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ANTIOXIDANT POTENTIAL OF MALAYSIAN MEDICINAL PLANT

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Abstract

The objective of this research is to study the phytochemical content of Malaysian medicinal plants (*Andrographis paniculata, Morinda Citrifolia* and *Ficus Deltoidea*). This study involves the investigation on total phenolic content, total flavonoid content and DPPH scavenging activity using different method of extraction - hot extraction and ultrasonic assisted extraction. From the experiments, it can be seen that *Ficus deltoidea* leaves has the highest amount of phenolic content, flavonoid content and better scavenging activity compared to *Andrographis paniculata* and *Morinda citrifolia*. Each extraction was carried out with different solvents - water, 80% methanol and 80% ethanol. It is found that the solvents used for extraction has significant effect on phenolic content ($p=0.0014$) and flavonoid content ($p=0.004$) for all three plants used in this study. 80% methanol has the highest effect on the extraction of phytochemicals followed by 80% ethanol and water. As for DPPH scavenging, solvents do not have any significant effect on the scavenging activity. Ultrasonic assisted extraction is significantly better in extraction compared to water under the same conditions.

Keywords: *Morinda citrifolia*, *Ficus deltoidea*, Andrographis paniculata, Antioxidant, Free radicals.

1. Introduction

It is expected that more than 366 million of the world population will be affected by diabetes mellitus by the year 2030 [1]. Diabetes mellitus is a disease caused by hyperglycemia and disruption in fat and protein which results from the defects in insulin secretion and insulin action [2]. Type 1 diabetes is caused by the destruction β-cells of the pancreas and abnormalities in carbohydrate, fat and protein metabolism which shows resistance to insulin [3]. People with type 1 diabetes

Abbreviations

ANOVA	Analysis of varience
CE	(+)- catechin equivalent
DPPH	2,2 diphenyl-picrylhdrazyl
DW	Dry weight
GAE	Gallic Acid equivalvent
HPLC	High Preformance Liquid Chromatograph
M	Molar
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UV	Ultraviolet
w/v	Weight by volume

require insulin for survival. Type 2 diabetes is the common type of diabetes with 90% of the total cases caused by the failure of the insulin to act against the glucose level [3]. Without insulin action in human body, the amount of glucose rises and poses more health threats like retinopathy, nephropathy and neuropathy [1].

Recent study has shown that oxidative stress has been linked with the development of diabetes mellitus [4]. Oxidative stress does play an important role in diabetes mellitus. Oxidative stress creates complications at microvascular and macrovascular level [3]. Oxidative stress contributes to the malfunction of two major mechanisms involved in maintaining glucose level – insulin resistance and insulin secretion [3]. Oxidative stress is basically the imbalance between the oxidants and the antioxidants [2]. When the ability of the oxidants is limited to defend the system from the oxidants, oxidative stress is exerted on the cells [3]. The damage created by oxidative stress is permanent [5]. Free radicals are electrically charged molecules where they have an unpaired electron [6]. Hence, these free radicals seek another electron to neutralize themselves [6]. After capturing the electron, another free radical is created in the process triggering a chain reaction producing thousands of free radicals within seconds [7]. These free radicals need to be deactivated using antioxidants [5]. If the antioxidants are unable to control the free radicals, these free radicals would cause damages to proteins, lipids and DNA [8]. This would lead to cell death or physiological dysfunctions such as diabetes and cancer [3].

Hence, there must be a balance between oxidants and antioxidants [2]. Halliwell et al. stated that when the antioxidants ability to deactivate the oxidants is limited, the damages to the cells are extensive [5]. Vaya and Avrim have identified two mechanisms of antioxidants – chain-breaking mechanisms and removal of ROS initiators [9]. Plant phenolic compounds have been proven to protect the cells from oxidants [10]. According to Kinsella et al., plant phenolic compound could actually protect against degenerative disease due to oxidative stress [11]. This is further validated by Fuente and Manzanaro as they have stated that plant phenolic compound aids the inhibition of polyol pathway which is responsible for diabetes [44]. Plant phenolic compounds have been proven to protect the cells from oxidants [10]. This is further validated by Fuente and Manzanaro as they have stated that plant phenolic compound aids the inhibition

of polyol pathway which is responsible for diabetes [12]. It has been proven that this phytochemical exists in all the three plants used in this research [13, 14, 15]

Phenolic compounds are divided into two major groups - flavonoids and non-flavonoids [16]. Flavonoids are used for anti-allergenic, anti-inflammatory, anti-bacterial and also anti-hepatotoxic purposes [17]. The flavonoid level in each plant differs as they are affected by various factors such as species, light and etc. [18]. In plants, it has been proven that to resist infection or radiation, plants produce more of flavone or flavonol which are the constituents of flavonoids [17]. This proves that the level of flavonoids in plants is vital for the resistance of external threats. This increase in flavonoid level is vital as it is capable of free radical scavenging [17]. This is the basis of DPPH free radical scavenging. DPPH is used to replicate the free radicals. Hence any impact on DPPH can be interrelated to oxidative damage caused by free radicals.

