Comparisons between the Antioxidant Activities of the Extracts of *Anacardium occidentale* and *Piper betle*

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Abstract

In this study, the antioxidant capacities of extracts of *Anacardium occidentale* were compared with those of *Piper betle.* Antioxidant activities were evaluated based on their ability to reduce ferric ions and scavenge DPPH·, $ABTS^{\cdot+}$ and nitric oxide radicals. Total phenolic content of these plants were measured by the Folin-Ciocalteau method. The plants were extracted with methanol, ethyl acetate and hexane. With the exception of the ethyl acetate and hexane extracts of *A. occidentale*, the other extracts showed significant radical scavenging and reducing properties. The ethyl acetate extract of *P. betle* contained the highest total phenolic content, showed the highest ferric reducing and DPPH· scavenging activities. However, the methanol extract of *A. occidentale* was potent at scavenging the $ABTS^{\cdot+}$ and nitric oxide radicals. Total phenolic content of the plant extracts showed close correlation with the antioxidant activities, suggesting that phenolic compounds present in the plants may be a major contributor of the observed antioxidant activities. In conclusion, the methanol extract of *A. occidentale* can be an alternative source of polyphenolics with potent antioxidant activities.

Keywords: Antioxidant activity, polyphenolics, *Anacardium occidentale, Piper betle,* radical scavenging, reducing activity.

Introduction

Research on natural products has gained a wide popularity due to the potential of discovering active compounds that may be the magic bullet towards curing or preventing certain conditions and diseases. In addition, plants have been used for thousands of years as alternatives to modern medicine. However, many of their uses remain anecdotal. The antioxidant properties contained in plants have been proposed as one of the mechanisms for the observed beneficial effect. Antioxidants such as the vitamins A, C and E and polyphenolic compounds such as flavonoids and resveratrol have been demonstrated to possess potent antioxidant activities and able to prevent oxidative damage, both *in vitro* and *in vivo* [1]. These antioxidant compounds are present in abundance in plants, hence promoting the research into potential use of antioxidants from natural sources. In view of this, numerous plants with claimed medicinal properties have been analysed for their antioxidant properties. Malaysia has a diverse flora and the potential for drug discovery of many of these plants remain largely unknown.

Numerous locally consumed plants and herbs have been screened for their antioxidant properties. Of these, *Piper betle* extracts have been demonstrated to possess significantly potent antioxidant properties [2, 3]. Another local plant that is less researched and less well-known is the shoots of *Anacardium occidentale,* or locally known as 'pucuk gajus'. The shoots of this plant are commonly consumed either raw or boiled as accompaniments to the main meal. *A. occidentale* has been used to treat various ailments including malaria and yellow fever [4] as well as diarrhea [5]. Although the antioxidant properties of the fruit of this plant had been measured, no data is available on the antioxidant activities of the shoots of this plant. In view of the potential of the shoots of *A. occidentale* to be a source of natural antioxidants, it was the aim of this study to measure the antioxidant activities of various extracts of the shoots of *A. occidentale* and to compare their activities to *P. betle.*

Materials and Methods

Chemicals

All reagents and solvents used in the experiments were of analytical grade and obtained from the usual suppliers. Water used was of Millipore quality.

Preparation and extraction of plant extracts

The shoots of *A. occidentale* and *P. betle* were purchased from the local wet market. The samples were washed and left to dry in an oven at 40˚C. The dried samples were ground to powder form and stored at -20°C until further analysis.

The powdered shoots were extracted with methanol, ethyl acetate and hexane at room temperature for 24 h at a ratio of 1:20 (g:ml). The extracts were evaporated to dryness on the rotary evaporator and the residues were re-dissolved in 10% DMSO. Extracts were kept at -20°C until the antioxidant analyses.

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Total phenolic analysis

Analysis for total phenolic content was performed based on the Folin-Ciocalteau method [6]. Gallic acid was used for the calibration curve and a concentration range of 50-200 mg/l was prepared. Results were expressed as mg gallic acid equivalents/g dried plant material. All experiments were done in triplicate.

Ferric reducing activity

The ferric reducing activity of the plant extracts was estimated based on the ferric reducing ability of plasma (FRAP) assay developed by Benzie and Strain [7]. The ferric reducing activity was estimated by measuring the change in absorbance in the 0 to 4 min time reaction and was related to the absorbance changes of a Fe (II) standard solution tested in parallel. All results were based on three experiments and results were expressed as mmole ferric reducing activity of the extracts per g of dried weight. Quercetin and rutin were used as positive controls.

