

Effect of *Garcinia Atroviridis* on Oxidative Stress and Atherosclerotic Changes in Experimental Guinea Pigs

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Abstract: Problem statement: Oxidative stress plays a major role in hypercholesterolemia induced atherosclerosis. *Garcinia atroviridis* has been shown to exhibit antioxidant properties. **Approach:** The present study aimed to investigate the effect of *Garcinia atroviridis* on the oxidative stress in guinea pigs fed with high cholesterol diet. Twenty-four male Guinea pigs were divided into four groups. Group I served as control while group II, III, IV were fed with 1% cholesterol diet in rabbit chow pellet, fed with both 1% cholesterol diet plus 50 mg body⁻¹ weight of *Garcinia atroviridis*, fed with *Garcinia atroviridis* only (50 mg mL⁻¹ water body⁻¹ weight), respectively. All animals were sacrificed by cardiac puncture and the blood was collected for the determination of malonaldehyde and DNA damage using comet assay and histology of the aorta was performed. **Results:** The results showed a significant increase in the percentage of DNA damage in the tail and in the tail moment (comet assay) in the group II compared to the group I. Group III showed pattern of reduction in the percentage of DNA damage by 42% and tail moment by 50% compared to the group II. There were no significant changes in the MDA levels in serum, liver and heart in all groups. Histological studies in group III showed a tendency in the reduction of the fat deposition in the aorta and number of foam cells compared to the group II. **Conclusion:** The supplementation with *Garcinia atroviridis* reduced the DNA damage and deposition of lipids in the wall of the aorta in hypercholesteromic guinea pigs.

Key words: Oxidative stress, *Garcinia atroviridis*, extract, atherosclerosis, aorta, histology

INTRODUCTION

Oxidative Stress (OS) is a term used to describe the steady state level of oxidative damage in cells, tissues, or organs caused by the Reactive Oxygen Species (ROS). It is imposed on cells as a result of an increase in oxidant generation, a decrease in antioxidant protection and a failure to repair oxidative damage. Oxidative Stress (OS) occurs when antioxidant defense mechanisms are overwhelmed by free radicals either due to increased free radical formation or decreased antioxidant capacity (Sohal and Weindruch, 1996).

There is an increase in evidence which shows that atherosclerosis is associated with damage of the DNA of both the circulating cells as well as the cells of the vessel wall (Andreassi, 2006). Reactive oxygen species are the most likely agents inducing DNA damage in atherosclerosis. DNA damage produces a variety of responses, including cell senescence; apoptosis and DNA repair (Kapoor and Prasad, 1994).

Garcinia atroviridis is a medium sized fruit tree which belongs to the family Gutiferae. In Malaysia it is popularly known as 'asam gelugur' or 'asam keping'. The plant contains citric, tartaric and ascorbic acid which have antioxidant activities. However, the most important bioactive compound is Hydroxyl Citric Acid (HCA) (Seon-Min *et al.*, 2001). *Garcinia atroviridis* extract exhibit strong antioxidant, antimicrobial, antitumour and anti-inflammatory activities (Mackeen *et al.*, 2000). The present study aimed to investigate the effects of *Garcinia atroviridis* on oxidative stress and atherosclerosis in guinea pigs administered with high cholesterol diet.

MATERIALS AND METHODS

***Garcinia atroviridis* extract preparation:** *G. atroviridis* fruits were purchased from a local supplier and were identified by a pharmacognosy expert in the

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Department of Pharmacy, Faculty of Allied Science. *G. atroviridis* fruits were prepared by air-drying and cutting them into small pieces (600 g), which were successively extracted with methanol (99.8%) using a Soxhlet extractor. The resultant solution was filtered and dried using a rotatory evaporator in a water bath at a temperature not exceeding 50°C.

Animal studies: Twenty-four male Dunkin Hartley guinea pigs weighing between 700-1000g were used. The cholesterol and lipoprotein metabolism in guinea pigs have remarkable similarities to that of human metabolism (Fernandez, 2001). We followed the previous protocol where guinea pigs were used as a model of atherosclerosis (Toshi *et al.*, 1991).

