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Gelam honey increases antioxidant enzyme activity in young rat cardiac mitochondria

Siti Maisarah Hasenan, Zulaikha Sahhugi and Zakiah Jubri*

Department of Biochemistry, The National University of Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia

QR Code

***Correspondence Info:**
Dr. Zakiah Jubri,
Department of Biochemistry,
The National University of Malaysia Medical Centre, Jalan Yaacob Latif,
Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia

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Abstract

Objective: The aim of this study is to determine the effect of gelam honey on antioxidant enzyme activity in cardiac mitochondria of young and aged rats.
Methods: Twenty-four male Spraque-Dawley rats were divided into young (2 months) and aged (19 months) groups. Each group was further divided into control (fed with plain water) and supplemented with 2.5 g/kg body weight of gelam honey for 8 months.
Results: Gelam honey increases cardiac manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) activities in young. Malondialdehyde (MDA) level was reduced in gelam honey supplemented young group. Gelam honey increases cytochrome oxidase activity in the young group compared to control.
Conclusions: Gelam honey reduces the oxidative damage and increase mitochondrial function by increasing antioxidant enzymes and cytochrome oxidase activity in the young group.
Keywords: Aging; Reactive oxygen species; Antioxidant enzymes; Oxidative damage; Gelam honey.

1. Introduction

Cardiac muscle requires a highly efficient energy production system to enable continuous pumping of the blood throughout the body. The main source of energy production within cell is mitochondrial oxidative phosphorylation which also producing various reactive oxygen species (ROS) [1]. The overproduction of ROS and low cellular level of antioxidant may rapidly oxidize important biomolecules such as DNA, protein and lipid. Thus, may result in cellular oxidative damage and cause mitochondrial dysfunction that further lead to cellular aging and disease.

Cardiac aging is due to decline in biochemical and physiological function and may cause cardiovascular diseases such as hypertension, coronary artery disease, hypertrophy, cardiomyopathy and heart failure. Many theories of aging have been proposed, but the most famous one is the free radical theory by Harman [2]. This theory proposing that aging is due to cumulative cellular damage caused by free radical. Previous studies in humans and animals have shown that aged hearts are more susceptible to stress and disease related challenge which lead to increased prevalence of heart failure and cardiovascular mortality in aged population [3].

Taking high antioxidant in the diet may help in clearing ROS and necessary for cell survival and to function normally. Antioxidant is found a lot in natural products and one of it is honey. Honey is a natural sweet substance derived from floral nectar and produced by honey bees [4]. It contains a variety of substances, including a mixture of sugars, vitamin, amino acids, mineral, protein, enzyme and phenolic compound [5].The presence of various constituentsis differing in different types of honey due to environmental factors, season, geographical floral origin and treatment of beekeepers [6].

There are several local honeys such as nenas,coconut, tualang and gelam honey. In this study, we focused on gelam honey because previous study reported that gelam honey has antioxidative effects due to high in flavonoids and phenolic contents as compared to other local honeys [7].Gelam honey is one of the Malaysian monofloral honey. It is produced by *Apis mellifera*beesfrom *Melaleuca cajuputi*tree nectar.The major phenolic compounds of gelam honey are caffeic acid, gallic acid, ferulic acid, chlorogenic acid, p-coumaric acid, ellagic acid, quercetin, chrysin, and hesperetin [8]. These polyphenols are reported is beneficial as a therapeutic purpose for a variety of degenerative diseases including diabetes, cardiovascular diseases and cancer [9-11]. Furthermore, these antioxidants have no side effects and more easily integrated into lifestyle changes as compared to conventional pharmaceutical drugs.

2. Materials and Methods

2.1 Gelam Honey

Gelam honey was obtained from the Department of Agriculture, Batu Pahat, Johor, Malaysia. Gelam honey was irradiated at the dosage of 25 kGy using ELORADO-8 with Cobalt-60 as its source for sterilization at Malaysian Nuclear Agency. The irradiated honey was kept in the dark place at room temperature.

2.2 Animal

A total of twenty-four male Sprague-Dawley rats were divided into two groups: 12 young rats (2 months old) with average weight 325 gramsand 12 aged rats (19 months old) with average weight 513 grams. Each group consisted of control groups (n=6) and treatment groups (n=6). The control group was force-fed with water 2.5 mL/kg body weight while treatment group was supplemented with 2.5 g/kg body weight of gelam honey for 8 months. The rats were obtained from Laboratory Animal Resource Unit, Faculty of Medicine, The National University of Malaysia. The experimental protocol was approved by The National University of Malaysia Animal Ethics Committee (FP/BIOK/2014/ZAKIAH/16-JULY/600-JULY-2014-JUNE-2016-NAR-CAT2).

