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Toxicity Screening and Hypocholesterolemic Effect Evaluation of Aqueous Extract of *Anacardium occidentale* Linn. in Hypercholesterolemic Induced Rabbits

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Abstract

Previous findings have supported to the ethnopharmacological use of *Anacardium occidentale* Linn. in folk medicine. In this study, the toxicity properties and the hypocholesterolemic effect of aqueous extract of *Anacardium occidentale* Linn. were evaluated in hypercholesterolemic induced rabbits.

Thirty Five male New Zealand White Rabbits were randomly assigned into five groups and fed with normal diet (NC), 0.5% high cholesterol diet (PC), 0.5% high cholesterol diet+10 mg/kg simvastatin (SC), 0.5% high cholesterol diet+100 mg/kg AOE (AOE100) and 0.5% high cholesterol diet+200 mg/kg AOE (AOE200). The study duration was set for 12 weeks. In vitro toxicity study has been performed using brine shrimp lethality test and MTT assay to determine the LC_{50} and IC_{50} values respectively while in vivo toxicity study has been evaluated in hypercholesterolemic induced rabbits. Blood samples were withdrawn at week 0 and 12.

Supplementation of 0.5% high cholesterol diet caused the elevation of TC, LDL and TG and also significantly rise ($p<0.05$) the level of liver enzymes compared to the normal control group. For in vitro toxicity screening, extracts demonstrated very low LC_{50} values and no IC_{50} value detected. For in vivo hypercholesterolemic induced rabbits, extracts were able to prevent the increment of liver enzymes: gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase compared to positive control group.

Aqueous extract of AO found to be not toxic and posses hypocholesterolemic and hepatoprotective effects in hypercholesteromic induced rabbits.

Introduction

Recently, there has been a resurgence of interest in herbal medicines capable of reducing and/or regulating serum cholesterol and triglycerides levels [1]. *Anacardium occidentale* Linn. (AO), a tree native to Brazil is presently cultivated in many regions of the world including Malaysia. Various parts of the tree have been submitted to chemical and pharmalogical screenings [2]. As reported in phytochemical tests, cashew leaves

contain various flavonoids, mainly quercetin glycosides. It is demonstrated that quercetin was able to act as antioxidant by inhibiting the lipid peroxidation process [3].

Although the incidence of side effects from natural products is relatively low, they are not entirely free of serious risks [4]. Hence to mitigate these risks, screening and monitoring for hepatic injury were put in place to assess the degree of damage to the tissue or whole body [5]. Several tests have been used to assess hepatic function or to monitor the progression of hepatic injury. The most general teats include the measurement of serum liver enzymes names AST, ALT and ALP [6] and GGT [7]. However none of these tests can individually confirm liver dysfunction as they often abnormal in the event of clinical problems other than liver dysfunction [8].

To date, there is no literature documented on the toxicity properties and hypocholesterolemic effect of the aqueous extract of AO in the event of hypercholesterolemia. Therefore, this study examines its toxicity properties in vitro via brine shrimp lethality test and MTT assay and in vivo by measuring some metabolic enzymes normally used as markers for liver functions and its hypocholesterolemic effects in hypercholesterolemic induced rabbits.

Materials and Method

Collection and Identification of Plant Materials

Fresh leaves of *Anacardium occidentale* Linn. were collected from Kelantan, Malaysia in January 2008. The leaves were identified and authenticated by a plant taxonomist in Institute of Bioscience, Universiti Putra Malaysia (Voucher Specimen Number: SK233)

Extract Preparation

The aqueous extract of AO 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 hours. Following extraction, the extract were filtered and the supernatant was subjected to freeze dried and was kept in the 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.

In vitro toxicity screening: Brine Shrimp Lethality Test

The procedure of brine shrimp lethality test was modified from the assay described by Solis (1993)[9]. Brine shrimp eggs (Artemia salina) obtained locally were hatched in artificial sea water (25 g/l) for 24 hours. Following 24 hours of hatching, 10 larvae were collected and transferred into 24-microwell plate followed by adding 200 µl (final volume 1 ml) of various concentration (0 – 1000 µg/ml) of extract. Following 24 hours of incubation, the number of death of larvae is counted with aid of stereo microscope and the lethal concentration (LC_{50}) was counted.

