J. Trop. Agric. and Fd. Sc. 51(2)(2023): 11 – 15

Bioactivity analysis of *Phyllanthus tenellus* extracted via different aqueous extraction techniques

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Abstract

This present study aims to investigate how different water based extraction techniques might affect the total phenolic, flavonoids, DPPH scavenging activity and cytotoxicity of *Phyllanthus tenellus*. Four common extraction techniques such as pressurised hot water extraction (PHWE), microwave assisted extraction (MAE), heat assisted sonication (HAS) and boiling were chosen in this study. The total phenolic content in all extracts were found to be insignificant through one way ANOVA analysis (p >0.05), but further T-test between individual extracts showed PHWE has the significant highest phenolic content from the rest. PHWE also registered the highest reading of flavonoids followed by MAE, boiling and HAS. In terms of DPPH scavenging activity, PHWE has the lowest IC_{50} value at 2.58 µg dried extract (DE), followed by MAE (7.12 µg DE), HAU (7.55 µg DE) and boiling (14.39 µg DE). The cytotoxicity of all the extracts followed the same pattern; they had a weak cytotoxicity (cell viability between 61% – 74%) at 500 µg/mL and high cytotoxicity (cell viability 16% – 17%) at 5,000 µg/mL. A method of increasing phenolic and flavonoids content without the usage of organic solvents offers vast possibility in the development of functional food products. This first ever study on various water based extraction techniques on *P. tenellus* could cater for the aforementioned course.

Keywords: *Phyllanthus tenellus,* aqueous extraction, bioactivities

Introduction

Phyllanthus tenellus (*Figure 1*) commonly known as *dukung anak*, is a herbaceous plant belonging to the family of Euphorbiaceae, is found abundantly in Southeast Asia and most tropical countries. Similar to other *Phyllanthus* sp. it is also known for its health benefiting properties. This plant is used to treat urolithiasis, inflammatory bowel disease, diabetes and hepatitis B (Silva et al. 2012). *Phyllanthus* sp. are known to contain high levels of hydrolysable tannins which have been frequently associated with its anti-viral activity (Liu et al. 2001, Tan et al. 2013). The genus *Phyllanthus* is also a rich source of phenolics, flavonoids, alkaloids, terpenoids and sterols (Zain and Omar 2018, Jantan et al. 2019) which associated with anti-oxidant, anti-inflammatory and analgesic effect.

One of the most important steps in the study of natural products is the extraction process. Various organic solvents with different polarities such as methanol, hexane, chloroform, acetonitrile, benzene and ethyl alcohol have been used over the years to optimise extraction of bioactive compounds (Zhang et al. 2018). However, these solvents are not only toxic for both humans and the environment; they are also relatively expensive in industrial scale extraction. In more recent years, extraction technique has advanced in using new and safer way such as microwave-assisted extraction, supercritical fluid extraction, pressurised liquid extraction and ultrasound-assisted extraction (Mushtaq et al. 2014, Pavlik et al. 2023). One of the cleanest and green technologies is just to use water as the solvent together with the aforementioned extraction techniques. Jusoh and colleagues in their 2019 study demonstrated on how *P. tenellus* extracted using pressurised hot water extraction (PHWE) exhibit shorter extraction time compared to methanolic and extraction at room temperature, lower costs of the extracting agent, and an environmentally compatible technique too. Unfortunately, hitherto other extraction techniques using water as the extraction solvent have not been tested on *P. tenellus*.

Article history
Received: 16.3.2023
Accepted: 31.5.2023

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Figure 1. Phyllanthus tenellus

In this study, *P. tenellus* was extracted with four different water based extraction methods which include pressurised hot water extraction (PHWE), microwave assisted extraction (MAE), heat assisted ultrasonication (HAU) and boiling. The extracts were compared for their total phenolic content, total flavonoid content, DPPH scavenging activity and cytotoxicity via cell based assay. Testing of other water based extraction techniques help to broaden the search of optimum and safer extraction method for phytochemical analysis.

Materials and methods

Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Sodium bicarbonate was purchased from Fisher Scientific (Leicestershire, UK). 2,2-diphenyl-1-picrylhydrazyl (DPPH), Aluminium (III) chloride and phosphate buffer saline (PBS) were obtained from Sigma-Aldrich (St. Louis, USA). Reagents used for cell culture studies such as powdered Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, L-glutamine, pyridoxine hydrochloride but without sodium bicarbonate and sodium pyruvate, antibiotic penicillin streptomycin, Fetal Bovine Serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TripLE^{TM} Express enzyme were purchased from Gibco (Life technologies, USA). Vero cell was purchased from American Type Culture Collection (ATCC).