Medicinal plants have been used for years to limit the effects of diabetes mellitus. In recent years, many studies have been carried out to find a safer cure for diabetes mellitus. More than 200 bioactive components from plants have been identified as glucose reducing components [19]. Some of the bioactive components identified are phenolic, flavonoids, triterpenoids and alkaloids [20, 21, 22]. These bioactive components play a crucial role as antioxidants. Flavonoids are the largest group of phenolic compound [16]. These two phytochemicals are often related to cure of diabetes mellitus [23]. Flavonoid is a major component involved in antidiabetic activity. This proved by a research carried out on type 2 diabetic rats strongly suggest that flavonoids is indeed an active component involved in antidiabetic [10]. This is also further validated by another study done – both in- vitro and in vivo studies shows that phenolics has positive effects on glucose homeostasis.

The medicinal plants chosen to carry out the experiments are Mengkudu (*Morinda citrifolia*), Mas Cotek (*Ficus deltoidea*) and Hempedu Bumi (*Andrographis paniculata*). These plants are chosen based on the following criteria. Firstly, these plants are listed as plants with potential anti-diabetic activity. [24]. Secondly, these medicinal plants are part of the priority species for the development programme in Malaysia [25]. Finally, the availability of the plants in Malaysia is also taken into account.

Flavonoids are the main chemical component in *Andrographis paniculata* which are responsible for the plants' biological activities [24]. Hence the plant exhibits antioxidant properties. *Ficus deltoidea* exhibits medicinal properties as they possess phenolic compounds in them [25]. Flavonoids are also a major component of Morinda citrifolia [26]. Total phenolic content and total flavonoid content for the leaves are low compared to the fruits [27].

For extraction of the extracts – water, methanol and ethanol are often used. It was found that methanol extraction is better compared to aqueous extraction and ethanol extraction as the total phenolic content and the total flavonoid content were the highest compared to other method of extraction. But DPPH scavenging activity was higher in aqueous extraction followed by ethanol extraction and methanol extraction [24]. Total phenolic content and total flavonoid content were higher in samples extracted by methanol [25]. DPPH scavenging activity was also higher in methanol extraction compared to aqueous extraction. Binary solvent

system is used in this study as it has been proven to maximize the active compounds in the plants [28].

It has to be understood that even though flavonoids are vital for health, flavonoids would not be as effective as a component on its own [13]. Flavonoids rely on different phytochemicals which gives flavonoids the ability to defend the cells from oxidants [13]. Rutin along with vitamin C and vitamin E when combined produced better free radical scavenging [14]. Combination of fruits actually yielded better antioxidant activity compared to just one fruit [29]. One single purified phytochemical can never replace the health benefits of the combination of natural phytochemicals which exists in plants [30].

Plant phenolic is heat sensitive hence using freeze drying would be a better choice compared to oven drying [31]. Freeze drying prevents the growth of microorganisms [31]. In addition to that, freeze drying slows down lipid oxidation significantly [31]. Freeze drying is the best method to preserve antioxidant activity and nutrient content of plants as it actually increases the level of phenolic compounds [32]. But it has been argued that freeze drying might cause reduction in antioxidant activity due to degradation of certain compounds (phenolics and flavonoids) [31]

The study is carried out to measure the antioxidant activity of *Andrographis paniculata, Ficus deltoidea* and *Morinda citrifolia* individually using the leaves of these plants for total phenolic content and total flavonoid content measurement and DPPH Free Radical Scavenging. These plants are chosen because Different solvents (water, 80% methanol and 80% ethanol) are used to extract the plants in two methods of extraction – water bath extraction and ultrasonic assisted extraction. The study is also carried out to study the effect of freeze drying on plant extracts with the highest antioxidant activity. The bioactive compounds are identified through HPLC analysis.

2. Research methodology

2.1. Material and method

2.1.1. Plant material

Hempedu Bumi (*Andrographis paniculata*), Noni (*Morinda citrifolia*) and Mas Cotek (*Ficus deltoidea*) were purchased from a plant supplier in Sungai Buloh, Selangor, Malaysia. The leaves are cleaned before cutting into small pieces using a clean scissors.