In vitro toxicity screening: MTT Assay

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from American Type Culture Collection (ATCC, Rockvilled, MD, USA). The cells were maintained in a T-25 flasks (Nunc, Roskilde, Denmark) containing M200 medium and Low Serum Growth Supplement (LSGS) (Cascade Biologies Inc, Sweden) in a humidified atmosphere of air with 5% CO2 at 37ºC and routinely subcultured in every two days as described by Chen (2004)[10] with slight modification. Uniform monolayers from the primary culture were formed after 6-8 days and TE (trypsin/EDTA) solution (a sterile, phosphate buffered saline solution containing 0.025% trypsin and 0.01% EDTA, with pH of 7.2 at room temperature) was used to harvest cells. Only 3rd-5th passage were used in the experiment.

In order to evaluate the toxicity potential of AO on HUVEC, firstly the cells (1 x 10^6 cells per well) were seeded in 96-well plates and incubated for 24 h. Following incubation, AO at concentration of 100, 200, 300, 400, 500, 600 and 700 µM is added and incubated for the MTT assay as previously described by Takahashi (2002)[11]. In brief, the cultures were washed with PBS, 20 µL of 5 mg mL^{-1} of MTT solution was added and the cells were incubated for 4 h. After that, the media were removed, 50 µL of Dimethyl Sulphoxide (DMSO) was added to each well. Absorbance at 570 nm was determined by a microplate ELISA reader (Grodig, Austria). The percent of cell viability was calculated according to the formula below:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance of experimental group}}{\text{Absorbance of blank control group}}$$

Animals and Experimental Protocol

Thirty Five healthy adult male New Zealand White Rabbits weighing 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 two 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 hours before venous blood samples were collected at week 0 and 12. 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, Universiti Putra Malaysia (UPM) Serdang, Selangor.

Lipid Profiles

Analysis of lipid profiles includes measuring of Total Cholesterol (TC), Triglycerides (TG), High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) using Roche Commercial Kits. All plasma samples were evaluated using Hitachi Chemistry Analyzer at the Pathological Chemistry Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All tests utilize the principle of enzymatic colorimetric assay.

In vivo toxicity screening: Liver Function Test

Analysis of liver function includes measuring of GGT, ALP, AST, ALT using Roche Commercial Kits. All plasma samples were evaluated using Hitachi Chemistry Analyzer at the Pathological Chemistry Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All tests utilize the principle of enzymatic colorimetric assay.

Result

In vitro toxicity screening: Brine Shrimp Lethality Test and MTT Assay

Aqueous extracts of *A. occidentale* L. with various concentration ranging from 0–1000 µg/ml and 0–700 µg/ml were tested in this toxicity screening using brine shrimp lethality test and MTT assay respectively and the results were shown in Table 1. The LC_{50} value is 226.67-$\pm$ 2.52 µg/ml while the IC_{50} value is not detected.

Lipid profiles

Compared with the normal diet, the high-cholesterol diet for 12 weeks produced a significant elevation ($p<0.05$) of all biochemical factors measured from rabbits (Table 2). The oral administration of simvastatin (10 mg/kg/day) and aqueous extract of *A. occidentale* (100 and 200 mg/kg/day) caused a significant decline ($p<0.05$)

in plasma level of TC, TG, LDL and MDA at week 12 when compared to PC group.

In vivo toxicity screening: Liver Function Test
Supplementation 0.5% enriched high-cholesterol diet for 12 weeks produced a significant elevation ($p<0.05$) of all biochemical factors measured from rabbits (Table 3). The oral administration of simvastatin (10 mg/kg/day) and aqueous extract of *A. occidentale* (100 and 200 mg/kg/day) caused a significant decline ($p<0.05$) in plasma level of GGT, ALP, ALT and AST at week 12 when compared to PC group.