Male Dunkin-Hartley guinea pigs (n = 24) were divided into four groups with six animals in each group (Table 1). Group I (control) consisted of animals fed with commercial rabbit chow. Group II were fed with 1% cholesterol diet in rabbit chow pellet only, to induce atherosclerosis (in 100 g of crushed rabbit chow pellet, 1 g of cholesterol powder was added and mixed with 50 mL of sterile distilled water and the mixture was made into pellet form and dried in an oven under 50°C overnight). Group III was fed with both 1% cholesterol diet plus 50 mg/body weight *Garcinia atroviridis*, while Group IV was fed with *Garcinia atroviridis* (50 mg mL⁻¹ water/body weight). All the treatment were given daily for 60 days. The diets were obtained from The Laboratory Animal Resource Unit Faculty of Medicine, University Kebangsaan Malaysia (UKM). The animals were housed in cages maintained under standard conditions (temperature 24°C, light/darkness cycles of 12 h) with free access to food and water, in addition to a twice weekly, vegetable diet of mustard leaves cabbage and carrot. *Garcinia atroviridis* was forced fed with a gastric lavage. The experiment was approved by the Animal Ethical Committee of University Kebangsaan Malaysia (UKM) and the animal care was given according to approved standards for laboratory animal care.

The animals were kept on their respective diets for 2 months. After two months of diet, the animals were fasted overnight in preparation for serum and tissue collection. In the morning, the animals were weighed, anesthetized by chloroform and the thoracic abdominal cavity was opened. Blood was collected by cardiac puncture and serum was separated by centrifugation at 3000 rpm for 5 min and serum was collected and kept in refrigerator at -76°C until needed. The heart together with the abdominal aorta (2-3 cm length) was excised from each animal. The aorta was cut at the origin and removed from the heart. A 2 mm section of the aorta of

each animal was soaked in a 10% (v/v) formal saline solution for H and E staining. The liver and heart were soaked in de-ionized water and homogenized for biochemical analysis.

Determination of malondialdehyde in serum, liver and heart:

Malondialdehyde levels in the supernatant and serum were measured as Thiobarbituric Acid Reactive substances (TBARs) according to a previously described method (Melli *et al.*, 2006; Ohkawa *et al.*, 1979). TBARs were extracted in a mixture of butanol:pyridine (15:1) which was separated by centrifugation. In brief, 0.5 ml each of sample was mixed with 2.5 mL of 20% Trichloroacetic Acid (TCA) (Sigma, Germany), vortexed and then left for 15 min. Then 1.5 mL of 0.2 g dL⁻¹ Thiobarbituric Acid (TBA) (Sigma, Germany) reagent was added and vortexed again. The mixture was heated in a boiling water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4ml of n-butyl alcohol and then vigorously vortexed for 4 min. The organic phase was separated by centrifugation at 3000 rpm for 10 min and absorbance was recorded at wavelength of 532 nm. A Malondialdehyde (MDA) solution made freshly by the hydrolysis of 1, 1, 3, 3-Tetramethoxypropane (TMP) (Sigma, Germany) was used as the standard. MDA was dissolved in 0.05 M sulphuric acid to prepare 10 mM stock solution. By diluting the stock solution, different concentrations (1-5 nmol mL⁻¹) of MDA were obtained in order to prepare a standard curve. Protein content of the sample was measured by Biuret method (Gornall *et al.*, 1949). The results are expressed as the nmol MDA mg⁻¹ protein.

Comet assay: The assay was performed immediately after blood withdrawal; in accordance with previously published methods (Lemay and Wood, 1999; Sun *et al.*, 2005) using the comet assay kit (Trevigen, Inc., Gaithersburg, MD) with some modification. The whole blood was taken and the blood cells were combined at a ratio of 1:10 with molten, low melting point agarose kept at 37°C. 75 µL of the mixture were immediately pipetted and evenly spread onto Comet slides that are specially coated with high melting point agarose.

Table 1: Experimental diet group each group contain six animals

Group	Diet/treatment
Group I	Control (rabbit chow diet)
Group II	Cholesterol diet (1% cholesterol in rabbit chow diet)
Group III	Cholesterol diet + <i>Garcinia atroviridis</i> (1% cholesterol diet supplemented with <i>Garcinia atroviridis</i> 50 mg body ⁻¹ weight)
Group IV	<i>Garcinia atroviridis</i> (rabbit chow diet supplement with <i>Garcinia atroviridis</i> 50 mg body ⁻¹ weight)