Planting material

P. tenellus was grown in the net house at Biotechnology and Nanotechnology Research Centre, MARDI in a semi-controlled environment. A voucher sample was sent to MARDI's herbarium for validation purposes (MD10525). Herb was irrigated twice daily with automated irrigation system in the net house. Upon maturation, all parts except the roots were harvested, washed under running tap water and dried with pepper towel to remove water droplets. About 40 g of the fresh herb was then allocated in six replicates each for subsequent extractions.

Extraction of phytochemicals from fresh *P. tenellus*

Pressurised Hot Water Extraction (PHWE)

Fresh sample (40 g) was allocated in a 500 mL Duran bottle and 200 mL of deionised water was added. The mixture was then autoclaved using the manufacturer's default set up (121 °C for 15 min). After that, the mixture was left to cool at room temperature before being filtered using Buchner set up (Buchner flask, Whatman paper No. 40 and vacuum pump). The collected extract was stored in -80 °C overnight before being freeze dried using freeze dryer (Labconco, vacuum of 0.120 mBar, temperature of –51 °C).

Microwave assisted extraction (MAE)

MAE was done using the similar approach as in PHWE, but the mixture was left to boil in the microwave (Sharp 25 L, Japan) for 15 min. The manufacturer's default heating set up was used for this extraction. After that, the mixture was left to cool at room temperature before being filtered. The collected extract was stored in –80 °C overnight before being freeze dried using freeze dryer.

Heat Assisted Ultrasonication (HAU)

HAU was done using an ultrasonic bath (Elmasonic Easy 60H, Germany). Ultrasonic bath was pre-heated to the maximum temperature (80 °C) prior to sonication. Sample was sonicated for 15 min and left to cool at room temperature before being filtered. Collected extracts were pre-frozen in –80 °C before freeze dried.

Boiling

The extraction method was similar to the rest of the extraction except the mixture (in Duran bottle) was heated to boiling point using a magnetic hot plate stirrer (Thermo, USA). Once the boiling point achieved, the mixture was left for 15 min on the top of the hot plate before the machine was turned off. The mixture was left to cool down before being filtered for its supernatant. Collected extracts were pre-frozen in –80 °C before freeze dried.

Total phenolic content (TPC) assay

TPC was carried out in accordance to the method used in Chandradevan et al. (2020). In general, 20 µL of the dissolved extract (20 mg/mL) was mixed with 100 µL of Folin-Ciocalteu (10-fold dilution) reagent in 96-well plate. After that, 80 µL of 7.5% sodium carbonate was added and the mixture was left in the dark for 15min prior to absorbance reading at 750 nm (SPECTRAmax PLUS, USA). Gallic acid was serially diluted ranging from 100 µg to 0.781 µg and was used as the reference standard

for this assay. The results were expressed as mg gallic acid equivalent (GAE) per 100 mg of dried extract.

Total flavonoid content (TFC) assay

TFC was performed in accordance to the method described in Chandra et al. (2014) with minor modification done on the volume and incubation time of the mixtures. In general, 20 µL of the dissolved extract (20 mg/mL) was mixed with 180 µL of aluminium (III) chloride (2% m/v) and incubated under the light for 15 min before absorbance was taken at 420 nm. Catechin was used as the reference standard, ranging from 100 µg to 0.781 µg. The results were expressed as mg catechin equivalent (CA) per 100 mg of dried extract.

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

DPPH scavenging assay was performed based from the method described by Chandradevan et al. (2020). In general, 50 µL extract (serially diluted from 1000 µg/mL to 31.25 µg/mL) was added with 100 µL of DPPH (0.15 mM, dissolved in ethanol) in 96-well plate and incubated in dark for 30min. Absorbance of the scavenging activity was measure at 515 nm. Blank sample was prepared by replacing DPPH with ethanol. Blank solvent was prepared by replacing the extracts with the solvents used to dissolve them. Quercetin hydrate was used as the positive control and all experiments were performed in triplicates. The result of DPPH scavenging activity was calculated as in percentage of inhibition based from the formula [(Abs_n – Abs_s)/ Abs_n] X 100%, where Abs_n is the difference of absorption between the negative control and blank solvent; and Abs_s is the difference of absorption between sample and blank sample. The final results were expressed as in amount (µg) of extract needed to scavenge 50% of DPPH (IC_{50}).