$ABTS^{\cdot+}$ radical scavenging activity

Determination of the 2,2'-azinobis-3-ethylbenzothiazoline -6-sulphonic acid ($ABTS^{\cdot+}$) radical scavenging effect of the plant extracts was performed according to the method of Re *et al.* [8] with some modifications. $ABTS^{\cdot+}$ radical was initially generated by reacting 7 mM $ABTS^{\cdot+}$ solution in water with 2.45 mM potassium persulfate in the dark for 12-16 h. The $ABTS^{\cdot+}$ radical scavenging activity of the extracts was measured using a Trolox standard curve and results were expressed as mmol Trolox equivalent antioxidant capacity per g dried plant sample. All determinations were performed in triplicate. Quercetin and rutin were used as positive controls.

DPPH· radical scavenging activity

Radical scavenging activities by antioxidants in the plant extracts were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radicals [9]. The DPPH· radical scavenging activity of the extracts was measured using a Trolox standard curve and results were expressed as mmol Trolox equivalent antioxidant capacity per g dried plant sample. All determinations were performed in triplicate. Quercetin and rutin were used as positive controls.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined based on the method by Rai *et al.* [10]. The nitric oxide scavenging activity of the extracts was estimated using a Trolox standard curve and results were expressed as mmol Trolox equivalent antioxidant capacity per g dried plant sample. Quercetin and rutin were used as positive control. All determinations were performed in triplicates.

Results and Discussion

Total phenolic content

The Folin-Ciocalteau assay is a relatively simple method and is widely used for estimation of polyphenolic content of plants. Polyphenolics are secondary plant metabolites and in addition to their various function in plants, some of these compounds act as anti-inflammatory, anti-microbial and anti-cancer agents in humans [11]. Polyphenolics in plants also have the potential to prevent oxidative-damage related diseases including cancer and atherosclerosis [12].

The highest total phenolic content was observed in the ethyl acetate extract of *P. betle* whereas the ethyl acetate extract of *A. occidentale* showed the lowest total phenolic content (Table 1). When total phenolic content of the plant extracts was related to the antioxidant activities, it showed a close correlation between the two whereby extracts with high total phenolic content demonstrated high antioxidant activities. Polyphenolics in plants have been shown to contribute towards the antioxidant activities of plants [13, 14] and as these compounds are ubiquitously found in plants, they can be important natural sources of potential antioxidants. The total phenolic content of our plant extracts was much higher when compared to several Thai indigenous plants (highest total phenolic content was 180.5 ± 1.3 mg GAE/g dried weight), suggesting these local plants can be a good source of polyphenolics [15].

Antioxidant activities

Antioxidants prevent oxidative damage caused by free radicals in several ways. These include by chelating metal ions, by scavenging free radicals through donation of hydrogen/electron, by inhibiting enzymes that produce free radicals such as cyclooxygenase and lipooxygenase or by stimulating the expression of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase. Various antioxidant assays have been developed for determination of the antioxidant capabilities of plants. These *in vitro* assays provided a useful measure of the potential of plants to act as antioxidants *in vivo.* In this study, the ability of antioxidants in the plant extracts to act as reducing agents and as radical scavengers was tested.

Ferric reducing activity

This assay measures the ability of antioxidants in plants to prevent reduction of ferric ions. Antioxidants present in the plant samples act as reductants while the reagent, containing excess ferric ions act as the oxidants. Formation of blue colour in the reaction was measured at a wavelength of 593 nm over a 4 minute time reaction. The high intensity of the blue colour indicates the high presence of reductants, hence high amount of antioxidants.

A dose-response curve was plotted for the plant extracts and positive controls. With exception of the ethyl acetate and hexane extracts of *A. occidentale*, the other extracts showed a linear relationship (Figure 1), indicating their reducing capacities increases with concentration. The ferric reducing activity of the plant extracts was compared with a standard curve of $FeSO_4.7H_2O$ (Table 1). The methanol extract of *A. occidentale* showed the highest ferric reducing activity compared to the ethyl acetate and hexane extracts of the same plant, approximately four to seven fold higher. In contrast, all three extracts of *P. betle* demonstrated considerable ferric reducing capabilities, with the ethyl acetate extracts having the strongest activity. When reducing activity was compared to positive controls, it showed quercetin to be much more potent than the plant extracts whereas the ferric reducing activity of rutin was almost similar to the ethyl acetate extract of *P. betle.*

