Changes in the Markers of Atherosclerosis Following Administration of Belimbing Dayak (*Baccaurea Angulata)* Fruit Juice in Experimental Rabbits Fed with Cholesterol Diet

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Abstract**—**The aims of this present study were to investigate the potential antioxidant, anti-inflammatory and plaque-reducing activity of *Baccaurea angulata* (BA) fruit as a new anti-atherogenic plant. Twenty five male rabbits of New Zealand strain used were randomly assigned to five groups. Rabbits were fed either a standard chow diet (group N) or a high-cholesterol diet (groups CH, C1, C2 and C3). Groups C1, C2 and C3 were also given 0.5ml or 1ml or 1.5ml/kg/day BA whole fruit juice respectively. BA juice had high antioxidant activities indicated by the increase in the SOD activity and total antioxidant capacity (TAC), when comparing the C1, C2 and C3 groups with group CH. In the group CH, there was statistically significant increase in IL-8 and IL-18 levels as compared to C1, C2 and C3 groups. Likewise, BA juice reduced plaque formation in rabbits' aorta. Therefore, BA juice is beneficial in preventing atherosclerosis.

Keywords**—**Antioxidant, anti-inflammatory, atherosclerosis, *Baccaurea angulata*.

I. Introduction

THE need for better and more available healthcare access is the main issue for most of the developing countries. Millions of people in developing countries die each year from diseases that have established cures, just because they lack access to the most basic essential healthcare services, including effective and reliable medicines that remain beyond their means. This is true in both the rapidly growing cities and in the rural areas [1].

More than half of the world's population is estimated by the World Health Organization (WHO) to have little or no access to essential health care needs. Apart from the access problem, many people are underinsured or may not get the care they need because they could not afford the present health care services [2]. Therefore, experimenting new and innovative therapeutic approaches are needed to tackle these major problems. Medicinal plants are known to have a wide array of potential therapeutic effects [3-4]. For the poor people, medicinal plants do not only provide affordable medicine or offer relief for a wide range of health needs, but also open rich employment opportunities.

Many traditional healing herbs and plant parts have been used as a remedy for several diseases and illnesses through much of human history, especially in the local communities [5-7]. A lot of people prefer medicinal herbs to drugs. According to WHO, around 80% of the population was estimated to rely mainly on plant based remedies for primary healthcare [8]. Most of these medically important plants are cultivated in home gardens, forests or habitat. Many countries have recently begun to raise interest on natural products to treat major conditions, such as, AIDS, CVDs and cancer. These medicinal plants have now advanced to a point that they continue to supplement limited public health facilities and the consequent increased demand due to the population expansion has put tremendous pressure on the natural supply. Several authors have highlighted the potential benefits of underutilized fruits to human health [9-13]. Hence, there is a need for more research efforts on more underutilized fruits to improve their utilization and nutrition, so as to serve as alternative treatments.

Baccaurea angulata (BA), locally known as 'belimbing dayak' or 'belimbing hutan', as one of the underutilized fruits is widely distributed in Borneo Island of Malaysia and several other regions of Indonesia [14]. The aims of this present study were to investigate the potential antioxidant, anti-

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inflammatory and plaque-reducing activity of *Baccaurea angulata* (BA) fruit as a new anti-atherogenic plant.

II. Materials And Methods

A. Materials

Superoxide dismutase (SOD) assay kit and total antioxidant capacity (TAC) assay kit were products of Abnova (Taipei City, Taiwan). Rabbit interleukin 8 (IL-8) ELISA kit and rabbit interleukin 18 (IL-18) ELISA kit were purchased from CUSABIO (Hubei Province, China). The aqueous solutions were prepared with quality ultracentrifuge water.

B. Fruits and Sample Preparation

Baccaurea angulata fruits were acquired from Bau, Sarawak, Malaysia. The fruits were collected directly from trees. Upon arrival, they were stored in –30°C until the moment of analysis. For juice preparation, everyday, only matured healthy looking fruits were chosen (without mechanical damages and bacterial infection), and juiced with an electric fruit juicer after a thorough cleaning with distilled water.

C. Animals and Diets

Twenty five male New Zealand white rabbits (9 to 10months old, 2.2 to 3.2kg) were purchased from A Sapphire Enterprise, Seri Kembangan, Selangor, Malaysia. The animals were individually housed in stainless-steel cages in an air-conditioned room (20 ± 1°C, 55 ± 5% humidity) with 12h light/dark cycle. After two weeks of acclimatization period, they were randomly divided into five groups:

Group CH: fed 1% cholesterol diet.