2.1.2. Chemicals

Distilled water, Methanol, Ethanol (96%), Sodium hydroxide, +(-)catechin, Sodium carbonate, Sodium nitrite, Folic-Ciocalteu reagent, Gallic acid, Aluminium chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH).

2.1.3. Apparatus

Water bath, Ultrasonic cleaner, 100 ml and 500ml measuring cylinder, Whatman No.1 filter paper, Beaker, Aluminium foil, Laboratory bottles.

2.2. Extraction

The conditions were standardised for both the extraction methods. This is done to study the efficiency of each method with different solvents. Solid to solvent ratio used is 1:15, the temperature used is 45℃ and the duration for extraction is 40 minutes. Three different solvents are used - distilled water, 80% methanol and 80% ethanol to extract the plants.

For hot water extraction and ultrasonic assisted extraction, the leaves were cut into pieces and placed into conical flasks and laboratory bottles respectively. The conical flask and laboratory bottles were then filled with 15 ml of solvent. The conical flask was then placed in a water bath (CloudLab Scientific Sdn. Bhd., Model MT-1034, Malaysia) at 45ºC for 45 minutes while the laboratory bottle was placed into the ultrasonic cleaner (Soluzioni Technologiche Inc., Model Sonica 5300EP S3, Italy) at 45ºC for 45 minutes. The extracts were then filtered using filter paper (Whatman International Ltd., England) into beakers. The extraction was done in triplicates for each solvent.

2.3. Phytochemical content

2.3.1. Total phenolic content

It is measured using Folin-Ciocalteu colorimetric which was described by Chong [34]. A total of 0.1 ml of plant extract is added 0.2 ml of Folin-Ciocalteu reagent. The mixture is then added with 2 ml of distilled water. The mixture is then left to rest at room temperature for 3 minutes. Then, 1 ml of 20% sodium carbonate is added to the mixture. The mixture is left to be incubated at room temperature for an hour. The absorbance of the mixture was measured at 765 nm using an UV-Spectrophotometer (Dynamica, Model RB-10). Blank sample was prepared as explained above except by replacing the plant extract with distilled water. Determinations of total phenolic content were carried out with the respect to gallic acid standard curve. Measurements were carried out in triplicates. The results were expressed in term of milligrams of gallic acid per 100 g dried weight (mg GAE/100 g DW). The total phenolic content (mg GAE/ 100 g DW) was gauged using the following the equation:

$$Total\ phenolic\ content\ (mg\ GAE/100\ g\ DW) = GAE \times \frac{V}{M} \times 100 \quad (1)$$

where GAE is gallic acid equivalence (mg/ml) attained from the standard curve, V is total volume of solvent used during the assay (ml), and M is the mass of plants used during the assay (g)

2.3.2. Preparation of standard curve for total phenolic content

Gallic acid with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were prepared. 0.1 ml of the gallic acid solution is added with 0.2 ml of Folin-Ciocalteu reagent. The mixture is the added with 2 ml of distilled water. Then, 1 ml of 20% sodium carbonate is added to the mixture. The mixture is left to be incubated at room temperature for an hour. The absorbance of the mixture was measured at 765 nm using an UV-Spectrophotometer. Blank sample was

preparing the mixture as explained above except by replacing the plant extract with distilled water. A standard curve is plotted - y-axis represents the absorbance while x-axis represents the concentration of the gallic acid. The calibrated equation obtained from the standard curve is y=2.5899x+0.5302 (R^2=0.8704).

2.3.3. Total flavonoid content

Total flavonoid content was measured using the method described by Ozsoy [35]. Plant extracts (0.25 ml) were mixed with 1.25ml of distilled water and 0.075 ml of 5% (w/v) sodium nitrite solution. The mixture was incubated at room temperature for 6 minutes and 0.15 ml of 10% (w/v) aluminium chloride was added to the mixture. 1 M of sodium hydroxide (0.5 ml) was added to the mixture and then 0.275 ml of distilled water was added. The absorbance of the mixture was measured at 510 nm using an UV-Spectrophotometer. Blank sample was prepared as explained above except by replacing the plant extract with distilled water. Determinations of total flavonoid content were carried out with the respect to (+)-catechin standard curve. Measurements were carried out in triplicates. The results were expressed in term of milligrams of (+)-catechin per 100 g dried weight (mg CE/100 g DW). The total phenolic content (mg CE/ 100 g DW) was gauged using the following the equation:

$$\text{Total flavonoid content (mg CE/100 g DW): CE} \times \frac{\mathbf{V}}{\mathbf{M}} \times 100 \quad (2)$$

where CE: (+)-catechin equivalence (mg/ml) attained from the standard curve, V: Total volume of solvent used during the assay (ml) and M: Mass of plants used during the assay (g)

