dried	11.02^{a} ± 0.13	5.35^{a} ± 0.25	7.45^{a} ± 0.27	0.56^{b} ± 0.13	8.65^{a} ± 0.21	68.07^{a} ± 1.63

Mean of triplicate ± standard deviation
The superscript letter is to compare the drying methods of *Phyllantus acidus.* Mean with different superscript letters are significantly different ($p < 0.05$).

Table 2. Extraction yield (%) of the three dried *Phyllantus acidus* fruits extracted with 50 and 100% ethanol

Drying	Ethanol ratio (%)	Extraction yield (%)
FD	50	59.09^{Aa} ± 4.12
	100	45.64^{Ab} ± 3.71
AD	50	40.09^{Ba} ± 5.25
	100	19.11^{Bb} ± 0.75
OD	50	65.75^{Ba} ± 5.07
	100	28.52^{Cb} ± 3.17

The first capital superscript letter is to compare the drying methods for the same ethanol ratio.
The second superscript letter is to compare the ethanol ratio for the same drying methods.
Mean with different superscript letters are significantly different ($p < 0.05$).
FD: Freeze drying, AD: Air drying, OD: Oven drying

be affected by several parameters including drying temperature, time contact, solvent-to-solid ratio, solvent composition, and the polarity of extracting solvents (Pinelo *et al*., 2005; Sulaiman *et al.*, 2011).

Total phenolic content (TPC)

The bioactivities of plant materials have majorly linked to phenolic compounds. The TPC of the three dried *P. acidus* fruit extracted with 50 and 100% ethanol ratios are shown in Figure 1. The TPC values were ranged from 10.04 to 28.39 mg GAE/g dried extract. As presented in Figure 1, 50% ethanolic extracts of all dried *P. acidus* fruit contained much higher TPC those of 100% ethanol extracts. The result obtained was consistent with a previous study where the TPC improved when the water content was increased (Spigno *et al*., 2007). This could be rationalized by better extracting efficiency of solvent mixture in comparison with a single solvent. In addition, the solubility of chemical constituents in the extracts might be disrupted due to the difference in polarities of extracting solvents (Sulaiman *et al*., 2011; Javadi *et al*., 2014).

Among 50% ethanol extracts, the highest TPC was achieved by OD with the value of 28.39 mg GAE/g dried extract, followed by FD and AD with 22.16 and 10.04 mg GAE/g dried extract, respectively. The lowest TPC of AD and FD extracts might be due to the binding of phenolic compounds with other components such as proteins, or because of a changed chemical structure caused by these drying methods (Sagrin and Chong, 2013).

In contrast, a different trend was observed in 100% ethanolic extracts, in which OD sample contained the lowest TPC value of 4.26 mg GAE/g. Notably, FD and AD of 100% ethanol extracts were slightly higher in TPC with values of 5.43 and 5.54 mg GAE/g dried extract, respectively. However, FD and AD possessed non-significant ($p > 0.05$) difference.

DPPH radical scavenging activity

Free radicals can be deactivated or stabilized by the antioxidants before they cause oxidative damage toward cellular structures (Lee *et al*., 2014). Therefore, the antioxidant activity of plant extract can be determined by using free radical scavenging activity. In this study, DPPH free radical scavenging activity was used to determine the antioxidant properties of *P. acidus* fruit extracts.

The result of DPPH free radical scavenging activity of fruit extract of *P. acidus* is presented in Figure 2. The percentage of DPPH inhibition with 50% ethanol of the three dried *P. acidus* fruit extracts varied from 35.87 to 64.17%, while for absolute ethanol was ranged from 11.87 to 28.35%. Similar to TPC, 50% ethanol extract of OD sample exhibited the highest DPPH scavenging effect with the inhibition percentage of 64.17, whereas OD

Table 3. Percentage of α-glucosidase inhibition and the IC_{50} values

Drying	Ethanol	Concentration (µg/mL)	Percentage %	IC_{50} (µg/mL)
FD	50%	5000	$98.99^{Aa} \pm 0.14$	$36.90^{a} \pm 0.10$
	100%	5000	$8.91^{Ba} \pm 1.06$	ND
AD	50%	5000	$84.71^{Ba} \pm 1.65$	ND
	100%	5000	$45.75^{Bb} \pm 3.27$	ND
OD	50%	5000	$99.24^{Aa} \pm 0.04$	$12.39^{b} \pm 0.56$
	100%	5000	$67.14^{Cb} \pm 2.89$	ND

The first capital superscript letter is to compare the drying methods for the same ethanol ratio.
The second superscript letter is to compare the ethanol ratio for the same drying methods.
Mean with different superscript letters are significantly different ($p < 0.05$).
FD: Freeze drying, AD: Air drying, OD: Oven drying