Group C1: fed 1% cholesterol diet + 0.5ml/kg/day BA whole fruit juice.

Group C2: fed 1% cholesterol diet + 1ml/kg/day BA whole fruit juice.

Group C3: fed 1% cholesterol diet + 1.5ml/kg/day BA whole fruit juice.

Group N: fed normal pellet.

Animals were fed diets at 50g/kg/day and allowed water ad libitum. Juices were administered orally. All procedures were approved by animal care and use committee of faculty of Medicine, International Islamic University Malaysia (IIUM) Kuantan campus, Pahang (ID NO. IREC 05; Meeting No. 4/2012).

D. Blood and Aorta Collection

Blood was collected prior to dietary treatment and at the end of the experiment (after the 12-week feeding period), from the marginal ear vein of unanaesthetized animals after being deprived of food for 12h. Serum was collected by centrifugation at 1800g and 4°C for 15min and stored at -80°C for subsequent antioxidant and anti-inflammatory assays. All rabbits were then sacrificed; their abdomens and chests were opened, the heart together with the aorta was excised from each rabbit. The aorta was cut at the origin and removed from the heart to access the atherosclerotic lesion area.

E. Effects of BA on Antioxidant Enzymes Activities

The activity of SOD was estimated spectrophotometrically using commercial assay kits. The SOD activity assay kit utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The total antioxidant capacity (TAC) Assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The net absorbance values of antioxidants are compared with a known uric acid standard curve. Absorbance values are proportional to the sample's total reductive capacity. The analyses were done according to the manufacturer's instructions.

F. Effects of BA on Inflammatory Biomarkers

The levels of plasma IL-8 and IL-18 were measured by specific ELISA kits according to the manufacturer's instructions (CUSABIO; Hubei Province, China). The assays employ the quantitative sandwich enzyme immunoassay technique. Antibodies specific for IL-8 and IL-18 have been pre-coated onto microplates. Standards and samples were pipetted into the wells and any IL-8 or IL-18 present is bound by the immobilized antibodies.

G. Analysis of Extent of Aortic Atherosclerosis

Aorta were rapidly dissected free from the root of aorta to the iliac bifurcation, washed out of blood using ice-cold (4°C) phosphate-buffered saline (pH = 7.4). Atherosclerotic plaque lesion was analyzed as described previously [15]. In brief, after as much of the external fatty layers and adherent connective tissue as possible were removed, aorta were opened longitudinally over the total length and stained with Sudan IV. Normal and lipid-containing atherosclerotic plaque were digitized. The areas covered by atherosclerotic lesions (sudanophilic lesions) on each aortic segment were quantified by using the image analysis software (ImageJ).

H. Statistical Analysis

Data were expressed as mean ± SD. The data were assessed by ANOVA using SPSS software (SAS institute, Cary, NC, USA) and the differences between the means were evaluated by Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

III. Results And Discussion

The purpose of this study was to evaluate the antioxidant, anti-inflammatory and plaque-reducing activity of BA fruit in hypercholesterolemic-fed rabbits. Oxidative stress is a physiological condition where there is a persistent imbalance

between the production of reactive oxygen species (ROS) and antioxidants [16-17]. Because of the potential harmful effects of ROS, which are thought to be involved in the development of many degenerative diseases, their excess must be promptly eliminated from cells by the variety of antioxidant defense mechanisms [18].

SODs are enzymes that catalyze the breakdown (dismutation) of superoxide (O_2^-) radicals into oxygen and hydrogen peroxide [19]. When disease is present, an imbalance between the amount of superoxide formed and the systemic manifestation of reactive oxygen species occurs. Muscoli *et al*. [20] reported that GM mice that lack SOD are more susceptible and responsive to the possible consequences of free radical damage and those that possess abundant SOD are resistant to cumulative damage done by free radicals. Furthermore, SOD has been shown to be protective in animal models of diseases, such as ischaemia-reperfusion injury in the heart, liver and brain [20]. In the animal model used, the hypercholesterolemic diet induced a significant decrease in serum SOD activity and recovered in rabbits fed cholesterol diet and BA fruit juice. As seen in table I, the mean serum SOD activity was significantly lower (P<0·05) in group CH than in groups C1, C2, C3 and N. These findings suggest that BA fruit juice may prevent superoxide related free radicals resulting from cholesterol.

