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Phytochemical Screening, *In vitro* and *In vivo* Antioxidant Activities of Aqueous Extract of *Anacardium occidentale* Linn. and its Effects on Endogenous Antioxidant Enzymes in Hypercholesterolemic Induced Rabbits

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Abstract: Oxidative stress has been shown to play important role in the development of various diseases. In this study, researchers investigated the existence of phytochemical constituents of *Anacardium occidentale* Linn. (AO) leaf and evaluate its *in vitro* and *in vivo* antioxidant activities in aqueous extract form. Phytochemical screening of AO was performed according to the standard method while *in vitro* antioxidant activities were performed via DDPH free radical scavenging and Ferric reducing antioxidant power assay. *In vivo* antioxidant activities were evaluated in hypercholesterolemic induced adult male New Zealand white rabbits. Phenolic, flavonoids, steroids and triterpenes were found in the leaf of AO. The freeze dried aqueous extract showed no significant different compared to BHT in *in vitro* antioxidant analysis when assessed using the FRAP assay. Supplementation of aqueous extract of AO (100, 200 mg/kg/day) to the hypercholesterolemic induced rabbits caused a significant decreased ($p<0.05$) of malondialdehyde and significant increased ($p<0.05$) of superoxide dismutase and catalase levels at the end of the study period compared to the groups received high cholesterol diet alone. Aqueous extract of AO possess the ability to act as an antioxidant *in vitro* and *in vivo* and also was able to increase the level of superoxide dismutase and catalase in experimental hypercholesterolemia. The presence of flavonoids in the extract could be attributed to the antioxidative effect of the plant.

Key words: *Anacardium occidentale*, antioxidant enzymes, flavonoids, DPPH, FRAP, Malaysia

INTRODUCTION

Hypercholesterolemia diet was reported to bring remarkable modifications in the antioxidant defense system (Fki *et al*., 2005) thus causing oxidative stress, the disturbances of the delicate balance between oxidants and antioxidants (Mazor *et al*., 1997). The consumption of a cholesterol enriched diet also has been documented to increases the degree of lipid peroxidation (Hakimoglu *et al*., 2007). However under normal conditions, the production of reactive oxygen species will be quickly eliminated by antioxidant defense mechanisms to prevent such event (Hsu *et al*., 2001). The antioxidant enzymes such Catalase (CAT), Glutathione Peroxidase (GPx) and the Superoxide Dismutase (SOD) was proposed to act as a 1st line of defense system to intercept the free radicals interaction (Rashtchizadeh *et al*., 2008).

Anacardium occidentale Linn. (AO), one of the commonly consumed traditional vegetables among Malays and indigenous community in Malaysia is a tree native to Brazil that is presently cultivated in many regions of the world including Malaysia. As reported in phytochemical tests, cashew leaves contain various flavonoids, mainly quercetin glycosides. Recently, two studies have reported the antioxidant activities of the leaves of AO (Roach *et al*., 2003; Abas *et al*., 2006). This present research examines the possibility of aqueous extract of AO to act as antioxidant agent by determining its antioxidative properties *in vitro* and *in vivo*.

MATERIALS AND METHODS

Collection and identification of plant materials: Fresh leaves of *Anacardium occidentale* Linn. were collected from Kelantan, Malaysia. The leaves were identified and authenticated by a plant taxonomist in Institute of Bioscience, Universiti Putra Malaysia (Voucher Specimen No: SK233)

Extract preparation: The methanol and chloroform extract of *Anacardium occidentale* Linn. were prepared by soaking 100 g of powdered leaves in 1000 mL methanol

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and 1000 mL chloroform, respectively at room temperature for 48 h. The aqueous extract was prepared by soaking 100 g of the powdered leaves in 1000 mL distilled water and incubated in shaking water bath at temperature of 60°C and incubation time of 6 h. Following extraction, the all extracts were filtered and the supernatant were concentrated using a rotary evaporator with the water bath set at 40°C for methanol and chloroform extract while the aqueous extract was subjected to freeze dried. The methanol and chloroform extract were kept in a desiccator while the freeze dried extract was kept in dark air tight container at -20°C until further used. The freeze dried powder was mixed with water (100 mg in 1 mL water) to dissolve it before administrated to the rabbits.