2.3.4. Preparation of standard curve for total flavonoid content

(+)-catechin with different concentrations (0.1, 0.3, 0.5 and 0.7 mg/ml) were prepared. Plant extracts (0.25 ml) were mixed with 1.25ml of distilled water and 0.075 ml of 5% (w/v) sodium nitrite solution. The mixture was incubated at room temperature for 6 minutes and 0.15 ml of 10% (w/v) aluminium chloride was added to the mixture. 1 M of sodium hydroxide (0.5 ml) was added to the mixture and then 0.275 ml of distilled water was added. The absorbance of the mixture was measured at 510 nm using an UV-Spectrophotometer Blank sample was prepared as explained above except by replacing the plant extract with distilled water. A standard curve is plotted - y-axis represents the absorbance while x-axis represents the concentration of the (+)-catechin. The calibrated equation obtained from the standard curve is y = 3.3216x + 7E-05 (R^2=0.9821) [36].

2.3.5. DPPH scavenging activity

DPPH scavenging activity is carried out using procedures described by Saha [37]. Plant extracts (0.1 ml) is added with 3.9 ml of ethanolic DPPH ($60x10^{-5}$ M). The mixture is then kept in the dark for 30 minutes at room temperature. This is done to block UV light as UV light would trigger free radicals [37]. This will disrupt the results of the experiment. The solution is then measured for absorbance using spectrophotometer at 517 nm. Due to the reaction, the colour of the solution will

change from deep violet to light yellow. Radical scavenging activity is calculated using the following formula:

$$\%\text{Inhibition} = \frac{A_B - A_A}{A_B} \quad (3)$$

where A_A= Absorption of extract , A_B= Absorption of blank sample

This is carried out for all the plant extracts including the combination of the extracts. Determinations are carried out in triplicates.

2.3.6. Freeze drying

The plant extract with the highest antioxidant activity will be freeze dried. After freeze drying, all the phytochemical analysis and the antioxidant activity will be carried out on the extract. This is carried out to determine the effectiveness of freeze drying on antioxidants. Freeze drying is carried out in Forest Research Institute Malaysia (FRIM). Freeze drying is carried out over a span of 3 days. The equipment used is a vacuum freeze dryer. The sample is placed in a sealed container. The container is then placed in the freeze dryer. The standard temperature used in FRIM is -50℃ and the pressure of vacuum is varies between 0.4 - 0.6 mB [38].

3. Results and Discussion

When the results are observed as shown from Table 1 to Table 6, it can be seen that *Ficus deltoidea* has the highest phenolic and flavonoid content when compared to *Andrographis paniculata* and *Morinda citrifolia*. DPPH scavenging of *Ficus deltoidea* is also more extensive compared to *Andrographis paniculata* and *Morinda citrifolia.* In addition to that, by comparing Table 1, Table 2 and Table 3 which illustrates water bath extraction with Table 4, Table 5 and Table 6 which illustrates ultrasonic assisted extraction, it is deduces that ultrasonic assisted extraction is a more efficient method compared to water bath extraction method as the values for phenolic content, flavonoid content and DPPH scavenging is higher. This is due to the working principle of ultrasonic. The cavitations from the ultrasonic waves created in the water medium collapses near the laboratory bottles sending shock waves to the cells [40]. This ruptures the cell wall of the plants thus releasing the contents of the cell even more compared to water bath. Water bath is not effective - the primary cell wall is tough to decompose as it is mainly made up of cellulose [41]. Cellulose decomposes at 500℃ [42]. The temperature used for water bath is not high to have an impact on the cell wall. The temperature could only disrupt the structure of the cell wall which allows the contents to escape. The secondary cell wall is made up of highly cross-linked phenolic molecules [41]. Hence, phenolic content, flavonoid content and DPPH scavenging activity for water bath extraction method is significantly lower than ultrasonic assisted extraction.

When compared between Table 1 to Table 6 for solvents, it can be seen that 80% methanol is the most efficient solvent in order to extract active compounds from the plants. The highest phenolic and flavonoid content are obtained when the plants were extracted with 80% methanol followed by 80% ethanol and finally water. Solvent do have a significant effect on the total phenolic content and the total flavonoid content for both water bath extraction method and ultrasonic assisted extraction method. The results for the effect of the solvent on total

phenolic content is p=0.014 (α<0.05) proving that solvents do affect the quantity of phytochemicals extracted. As for total flavonoid content, the results obtained from ANOVA two way is p=0.004 (α<0.05) also proving that solvents do affect the quantity of phytochemicals extracted. For DPPH scavenging, the results obtained from ANOVA- two way is p=0.076 (α<0.05). The result shows that the solvents do not affect the DPPH scavenging properties of each plant extract.