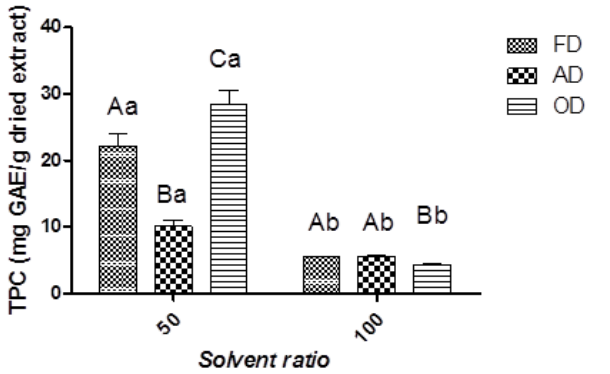

Figure 1. Total phenolic content of *Phyllanthus acidus* extracted using various ethanol ratios.
The first capital superscript letter is to compare the drying methods for the same ethanol ratio.
The second superscript letter is to compare the ethanol ratios for the same drying methods.
Mean with different superscript letters are significantly ($p < 0.05$) different.

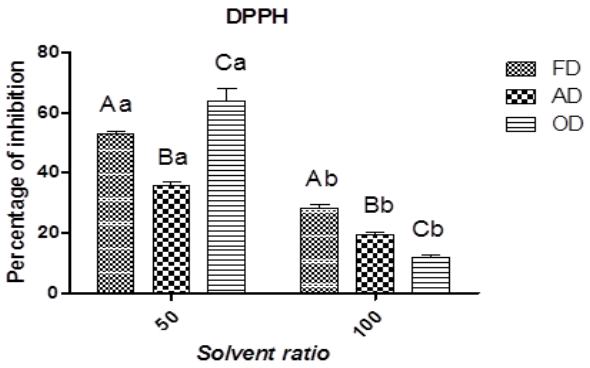

Figure 2. DPPH radical scavenging activity of *Phyllanthus acidus* extracted using various ethanol ratios.
The first capital superscript letter is to compare the drying methods for the same ethanol ratio.
The second superscript letter is to compare the ethanol ratios for the same drying methods.
Mean with different superscript letters are significantly ($p < 0.05$) different.

sample of 100% ethanol extract exhibited lowest DPPH scavenging effect at inhibition percentage of 11.87. This result showed that the fruit of *P. acidus* extracts has moderate antioxidant activity. The better performance of 50% ethanol extracts could be explained by their higher TPC in comparison with 100% ethanol extracts as phenolic compounds are the main source of antioxidant (Lim and Murtijaya, 2007).

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity of *P. acidus* extracts is presented in Table 3. Result obtained showed that the *P. acidus* possessed potent α-glucosidase inhibitory activity. 50% ethanol extracts of FD and OD sample were found to significantly inhibit α-glucosidase enzyme with the inhibition percentage of 98.99 and 99.24%, respectively, whereas other extracts were poorly inhibiting α-glucosidase at their concentrations of 5000 µg/mL. Further evaluation revealed that the IC_{50} of OD sample with of 12.39 µg/mL was 3-fold better than FD sample. The potent α-glucosidase inhibitory activity of 50% ethanol extracts of OD and FD samples might be contributed by TPC as both OD and FD samples showed the highest in TPC among all samples. Phenolic compounds could provide additional hydrogen bonding which may possibly enhance the activity of the compound due to stronger interaction with targeted enzyme.

In this study, TPC was positively correlated with DPPH and the α-glucosidase inhibitory activitieswith R values of 95.0 and 73.8%, respectively. Meanwhile, DPPH and α-glucosidase inhibitory activities were also positively correlated with R value of 63.3%. These correlations suggested that the higher the TPC, the better the antioxidant and anti- α-glucosidase activities. Taken all together, these results suggesting that 50% ethanolic extract of OD *P. acidus* fruits was the best extract in preparing bioactive *P. acidus.*

Conclusion

The results of the current study showed that *P. acidus* fruit extracts consisted of a potential range of phenolic compounds and exhibited potential antioxidant and α-glucosidase inhibitory activities. The results of proximate analysis showed that fresh and all the dried *P. acidus* fruit exhibited valuable nutrient contents. The present study also demonstrated that drying methods and ethanol ratios had different effects on the biological activity and chemical profile of *P. acidus*. Among the fruit extracts subjected to FD, AD and OD with 50 and 100% ethanol, the best in terms of TPC, DPPH free radical scavenging activity and α-glucosidase inhibitory was represented by 50% ethanolic extract of OD sample. The current study revealed that phenolic compounds could be the main contributors to the DPPH antioxidant and α-glucosidase inhibition activities with R values of 95.0 and 73.8%, respectively. This result suggests that phenolic compounds of *P. acidus* fruit extracts may contribute to antioxidant and α-glucosidase inhibitory activities. Therefore, the *P. acidus* fruits can be classified as food that contains health benefit beyond basic nutrition. However, more extensive studies need to be carried out to identify and purify the bioactive compounds.

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