The slides were kept at 4°C for 30 min to allow the agarose to solidify and then immersed in prechilled lysis buffer solution for 60 min at 4°C. Subsequently, the slides were immersed into freshly prepared alkali solution (300 mM NaCl and 1 mM EDTA) for 20 min. The slides were then transferred to an electrophoresis chamber and fresh alkali solution was poured to sufficiently cover the slides. Electrophoresis was done at 300 mA, 27 V (constant voltage) for 20 min. Subsequently, the slides were rinsed in Tris-EDTA (TE) solution [10 mM Tris (pH 7.5) and 1 mM EDTA] and then fixed in 100% ice-cold ethanol for 5 min. The samples were air-dried at room temperature and the cells were viewed by staining with 50 µL of ethidium bromide. The blood cells were viewed under a fluorescence microscope (excitation/emission at 510/515 nm; Zeiss Corporation, USA) with camera axiocam MRC Zeiss, USA. The results were interpreted as quantitative analysis of DNA damage by counting one hundred comets on each slide and were analyzed using epifluorescence microscope equipped with 515 barrier filter and 560 emission filter with Computerized image analysis with software comet score (USA). The program was designed to differentiate comet head from tail and to measure a variety of parameters including tail length, %DNA in tail, comet area, head diameter, tail area and tail moment.

Histological analysis of aorta: The aorta was embedded in paraffin as the final process of making tissue blocks, which were then trimmed and sectioned with a microtome (Leica, Wetzlar, Germany) at room temperature to obtain 4 µm sections. The ribbons of sections floated in a 50°C water bath (leica bath H1210), were “fished” and mounted onto the poly-L-lysine-treated glass slides. The sections were then deparaffinised on a 60°C hot plate. This was followed by a hydration process, which included immersion in xylene for 2 min and a series of steps of decreasing ethanol concentrations beginning with absolute ethanol (2 min), 95% ethanol (2 min) and 80% ethanol (2 min). The slides were then dipped in 3% hydrogen peroxide for 15 min and then examined under a light microscope for observing the histology of the aorta. The sections were examined blindly using scoring system (Kannel *et al.*, 1979). The scores were given as 0 (if no lesion is detected), 1 (about 33% of lesion detected), 2 (about 33-66% of lesion detected) and 3 (above 66% of lesion detected).

The parameter values were all expressed as the mean ± SD. Normal distribution of all variables was

examined by Kolmogorov-Smirnov test. Significant differences among the groups were determined by one-way ANOVA using SPSS 12.0 software package program. The results were considered significant if the value is $p < 0.05$.

RESULTS

Serum and tissue MDA: The MDA content of the serum and tissue at 60 days from the four experimental groups were summarized in (Fig. 1). There were no significant differences between MDA in serum, liver and heart. There were no correlation between MDA in serum, liver or heart in the all groups.

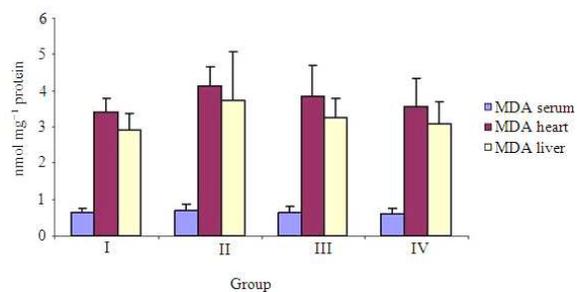


Fig. 1: MDA serum/heart/liver in different groups all result by mean ± SD

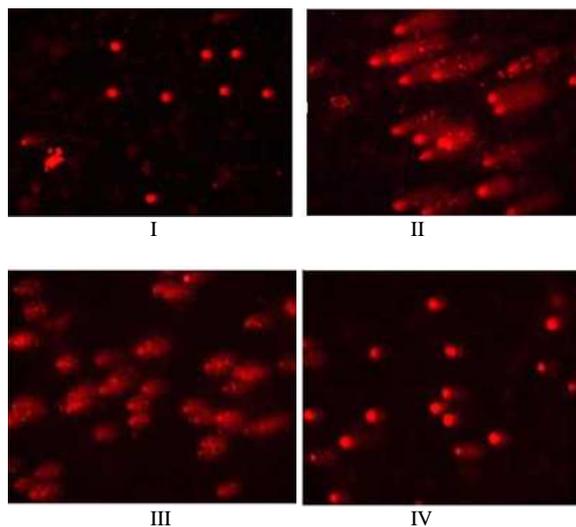


Fig. 2: Ethidium bromide-stained in Comet assay test in all group. Long tail with clear head Image of damaged cell. Comet image representative of damaged cell. After electrophoresis the stained DNA results in a Comet-like image

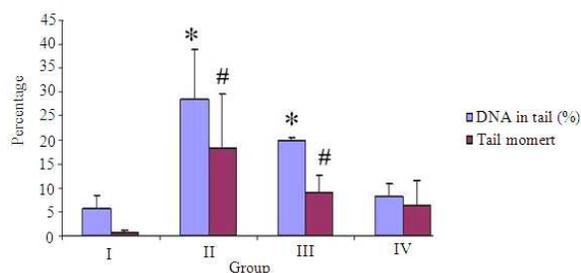


Fig. 3: DNA in tail (%) and Tail moment (comet assay) in the different groups. $p < 0.05$ compared to I and IV; $p < 0.01$ compared to I and IV