Discussion

In vitro toxicity screening of AO extracted at 60ºC and 6 hours incubation time was evaluated using the brine shrimp lethality test [12] and MTTT assay [11]. From brine shrimp lethality test, the extract exhibited very low toxicity properties as the LC_{50} value was greater than 100 g/ml [13]. Furthermore from MTT assay, incubation of HUVEC with AO at all concentration range for 24 hours was showed to be not toxic as any cell death observed. Therefore no IC_{50} value was obtained. Previous toxicity study by Tedong (2007)[14] on hexane extract of AO shown that the LD_{50} of the extract in mice of both sexes after oral administration was 16 g/kg.

Our study indicated that feeding rabibits with 0.5% high cholesterol diet increase the TC level in circulating blood, similar as demonstrated in the previous study [15]. The TC level in SC group significantly dropped 4.54 times at the end of the study period compared to the previous week. The reduction of TC level upon the supplementation of simvastatin has also been found in the previous study [16] thereby justifying the usage of statin in the treatment of hypercholesterolemia [17]. It was observed a slight increased of TC level recorded at week 4 compared to week 0 in all treatment groups with AO. The slight increased of TC level in all treatment groups compared to significant increased of TC level in PC group proves that AO can suppress the accumulation of cholesterol in plasma as demonstrated by Bassumillik (1994)[18] with the usage of guava pulp. The continuous increment of TC level in all treatment groups (AOE100 and AOE200) at week 8 from week 4 and at week 12 from week 8 was found to be similar as previously demonstrated [19].

Triglyceride level in PC group was found to be significantly high than the baseline level and is the highest compared to other groups at the end of the study period, similar with a previous study [20] but contradicted with other studies [19]. Our study showed that supplementation of AO to the high cholesterol diet fed rabbits resulted with a significant decreased of TG level against PC group at the end of study period. The excessive load of cholesterol to the liver above the acceptable level of its normal process causes the system to be unable in metabolising the lipids hence resulting in high cholesterol return in the form of LDL in the circulating blood [21]. In all AO treatment groups, LDL level was found to be significantly lower than PC at week 12.

Hypercholesterolemia was well documented to induce the production of free radicals [22] which later lead to the oxidative damage to biomolecules such as lipids, DNA, and proteins [23]. Oxidative damage is considered to be involved in the pathogenesis of liver damage [24], responsible for cell membrane damage and consequent release of marker enzymes of hepatotoxicity. Elevation of ALP, ALT and AST levels reflects the severity of liver injury [25].

The present study revealed an increased in ALP level in hypercholesterolemia-induced rabbits, consistent with the previous report [26]. Increased in the activity of ALP in blood might be due to the necrosis of liver [27,28]. Current findings also showed that GGT and AST level increased while ALT level do not much change in hypercholesterolemia-induced rabbits, partly consistent with the study done by Murata (2003)[29] who demonstrated that the level of AST, ALT and GGT among human subjects

increases as the number of components of metabolic syndrome increased.

Supplementation of 100 and 200 mg/kg/day AO to the 0.5% high cholesterol diet-fed rabbits demonstrated significantly low level of GGT, ALP and AST compared to PC group following 12 weeks experimental period. The presence of AO in the event of hypercholesterolemia alleviated its harmful effect on most all above measured parameters and the corrected level of these parameters were observed likely to near the normal values as the control, implying that the extract prevents liver damage. This finding revealed the hepatoprotective and safety of AO by causing no abnormalities of liver enzymes level.

In conclusion, the present results revealed that the aqueous extract of AO is found to be not toxic in vitro and in vivo and exerts a considerable hypocholesterolemic effects by demonstrating a significant decreased of TC, TG and LDL in hypercholesterolemic induced rabbits receiving AO. These findings could highlight the safety usage of AO as the hypocholesterolemic agent.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Authors' Agreement

All authors have made substantial contributions and final approval of the conceptions, drafting, and final version.

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