3.1. Hot water extraction

From Table 1, 2 and 3, by using ANOVA two-ways ($\propto = 0.05$), it is found that individual plants affect phenolic content, flavonoid content and DPPH scavenging where p = 0.001. It is also found that solvents are insignificant with phenolic recovery content (p = 0.089). As for flavonoid content and DPPH scavenging, p =0.003 which indicates the significance of solvent.

Table 1. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using water as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	6.159	3.984	9.556
Andrographis paniculata	90.546	42.519	34.812
Ficus deltoidea	96.224	22.123	87.031

Table 2. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using 80% methanol as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	33.17	18.209	31.399
Andrographis paniculata	104.989	74.959	93.174
Ficus deltoidea	225.917	90.237	93.515

Table 3. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using 80% ethanol as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	29.603	24.004	17.065
Andrographis paniculata	125.908	92.870	80.887
Ficus deltoidea	264.765	99.871	89.761

3.2. Ultrasonic assisted extraction

From both method of extractions, it is calculated that the method of extraction for phenolic recovery is insignificant where the value of p is 0.222. Method of extraction is also insignificant for flavonoid recovery (p=0.187) and DPPH scavenging (p=0.428). Although the method of extraction is calculated to be insignificant, but high values of phenolic, flavonoid and DPPH scavenging activity is necessary. Hence, it can be deduced that ultrasonic assisted extraction is suitable for recovery of phenolic and flavonoids.

Table 4. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using water as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	24.405	6.468	8.873
Andrographis paniculata	134.972	84.141	9.215
Ficus deltoidea	275.822	61.185	83.618

Table 5. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using 80% methanol as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	72.019	25.434	75.768
Andrographis paniculata	159.377	108.226	86.007
Ficus deltoidea	287.476	106.699	91.809

Table 6. Total phenolic content, total flavonoid content and DPPH scavenging activity of each plant using 80% ethanol as solvent for extraction.

Plants	Total phenolic content (mg GAE/100 g DW)	Total flavonoid content (mg CE/100 g DW)	DPPH scavenging activity (%)
Morinda citrifolia	72.119	47.863	50.512
Andrographis paniculata	171.429	95.431	88.396
Ficus deltoidea	298.832	132.612	90.102

3.3. Freeze drying

Ficus deltoidea which was extracted using ultrasonic extraction using 80% methanol has the highest scavenging activity. Hence, it was subjected to freeze drying. It was found that the scavenging activity increased from 91.91% to 93.62% after freeze drying. Using t-test ($\alpha = 0.05$), it is established that freeze

drying has a significant effect on scavenging activity ($p = 0.002817$). This proves that freeze drying will be a suitable preservation method for plant extracts. By using freeze drying, it will preserve the quality of phenolics and flavonoids in the plant better as compared to not using freeze drying.

4. Conclusion

As a conclusion, the objectives of this study are to measure phenolic content, flavonoid content and DPPH scavenging activity of *Andrographis paniculata, Ficus deltoidea* and *Morinda citrifolia*. The extraction methods – water bath method and ultrasonic assisted extraction used are different to observe the efficiency of each method in extracting the phytochemicals from the leaves. Different solvents are used in this study to extract the leaves – 80% ethanol, 80% methanol and water. From the study, it is found that the ultrasonic assisted extraction is a better method to extract compared to water bath extraction. In addition to that, the best solvent to extract the leaves would be 80% ethanol. The best plant to use as a remedy for diabetes among these three plants would *Ficus deltoidea*.

The finding from this study would be useful as the comparison is done for three different plants and two different methods of extractions. The solvents used for extraction are also different.. By using the proper method of extraction and proper binary solvent system, the pharmaceutical industry can maximize the extraction of the leaves thus increasing the phenolic content and flavonoid content and subsequently increasing the scavenging ability of each plant.

There are a few recommendations for the future work of this study. The study should be further improved by adding more chemical analysis. Sulforhodamine B (SRB) Cytotoxicity Assay should be carried for the plant extracts to observe the efficiency of the extracts on human cells. This would properly show the level of effectiveness of phenolics and flavonoids. Superoxide scavenging activity should also be carried out to further study the effect of phenolics on superoxide anion. In mitochondria, 3% of oxygen content is completely changed into superoxide anion which is a major source for free radicals. Hence testing of phenolics and flavonoids on superoxide anion should be carried out.

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