The TAC Assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. The present study showed that TAC decreased in cholesterol-fed rabbits and recovered in rabbits fed both cholesterol and BA fruit juice. Groups C1, C2 and C3 had significantly higher (P<0·05) serum total antioxidant capacity than did group CH, and the capacities seen in groups C1, C2 and C3 were higher than that of group N (Table I). These results suggest that BA fruit juice may overcome the oxidative stress induced by cholesterol and may thus prevent atherosclerosis and other heart related diseases. There have been numerous studies that show the importance of antioxidants in protecting the population from the modern scourge of heart disease [21-22].

It has become abundantly clear that inflammation plays a key role in the development of atherosclerosis. The current study showed that treatment with BA fruit juice for 12 weeks, revealed a significant decrease in serum IL-8 and IL-18 production. Feeding cholesterol alone (group CH) resulted in significantly higher (P<0·05) serum IL-8 and IL-18 levels. Whereas, Groups C1, C2 and C3 had significantly lower (P<0·05) serum IL-8 and IL-18 than did group CH (Table II). These results suggest that BA fruit juice may prevent atherosclerosis by lowering serum levels of inflammatory biomarkers. Interestingly, there is a finding which showed that reducing inflammatory biomarkers can inhibit atherosclerosis independently of lowering plasma cholesterol levels [23].

TABLE I
SOD AND TAC IN CHOLESTEROL-FED RABBITS

Groups	SOD (%)*	TAC
CH	34.86±3.42[a]	125.46±8.75[a]
C1	71.03±8.80[b]	246.06±92.38[b]
C2	59.29±14.72[b]	236.28±54.78[b]
C3	69.07±16.67[b]	300.39±63.29[b]
N	80.06±14.57[b]	207.52±24.07[b]

* SOD Activity (% inhibition rate)
[a,b] Mean values within a column with unlike superscript letters are significantly different (P<0·05).

TABLE II
IL-8 AND IL-18 IN CHOLESTEROL-FED RABBITS

Groups	IL-8 (pg/ml)	IL-18 pg/ml)
CH	111.94±9.63[a]	27.06±1.15[a]
C1	50.00±5.46[b]	16.33±2.10[b]
C2	74.85±16.37[c]	17.84±4.70[b]
C3	69.07±19.39[b,c]	18.71±2.68[b]
N	56.47±7.38[b,c]	18.91±2.34[b]

[a,b] Mean values within a column with unlike superscript letters are significantly different (P<0·05).

Furthermore, BA fruit juice clearly reduced atherosclerosis progression in cholesterol fed rabbits, possibly as a consequence of the observed antioxidant and anti-inflammatory activities. The plaque-reducing activity of BA fruit juice was dose-dependent. In the C, C1, C2, C3 and N groups, 96%, 55%, 49%, 22% and 0% respectively of the entire aorta were covered with plaque (Fig. 1). These findings showed that BA fruit juice had modulating effects on atherosclerotic plaque formation in rabbits fed a high-cholesterol.

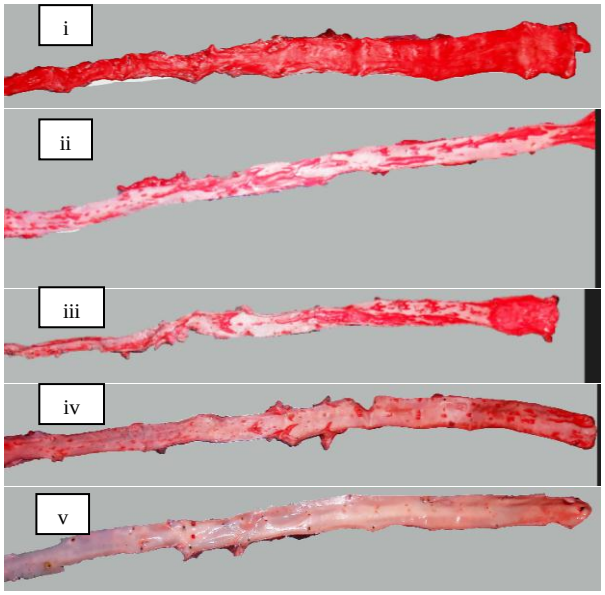

Fig. 1 Representative aortas stained with Sudan IV from group CH (i), group C1 (ii), group C2 (iii), group C3 (iv) and group N (v) rabbits are shown here.

IV. CONCLUSION

Our results show that treatment with BA fruit juice positively changed plasma antioxidant enzyme activities, inhibited inflammatory biomarkers and reduced

atherosclerotic plaque in cholesterol-fed rabbits, and thus could protect against oxidative stress linked atherosclerosis and decrease the atherogenic index.

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