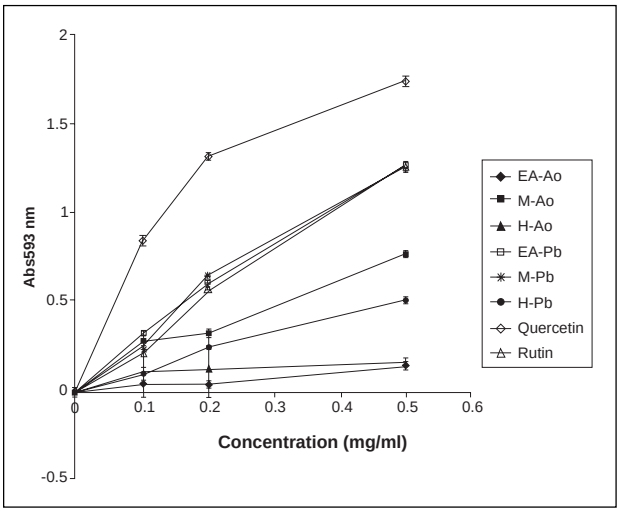

Figure 1: Dose response of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *Piper betle* in the ferric reducing activity assay. The ferric reducing activity assay was conducted over the concentration range of 0.1-0.5 mg/ml and the reducing capabilities of the methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle* were measured at an absorbance of 593 nm. Results are average of three readings ± S.D. EA-Ao: ethyl acetate *(A. occidentale)*; M-Ao: methanol *(A. occidentale)*; H-Ao: hexane *(A. occidentale)*; EA-Pb: ethyl acetate *(P. betle)*; M-Pb: methanol *(P. betle)*; H-Pb: hexane *(P. betle)*.

Gallic acid and ferrulic acid have been reported to be present in the leaves of *A. occidentale*, suggesting that these polyphenolics may contribute towards the ferric reducing activity of this plant [16].

$ABTS^{\cdot+}$ radical scavenging activity

This assay measured the ability of antioxidants in the plant extracts to scavenge the synthetic free radical,

Table 1: Total phenolic content and antioxidant activities of the methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle*.[a]

Extracts / Antioxidant Activities	Total Phenolic Content (mg GAE/g)[b]	Ferric Reducing Activity (mmol/g)	DPPH Radical Scavenging Activity (mmol Trolox/g)	ABTS Radical Scavenging Activity (mmol Trolox/g)	Nitric Oxide Scavenging Activity (mmol Trolox/g)
A. occidentale (methanol)	264.29 ± 31.28	4.25 ± 0.23	1.59 ± 0.02	2.11 ± 0.02	2.72 ± 0.06
A. occidentale (ethyl acetate)	69.97± 9.23	0.58 ± 0.06	0.53 ± 0.006	0.46 ± 0.01	0.70 ± 0.18
A. occidentale (hexane)	118.51 ± 4.93	0.88 ± 0.1	1.24 ± 0.02	0.51 ± 0.006	0.63 ± 0.10
Piper betle (methanol)	441.01 ± 65.04	4.64 ± 0.16	1.63 ± 0.006	1.95 ± 0.04	2.52 ± 0.26
Piper betle (ethyl acetate)	596.6 ± 43.42	5.15 ± 0.02	1.69 ± 0.02	1.99 ± 0.05	2.6 ± 0.04
Piper betle (hexane)	263.97 ± 3.51	2.6 ± 0.05	1.21 ± 0.01	0.87 ± 0.01	1.1 ± 0.28
Quercetin	-	12.35 ± 0.04	4.02 ± 0.01	2.09 ± 0.01	3.2 ± 0.05
Rutin	-	5.81 ± 0.07	3.79 ± 0.29	1.4 ± 0.04	3.31 ± 0.08

[a] Results expressed were average of triplicate ± S.D.
[b] The total phenolic content was expressed as mg gallic acid equivalent (GAE) in 1 g of dried weight ± S.D.
- Not measured.

$ABTS^{\cdot+}$, which was generated by reacting $ABTS^{\cdot+}$ stock solution with potassium persulfate. Scavenging of the $ABTS^{\cdot+}$ radicals by antioxidants caused a decrease in absorbance which was measured at 734 nm. The activity of the plant extracts and positive controls was expressed as Trolox equivalent antioxidant capacity (TEAC).

Of all the three extracts of *A. occidentale*, only the methanol extract showed significant radical scavenging activity while the ethyl acetate and hexane extracts had minimal activities (Table 1). The methanol and ethyl acetate extracts of *P. betle* had similar radical scavenging activities and these values were comparable to the methanol extract of *A. occidentale*. When the ABTS·+ radical scavenging activities of the plant extracts were compared to positive controls, the methanol extract of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle* were shown to have higher scavenging activities than rutin, and similar scavenging activities as quercetin, suggesting potency of these extracts.