Cytotoxicity of **P. tenellus** *extracts (PHWE, MAE, HAU and Boiling) via MTT assay*

Cytotoxicity test or also known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the toxicity of the plant extract, if any presence in all *P. tenellus* extracts. Prior to MTT assay, vero cells were incubated with *P. tenellus* extract at high (5000 µg/mL), medium (1000 µg/mL) and low dosage (500 µg/mL) for 72 hours at 5% CO_2 concentration, 37 °C. After 72 hours, 96-well plates were taken out from incubator and all media was discarded. Fresh 100 µL DMEM was added into the well followed by 20 µL of MTT (dissolved in 1x PBS buffer). The plates then were incubated for 4 hours at 37 °C in 5% CO_2 concentration. After that, all media was discarded again and 100 µL of DMSO was added accordingly. The plates were left for 15 min in dark at room temperature before absorbance was taken at 570 nm. The percentage of viability of cells was calculated by comparing the absorbance of treated cells with the control group (untreated cells).

Statistical analysis

All bioassay results were reported as mean of six biological replicates with standard deviation. One-way analysis of variance (ANOVA) and T-test were performed with significant difference between the collected data was set at confidence interval 95%. All calculations were carried out using GraphPad Prism version 5.01 for Windows (San Diego, CA, USA).

Results and discussion

Extraction technique plays a crucial role in the analysis of natural products. Usages of different extraction buffers coupled with extraction techniques ensure the maximum extraction of phytochemicals. Often, organic solvents were preferred as the extraction solvents since the ability of organic solvent to extract out least polar phytochemicals such as phenolic acids from the matrix of herbs cytoplasm (Chandradevan et al. 2020). Unfortunately, the idea of using organic solvent is not feasible when extraction is being carried out in large industrial scale due to the toxicity and corrosive nature of organic solvent. As such a more environmental friendly and safe approach needed for the extraction to be carried out, especially when developing herbal based functional foods.

As to certain this, water based extraction techniques were put into test in this study. *Phyllanthus* sp. had been an important choice of herb since the introduction of this herb in NKEA (Farizah et al. 2015). In MARDI, studies on various *Phyllanthus* sp. especially *P. tenellus* have been carried out for more than a decade and its health benefiting properties have been proven scientifically. In order to commercialise *P. tenellus* as a functional food, a safer extraction solvent has to be used. *Table 1* summarised the total phenolic content, total flavonoid content, DPPH scavenging activity and the toxicity of the extracts by means of cell assay. One way ANOVA at confident interval 95% indicated that there is not significant different (p >0.05) between the phenolic content among the four extraction techniques. However, further T-test between the individual extraction techniques shows that the phenolic content in PHWE was significant (4725 µg GAE) compared to the rest of the extraction techniques.

The opposite trend was observed for total flavonoid content assay. One way ANOVA between the extracts of different extraction techniques were significant (p <0.05); with PHWE registered the highest flavonoids (3.12 µg CE) followed by MAE (2.03 µg CE). Total flavonoid content between HAU and boiling was not significant when T-test was applied between these two extraction techniques. Higher yield of phenolic and flavonoids in PHWE was anticipated even though the rest of the extraction techniques utilised heating. PHWE which uses

Table 1: Compilation of total phenolic content (TPC), total flavonoid content (TFC), DPPH scavenging activity and cytotoxicity (MTT) of *P. tenellus* extracted at different water based extraction protocols

Extraction protocol	TPC (μg GAE/100 mg DE)	TFC (μg CE/100 mg DE)	DPPH (IC_{50}, μg DE)	MTT (% cell viability)	
PHWE	$4725.86 \pm 28.34^{a}{}_{1}$	$3.12 \pm 0.44^{a}{}_{1}$	$2.58 \pm 0.47^{a}{}_{1}$	High dose	17.72
				Medium dose	40.39
				Low dose	74.83
MAE	$4493.21 \pm 99.37^{b}{}_{1}$	$2.03 \pm 0.21^{b}{}_{2}$	$7.12 \pm 1.43^{b}{}_{2}$	High dose	17.58
				Medium dose	54.24
				Low dose	67.12
HAU	$4483.42 \pm 108.44^{b}{}_{1}$	$1.76 \pm 0.15^{c}{}_{3}$	$7.55 \pm 1.11^{b}{}_{3}$	High dose	16.21
				Medium dose	47.45
				Low dose	63.79
Boiling	$4546.48 \pm 69.20^{b}{}_{1}$	$1.86 \pm 0.08^{c}{}_{4}$	$14.39 \pm 5.49^{c}{}_{4}$	High dose	17.45
				Medium dose	47.69
				Low dose	61.62