Phytochemical analysis: The presence of possible phytochemicals constituents in the extract were evaluated qualitatively (alkaloids, saponins, steroid and triterpenoids, tannins, flavonoid).

Test for alkaloids: About 1 mL of chloroform extract of AO added with 1% of hydrochloric acid on steam bath and then filterated. About 1 mL filtrate then was added with 6 drops of Mayer's reagent. Appearance of cream white precipitate indicated the presence of alkaloids (Harborne, 1996).

Test for saponins: About 1 mL of methanol extract of AO added with 9 mL of distilled water was shaken vigorously and appearance of stable froth for at least 15 min indicated the presence of saponins (Harborne, 1996).

Test for steroids and triterpenoids: About 2 mL of chloroform extract of AO added was with 1 mL of chloroform and a few drops of acetic anhydride and a few drops of concentrated sulfuric acid. Appearance of blue or green color indicated the presence of steroids and appearance of red, brown color indicates the presence of triterpenoids (Harborne, 1996).

Test for tannins: The methanolic extract of AO was mixed with a few drops of 1% ferric chloride solution. Formation of blue-black colour indicates the presence of hydrolysable tannins while the formation of blue-green precipitate indicates the presence of condensed tannins (Trease and Evans, 2002).

Test for flavonoids: A portion of the methanolic extract of AO was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids (Sofowora, 1982).

Test for phenolic content: About 200 μL (0.2 mg mL^{-1}) aqueous extract solution was mixed with 0.75 mL 10-fold diluted Folin-ciocalteu reagent. Following 5 min of incubation, 0.75 mL of 6% Na_2CO_3 solution was added and the mixture was allowed to stand for 90 min at room temperature. Brown colour indicates the presence of phenolic compounds (Velioglu *et al*., 1998).

***In vitro* antioxidant analysis**

DPPH free radical scavenging: The potential antioxidant activity of the aqueous extracts of AO was assessed on the basis of scavenging activity of the stable 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical according to the previous described procedure (Yen and Hsieh, 1998). For the control, 1 mL of 0.45 mM DPPH is added into 0.5 mL absolute ethanol. For the sample, 1 mL of 0.45 mM DPPH were added with 0.5 mL extract (5 mL). The step is repeated by replacing extract with BHT (5 mg mL^{-1}). All samples were incubated for 30 min and following incubation, absorbance is read at 517 nm. The percentage of inhibition of the sample against DPPH radicals was calculated from the following equation:

$$\text{Inhibition (\%)} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{\text{Absorbance of control}} \times 100$$

Ferric Reducing Antioxidant Power (FRAP): The ferric reducing ability of *A. occidentale* Linn. aqueous extract was evaluated following the method described by Benzie and Strain (1996). About 100 μL of sample (studied extracts) were mixed with 300 mL distilled water and 3 mL of FRAP reagent (2.5 mL of 10 mM of 2, 4, 6-Tris (2-Pyridyl)-1, 3, 5-Triazine (TPTZ) solution in 40 mM HCl added with 2.5 mL of 20 mM $FeCl_3$ added with 25 mL of 0.3 M acetate buffer, pH 3.6) was mixed. Absorbance was read at 593 nm. The final results were expressed as the concentration of antioxidants having ferric reducing ability equivalent to that of 1 mM $FeSO_4$, particularly expressed in milimolar per liter.