A graph was plotted on the percentage inhibition of the $ABTS^{\cdot+}$ radicals with increasing concentration of the plant extracts (Figure 2). At the highest concentration, the methanol extract of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle* exhibited almost 100% inhibition of the radicals, similar to quercetin and rutin, further demonstrating the potency of these three extracts. The percentage inhibition of the $ABTS^{\cdot+}$ radicals by these three extracts had almost leveled off at the highest concentration tested whereas this was not seen with the other extracts.

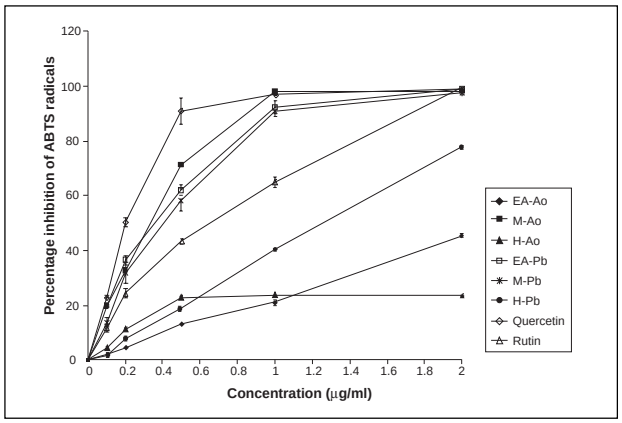

Figure 2: $ABTS^{\cdot+}$ radical scavenging capacity of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle*. The radical scavenging ability of varying concentrations (0-2000 µg/ml) of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle* was analysed based on their inhibitory effects on the $ABTS^{\cdot+}$ radicals at 734 nm the absorbance. Analyses were performed in triplicates and results are expressed as % inhibition of the absorbance of $ABTS^{\cdot+}$ radicals ± S.D. EA-Ao: ethyl acetate *(A. occidentale)*; M-Ao: methanol *(A. occidentale)*; H-Ao: hexane *(A. occidentale)*; EA-Pb: ethyl acetate *(P. betle)*; M-Pb: methanol *(P. betle)*; H-Pb: hexane *(P. betle)*.

DPPH· radical scavenging activity

This assay measures the ability of antioxidants in the plant extracts to scavenge the synthetic radical, DPPH·. Scavenging occurs by donation of hydrogen atom or electron by the antioxidants to DPPH·. The radical scavenging activities of the plants were expressed as TEAC. The methanol and hexane extracts of *A. occidentale* showed considerable activities although much lower than quercetin and rutin (Table 1). All three extracts of *P. betle* were equally able to scavenge DPPH· radicals with TEAC values that were similar to the methanol extract of *A. occidentale.* Dasgupta and Re [2] measured the DPPH· scavenging activities of *P. betle* extracted with boiling water. The percentage inhibition obtained was comparable to our ethyl acetate and methanol extracts.

Figure 3 shows the percentage inhibition of the DPPH· radicals plotted against concentrations of the plant extracts. The methanol extract of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle* showed more than 50% inhibition of the radicals at the highest concentration. Calculation of the IC_{50} (concentration in µg/ml that caused 50% inhibition of DPPH· radicals) showed values of between 73-84 µg/ml for the methanol extract of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle.* These values were lower when compared to IC_{50} values of several culinary herbs and spices such as basil, ginger and cumin indicating our plants are better radical scavengers [17].