Values are the mean ± standard deviation of six biological replicates. The subscript numbers represent one way ANOVA set at confidence interval 95% among extracts of the same assay. Subscript of different numbers indicates significant data ($p<0.05$). The superscript alphabets represent T-test done between any two samples within the same assay. Superscript of different alphabets indicates significant data ($p<0.05$). (GAE: gallic acid equivalent, CE: catechin equivalent, DE: dried extract, PHWE: pressurised hot water extraction, MAE: microwave assisted extraction, HAU: heat assisted ultrasonication)

autoclave at temperature beyond boiling point (121 °C) could play an important role. At higher temperature, the breakdown of plants' cell wall would be more easier compared to boiling at 100 °C or sonication at 80 °C, which cater the release of phytochemicals from the matrix into the solvent. PHWE also utilised the high pressure (15 psi) due to the increased of temperature in the closed system which not applied to other extraction techniques. A combination of high temperature and pressure seems to be the key role for the above results (Brunner 2005).

In addition to both phenolic and flavonoid content, the antioxidant activity of the extracts was tested via DPPH scavenging assay. The lower the value of IC_{50}, the stronger is the scavenging ability of the DPPH free radicals. The scavenging property between the extracts were significant (p <0.05) with PHWE registered the lowest IC_{50} value (2.58 μg DE) among the rest. Boiling registered the highest IC_{50} (14.39 μg DE) and the scavenging activity between MAE and HAU were insignificant (p >0.05). Scavenging activity of DPPH free radicals is closely related to the phytochemicals extracted. The presence of higher phenolic and flavonoids in PHWE could explain the significant scavenging activity too. Phenolic acids and flavonoids have been proven scientifically on their ability as an antioxidant. Besides, higher temperature and pressure at 15 psi leads to the breakdown of larger hydrosable tannins such as geraniin into small catabolites such as gallic acids, corilagin, brevifolin and possibly urolithin derivatives from *P. tenellus* (Jusoh et al. 2019). This breakdown is unique since it was observed to take place in human intestines with the facilitation of guts micro-biomes (Ito et al. 2008). PHWE has somehow offered an alternative method to ease the catabolism of large molecular phytochemicals without the needs of guts micro-biomes. Yield of more catabolites due to the breakdown of larger phenolic acid increase the capability of the extract as an antioxidant overall.

Cytotoxicity of the extracts needs to be determined considering the possibility of developing herbal based functional foods from *P. tenellus*. Vero cells were chosen since this cell is commonly used to cytotoxicity related studies. The cells were pre-grown in dried extract dissolved in DMEM media at three dosages and kept for 72 hours to check on cell viability. Cell viability of more than 80% is considered an extract to be non-toxic, 80 – 60% as weak, 60 – 40% moderate and below 40% as highly toxic (ISO 10993-5:2009). However, some researchers would consider the non-toxic percentage to be above 95% as to affirm an extract is utterly and totally safe for consumption (Thermofisher).

In this study, the cytotoxicity of *P. tenellus* extracted at various water based extraction techniques projected a similar pattern; the cytotoxicity was dosage dependent. The cell viability reduced as the concentration of extracts increased after 72 hours of incubation. All extracts were in the range of weak toxicity at low dosage (500 μg/mL) but highly toxic at high dosage (5000 μg/mL). The cytotoxicity results clearly indicate *P. tenellus* extract can impose mild toxicity if taken without proper guidance. Another pre-clinical study using PHWE of *P. tenellus* suggested that the maximum dosage that can be administrated to rat model was 1000 mg/kg of body weight. At 3,000 mg/kg of body weight, signs of liver toxicity were noticed (Yeap et al. 2021).

Conclusion

This study offers the first insight look on how different water based extraction techniques affect the phytochemicals content from *P. tenellus*. PHWE has shown its potentiality not only in increasing phenolic and flavonoids but also posed strong DPPH scavenging activity. A combination of high temperature and pressure had a remarkable effect in eluting phytochemicals from plant's matrices. However, mild toxicity of the extract at low dosages should be taken into consideration even though the extraction buffer used was water. The present study also agrees with previous findings indicating the potentiality of *P. tenellus* to be used as a traditional treatment for various illnesses such as jaundice, diarrhea, fever and lowering blood glucose level.

Acknowledgment

The authors of this paper would like to acknowledge MARDI for funding this project under the MARDI's special grant (KSR 999).

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