Animals and experimental protocol: Total 35 healthy adult male New Zealand White rabbits weighting between 1.8 and 2.0 kg were used in the experiments (East Asia Rabbits Corporation Sdn Bhd). The animals were randomly housed in an individual cage with free access to food and water in standard conditions of lighting, temperature and humidity for 2 weeks for acclimatization. Following acclimatization, the rabbits were divided into five groups (n = 7) and were fed accordingly; Normal Control group (NC) rabbits was fed the standard diet, atherogenic rabbits group (PC) was fed the standard diet

enriched with 0.5% cholesterol, Simvastatin group (SC) rabbits was fed the standard diet enriched with 0.5% cholesterol with 10 mg/kg/day simvastatin, treatment groups (AOE100, AOE200) were fed the standard diet enriched with 0.5% cholesterol with different doses of water extract of AO (100, 200 mg/kg/day). Animals were fasted for 12 h before venous blood samples were collected at week 0, 4 and 8th. At the end of the experimental period, the animals were then sacrificed via exsanguinations and aorta was collected for histological study. The animals handling procedure in this study was approved in strict accordance with the Animal Care and Use Committee of Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM) Serdang, Selangor.

Endogenous antioxidant enzymes analysis: There are four antioxidant enzymes evaluated; Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), Total Antioxidant Status (TAS) and Catalase (CAT). Quantitative determination of SOD, GPx and TAS were done using commercial kits from Randox (UK) while Catalase from Cayman's (Canada).

Measurement of lipid peroxidation index malondialdehyde: The lipid peroxidation index is measured using Thiobarbituric Acid (TBA) test on Malondialdehyde (MDA) according to method described by Ledwozyw *et al*. (1986) and determination of protein concentration in the sample was based on the method of Lowry protein assay (Lowry *et al*., 1951).

Statistical analysis: The data obtained were analyzed using the Statistical Package for Social Science (SPSS) program Version 15. After confirming the normality of data and the homogeneity of variance of data, the significance of the differences between means of the tests and control studies was established by one-way Analysis of Variance (ANOVA) coupled with post hoc Tukey HSD for multiple group comparison and $p<0.05$ was used to denote of statistically significant. Results are expressed as a mean±SD.

RESULTS AND DISCUSSION

Phytochemical analysis: Phytochemical study of AO revealed the presence of phenolic, flavonoids, steroids and triterpenes while other constituents such as alkaloids, saponins and tannins were not detected (Table 1). Several studies have reported a significant correlation between antioxidant activities present in some tropical vegetables with their total phenolic content (Maisutthisakul *et al*., 2008). Study by Kogel and Zech (1985) reported the presence of several phenolic acids in the leaves of AO, mainly gallic acid, protocatechuic acid, p-hydroxybenzoic acid, cinnamic acid, p-coumaric acid and ferulic acid. Flavonoids and tannins are phenolic compounds, a major group of compounds that act as primary antioxidants or free radical scavengers. They also posses redox properties therefore, allowing them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Pietta *et al*., 1998) and to some extent, metal chelator (Kumarasamy *et al*., 2004). Other researchers, Luzzi and Maiani (1999) suggested that excess flavonoids can be stored in body tissues and mobilized in response to physiological requirements.

***In vitro* antioxidant analysis:** As a complex matrix of interacting factors therefore, it has become more accepted to evaluate the antioxidant capacity of foods to give an index of healthiness (Van Boekel and Jongen, 1997). One of widely used techniques to assess the antioxidant capacity of plants is DPPH free radical scavenging activity. In this technique, the radical scavenging abilities and the reducing potentials of the antioxidant constituent of plant extract towards the stable radical DPPH was evaluated (Sanchez-Moreno, 2002). Previous study indicated that methanol extract of AO with IC_{50} value of 72 μg dried extract mL^{-1} were able to inhibit the formation of DPPH radicals with a percentage inhibition of 90.7+0.2% at the concentration of 400 μg mL^{-1} (Razali *et al*., 2008). The study showed that the percentage of inhibition of aqueous extract of *A. occidentale* L. against the DPPH radicals is 78.325+0.241% (Table 2). The reducing ability of *A. occidentale* L. aqueous extracts against the ferric ion which acts as the oxidants is shown in Table 2.