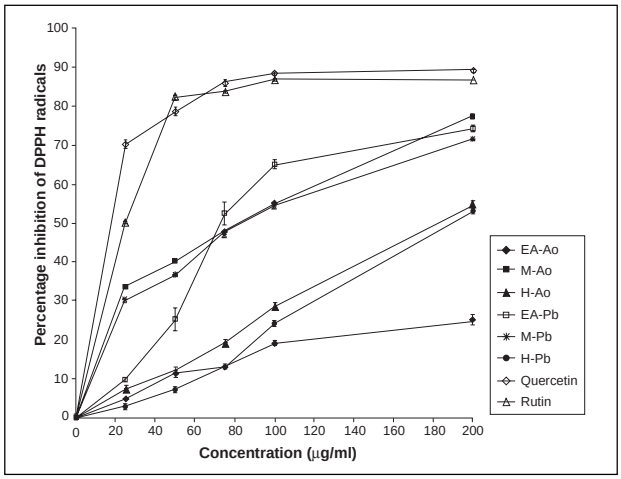

Figure 3: DPPH· radical scavenging capacity of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle*. The radical scavenging ability of varying concentrations (0-200 µg/ml) of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle* was analysed by measuring their inhibitory effects on the absorbance of the DPPH· radicals at 517 nm. Reaction was performed in triplicates and results are expressed as % inhibition of the absorbance of the DPPH· radicals ± S.D. EA-Ao: ethyl acetate *(A. occidentale)*; M-Ao: methanol *(A. occidentale)* ; H-Ao: hexane *(A. occidentale)*; EA-Pb: ethyl acetate *(P. betle)*; M-Pb: methanol *(P. betle)*; H-Pb: hexane *(P. betle)*.

Nitric oxide radical scavenging activity

Nitric oxide is a physiological messenger but excess amounts can cause oxidative damage. Nguyen *et al* [18] reported DNA damage and mutations in a human lymphoblastoic cell line exposed to nitric oxide in the presence of oxygen. In this assay, nitric oxide was generated from sodium nitroprusside and reacted with oxygen to produce nitrite ions. Antioxidants in the plant samples acted as scavengers of nitric oxide, thus producing less nitrite ions which can be measured using Griess reagent.

The methanol extract of *A. occidentale* had the highest nitric oxide radical scavenging activity (approximately, four fold higher) compared to the other two extracts of the same plant (Table 1). The methanol and ethyl acetate extracts of *P. betle* had similar scavenging activities as the methanol extract of *A. occidentale* whereas the hexane extract was less reactive. The nitric oxide scavenging capacities of the methanol extracts of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle* were comparable to quercetin and rutin, albeit slightly lower. This observation indicates the potential of these plants to protect cells and tissues from damage caused by nitric oxide *in vivo.*

There was a steady increase in the percentage inhibition of nitric oxide by the extracts up to a concentration of 100 μg/ml, after which there was a leveling off (Figure 4). At the highest concentration, the methanol extract of *A. occidentale* and the methanol and ethyl acetate extracts of *P. betle* inhibited more than 60% of nitric oxide whereas the other extracts only showed less than 30% inhibition.

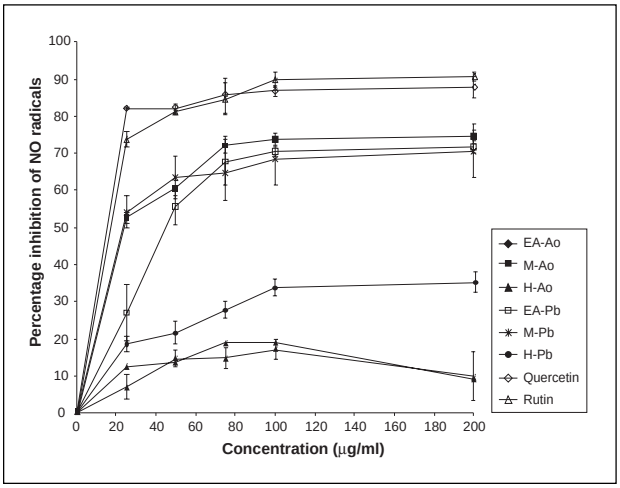

Figure 4: Percentage inhibition of nitric oxide radicals with different concentration of extracts of *A. occidentale* and *P. betle.* The radical scavenging ability of varying concentrations (0-200 μg/ml) of methanol, ethyl acetate and hexane extracts of *A. occidentale* and *P. betle* were analysed by measuring their inhibitory effects on the absorbance of the nitric oxide reaction product at 540 nm. Reaction was performed in triplicate and results are expressed as % inhibition of the absorbance of nitric oxide reaction product ± S.D. EA-Ao: ethyl acetate *(A. occidentale)*; M-Ao: methanol *(A. occidentale)*; H-Ao: hexane *(A. occidentale)*; EA-Pb: ethyl acetate *(P. betle)*; M-Pb: methanol *(P. betle)*; H-Pb: hexane *(P. betle)*.

Conclusion

Results obtained from the various antioxidant assays revealed that the antioxidant activities of the methanol extract of *A. occidentale* are comparable to the extracts of *P. betle*, suggesting that *A. occidentale* could be used as a source of antioxidant polyphenolics. The mostly low antioxidant activities observed in the ethyl acetate and hexane extracts of *A. occidentale*, despite the presence of polyphenolics, suggest that the antioxidants or polyphenolics present in these extracts are weak radical scavengers and reducing agents. Further chemical and pharmacological studies on this plant are needed to further ascertain its potential benefits.

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