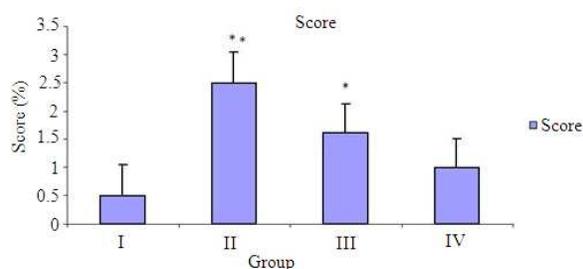


Fig. 4: Scoring for aorta histology in nt groups the values appears as mean \pm SD. **: $p < 0.01$ compared to I and IV; *: $p < 0.05$ compared to I and IV

Comet assay: The majority of cells examined by the alkaline comet assay had a normal morphology in group I (no tail or DNA damage) (Fig. 2). However, the DNA damage in tail was significantly higher in the group II compared with the group I. Each cell had the appearance of a comet, with a brightly fluorescent head and a tail with an intensity that was related to the amount of damage sustained by the cell. In the group III there was a trend of DNA damage reduction in tail comet which was insignificant compared to group II (Fig. 3).

Histological analysis: Although the group II and III both showed significant increase in the percentage score of atheromatous plaque compared to group I, but the G+C was showed a tendency to decrease the score by 56% compare to group II (Fig. 4).

DISCUSSION

Hypercholesterolemia has been documented as an important risk factor of atherosclerosis (Chen *et al.*, 1999). Cardiac morbidity and mortality are directly related to serum cholesterol level. The consumption of a cholesterol-enriched diet increases the degree of lipid peroxidation, which is also one of the early stages in the progression of atherosclerosis (Chen *et al.*, 1999). It has

been reported that some antioxidants can prevent atherosclerosis by protecting LDL from oxidation and are also associated with an anticholesterolemic effect (Freyschuss *et al.*, 2001; Ghasi *et al.*, 2000). Many plant extracts have been shown to exhibit hypocholesterolemic activity in guinea pigs and rabbits (Vasu *et al.*, 2005; Prasad, 1999).

In the present study, levels of serum and tissue MDA were elevated in guinea pig on high cholesterol diet and *Garcinia atroviridis* when administered only without cholesterol, reduced the serum and aortic MDA but it was insignificant. Increase in the level of MDA in tissue and serum in high cholesterol-fed have been reported earlier (Henrikssen *et al.*, 1987; Prasad and Lee, 2003; Nor Fadilah *et al.*, 2005). In the present study group IV reduced the serum, liver and heart tissue MDA by 10, 14 and 8% respectively as compared to the group II but the reduction was insignificant. *Garcinia atroviridis* contain hydroxycitric acid which has antioxidant activities which could reduce MDA level. In the present study, the concentration and duration of *Garcinia atroviridis* probably was not enough to reduce the MDA level, significantly.

This study showed that group III reduced DNA damage by 42 and 50% decrease in tail moment compared to group II but both were insignificant. Our results are in accordance with another study which reported that *Garcinia atroviridis* reduced the percentage of cells with DNA damage over a period of time (Andreassi and Nicoletta, 2003). Previous studies demonstrated that the DNA damage was significantly associated with the development and the progression of atherosclerosis (Amran *et al.*, 2009; Recep *et al.*, 2005). It was also demonstrated that DNA damage increased in patients with Coronary Artery Disease (CAD) (Singh *et al.*, 1988).

Histological study demonstrated that ethanol extract of *Garcinia atroviridis* had a tendency to reduce atherosclerotic lesions in the aorta. The descriptive results of the histological studies have been reported earlier (Wim *et al.*, 2002). Several epidemiological studies showed that increased dietary intake of natural antioxidants correlates to reduced coronary heart disease. Food rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancer and neurodegenerative disease (Gerber *et al.*, 2002; Di and Esposito, 2003). Antioxidants are known to alleviate oxidative stress which is generally perceived as one of the major causes for the accumulation of mutations in the genome.

CONCLUSION

In conclusion, supplementation of *Garcinia atroviridis* to hypercholesterolemic guinea pigs has a

tendency to reduce the progression of atherosclerosis by reducing the oxidative stress. Hence, in the future *Garcinia atroviridis* may be used as a supplement in the treatment of atherosclerosis.

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