Table 1: Phyto-chemical analysis

Constituents	Results
Alkaloids	ND
Saponins	ND
Flavonoids	+++
Tannins	ND
Triterpenes	+
Steroids	++
Phenolic	+++

ND = Not Detected; + = Weak colour; ++ = Mild colour; +++ = Strong colour

Table 2: *In vitro* antioxidant evaluation

Extracts	Values
DPPH free radical scavenging	
Freeze dried aqueous extract (%)	78.325±0.241[a]
BHT (%)	91.893±1.792[b]
Ferric reducing antioxidant power	
Freeze dried aqueous extract (mmol L^{-1})	1.811±0.089[a]
BHT (mmol L^{-1})	1.977±0.005[a]

Each value represents the mean±SD. The same alphabet are not significantly different ($p<0.05$) within groups

The assay shows that Butylated Hydroxytoluene (BHT) which acts as standard had the highest antioxidant activity of 1.977±0.005 mmol L^{-1}. Freeze dried extract of AO demonstrated FRAP value of 1.811±0.089 mmol L^{-1} with no significant different denoted between BHT and the extract. Hence, the reductive ability of the extracts suggests that the extracts were able to donate electron therefore suggesting that they may be able to donate electrons to free radicals in actual biological systems. Previous study demonstrated that in the leaves itself, pure gallic acid and ferullic acid which were detected previously (Kogel and Zech, 1985) and were found to be able to reduce ferric ions when assessed by FRAP assay (Soobrattee *et al*., 2005) thus suggesting that the results obtained may have partly been contributed by these phenolics.

***In vivo* antioxidant analysis:** It is previously reported that the GPx activity is decreased while CAT activity is increased (Prasad and Kalra, 1993: Mahfouz *et al*., 1997) in hypercholesterolemic induced rabbits compared to control group at the end of the study period. However, Mahfouz *et al*. (1997) does not observe any significant change in the SOD activity which contradicted with that of Prasad and Kalra (1993). The present study revealed no significant changes in GPx and SOD activity but significant increased of catalase activity of blood from cholesterol-fed rabbits (Table 3), contradicted to what has been reported. The differences could be due to the duration of the study period and the amount of cholesterol loaded.

Supplementation of *Anacardium occidenlate* Linn. (AO) in hypercholesterolemia event of all doses, however did not improved the level of GPx activity in cholesterol-fed rabbits at the end of the study period compared to PC group. However, SOD and catalase levels at the end of study period were significantly higher ($p<0.05$) compared to PC group. The decreased in GPx level is possibly due to its increased utilization in combating excessive erythrocytes oxidative stress in hypercholesterolemic rabbits. The decreased in GPx would lower the erythrocyte capacity to deal with H_2O_2 and possibly lead to an increased in catalase activity as an adaptation process (Mahfouz *et al*., 1997). Flavonoids compounds have the ability to decrease oxidative stress by promoting the cellular consumption of glutathione by inactivating selenium-dependent glutathione peroxidase (Cai *et al*., 1997) and increase the expression of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Rohrdanz *et al*., 2002).

Lipid peroxidation index malondialdehyde analysis: Malondialdehyde (MDA), one of end product of lipid peroxidation has become the principal and the most studied product of polyunsaturated fatty acid peroxidation (Del Rio *et al*., 2005). The MDA level in plasma in PC group at the end of the study period was significantly higher than other groups (Table 4). The increased of MDA in cholesterol-fed rabbits agrees with previous reports (Mahfouz *et al*., 1997). In AO treatment groups, MDA level in plasma significantly low ($p<0.05$)

Table 3: Comparison of *in vivo* antioxidant enzymes levels at week 0, 4 and 8th of experimental period

	Groups				
Bio-chemical factors	NC	PC	SC	AOE 100	AOE 200
SOD (U L^{-1})					
w = 0	2.65±0.0300^{a,1}	2.66±0.0300^{a,1}	2.61±0.0400^{a,1}	6.74±0.300^{b,1}	6.70±0.260^{b,1}
w = 4	2.65±0.0100^{c,1}	2.69±0.0200^{c,1}	2.69±0.0400^{c,1}	1.32±0.060^{d,2}	1.33±0.050^{d,2}
w = 8	4.48±0.5400^{e,2}	5.02±0.1400^{e,2}	3.45±0.0800^{f,2}	9.73±0.240^{g,3}	9.93±0.300^{g,3}
GPx (U L^{-1})					
w = 0	657.68±52.880^{a,1}	409.47±46.760^{b,1}	432.88±41.140^{b,1}	670.01±14.85^{a,c,1}	698.10±22.380^{a,}
w = 4	634.21±16.390^{d,1}	824.02±103.17^{d,e,2}	825.37±4.0000^{e,f,g,2}	732.90±33.47^{d,e,f,2}	895.77±37.410^{g,}
w = 8	699.57±102.40^{h,1}	827.96±79.370^{h,2}	922.00±38.720^{i,3}	404.99±13.36^{i,3}	477.37±52.750^{i,3}
CAT (nmol/min/mL)					
w = 0	0.026±0.004^{a,1}	0.025±0.004^{a,1}	0.027±0.004^{a,1}	0.028±0.004^{a,1}	0.026±0.004^{a,1}
w = 4	0.030±0.002^{b,1}	0.037±0.004^{c,2}	0.032±0.002^{b,c,1}	0.030±0.004^{b,1}	0.030±0.003^{b,c,1}
w = 8	0.026±0.003^{d,1}	0.040±0.003^{e,2}	0.027±0.004^{d,1}	0.060±0.004^{f,2}	0.071±0.004^{g,2}

Table 4: Comparison of lipid peroxidation index: TBARs levels at week 0, 4 and 8th of experimental period

	Groups				
Bio-chemical factors	NC	PC	SC	AOE 100	AOE 200
MDA (nmol mg^{-1} protein)					
w = 0	0.776±0.078^{a,1}	0.722±0.040^{a,1}	0.786±0.065^{a,1}	0.728±0.021^{a,1}	0.792±0.037^{a,1}
w = 4	0.834±0.055^{b,1}	1.771±0.121^{c,2}	1.025±0.088^{b,2}	1.174±0.211^{b,2}	1.069±0.446^{c,1}
w = 8	0.745±0.041^{d,1}	1.348±0.323^{e,2}	0.919±0.072^{d,e,1,2}	1.057±0.341^{d,e,1,2}	0.727±0.073^{d,1}

Each value represents the mean±SD; the same alphabet are not significantly different ($p<0.05$) between groups at specific time interval; the same number are not significant different ($p<0.05$) within groups

compared to PC group at the end of the study period. It was previously demonstrated that the addition of free radical scavengers to a cholesterol supplemented diet significantly decreased the plasma lipid peroxidation products (Matz *et al*., 1994). Flavonoids can potentially prevent free radical related injury such as lipid peroxidation as they exhibit powerful antioxidant activities *in vitro*. They are able to scavenge a wide range of reactive oxygen, nitrogen and chlorine species (Santos and Mira, 2004; Sadeghipour *et al*., 2005) as well as being able to inhibit the production of such reactive species (Selloum *et al*., 2001).

CONCLUSION

The results showed the ability of aqueous extract of AO to act as antioxidant *in vitro* demonstrated by the high FRAP value and the high percentage of inhibition of DPPH free radical and *in vivo* demonstrated by the low MDA level. The study also found that the extract was able to increase the level of certain antioxidant enzymes activity such SOD and catalase thereby suggesting the potential beneficial usage of the plant to treat various diseases related to the oxidative stress. The presence of flavonoids compounds could be attributed for such effects.

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