2.3 Cytosol and mitochondria isolation

After 8 months of gelam honey treatment, rats were sacrificed. The cardiac was removed and washed with ice-cold 1.15% NaCl (Sigma, St Louis, USA) (pH 7.2) before immediately frozen in liquid nitrogen. The mitochondria isolation was following procedure from AMSBIO, UK. The tissue was weighed about 100-200 mg and minced into smaller pieces. The tissue was homogenized using Ultra Turrax T25 Homogenizer (IKA Labortechnik, Germany) and then centrifuged at 600 g for 10 minutes at 4°C using Bench Top Refrigerated Centrifuge Sorvall RC-5B. The supernatant was taken and centrifuged again at 12 000 g for 15 minutes at 4°C. The final supernatant and pellet was a cytosolic and mitochondria fraction respectively. The pellet was resuspended in isolation buffer and was centrifuged again at 600 g and 12 000 g for 10 and 15 minutes respectively to purify the mitochondria. All the procedures were carried out on the ice.

2.4 Manganese Superoxide dismutase activity assay

Manganese superoxide dismutase (MnSOD) activity was measured using a SOD assay kit (Cayman, USA) in 96-well plate. Briefly, serial dilutions of SOD stock solution were made to get a standard curve. For each standard solution, 10 µl were added in 200 µl diluted radical detectors in a designated standard well. Meanwhile, 10 µl samples were also mixed with 200 µl diluted radical detector in the sample well. The reaction was initiated by adding 20 µl diluted xanthine oxidase to all wells. After mixing for a few seconds, the plate was incubated for 20 minutes before the absorbance measured at 450 nm. This kit utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme required to exhibit 50% dismutation of superoxide radical.

2.5 Mitochondrial glutathione peroxidase activity assay

Mitochondrial glutathione peroxidase (GPx) activity was measured using GPx assay kit (Cayman, USA) in 96-well plate. Background well contained 120 µl assay buffer and 50 µl co-substrate mixture. While, positive control well contained 100 µl assay buffer, 50 µl co-substrate mixture and 20 µl diluted GPx (control). To sample well, 20 µl samples were added together with 100 µl assay buffer and 50 µl co-substrate mixture. The reaction was initiated by adding 0.02 ml of cumene hydroperoxide to all wells. The conversion of NADPH to $NADP^+$ was followed by measuring the change in O.D/min at 340 nm. Changes in absorbance (ΔA_{340}) were determined by plotting graph to obtain the slope (rate) of the linear portion of the curve. Rate of ΔA_{340}/min for the sample was subtracted with the background. One unit of GPx activity was defined as the amount of enzyme required to oxidize 1.0 nmol NADPH to $NADP^+$ per minute.

2.6 Level of cardiac malondialdehyde

Cardiac malonyldialdehyde (MDA) level was determined using high performance liquid chromatography (HPLC) with photodiode array detector (Shimadzu, Japan) as described by Pilz *et al* [12] with some modifications. Briefly, 50 µl samples were mixed with 200 µl of 1.3 M NaOH and incubated at 60°C for 30 min. After cooling the mixture, 100 µl of 35% $HClO_4$ was added and centrifuged at 10 000 g for 10 min. Supernatant of the samples (300 µl) was transferred into 1.5 ml of HPLC tubes and 5 mM of DNPH solution (50 µl) was added into the mixture and

incubated for 30 min at room temperature. Then, samples (40 µl) were injected into the HPLC. The amount of MDA was expressed as the concentration of MDA in nmol per ml cytosol.

2.7 Level of protein carbonylation in cardiac tissues

The level of protein carbonylation in cardiac tissues was determined using protein carbonyl colorimetric assay kit (Cayman, USA). These kits quantitate carbonyl content by reaction between DNPH and protein carbonyl, which formed a schiff base to produce hydrazone and analyzed by spectrophotometer. Briefly, 200 µl samples was mixed with 800 µl DNPH in a designated sample tube. While, control tube contained 200 µl samples with 800 µl 2.5 M HCI. Each tube was incubated for one hour before mixed with 1 ml 20% TCA. Then the tubes were centrifuged and the pellet was resuspended in 1 ml 10% TCA before centrifuge again at 10 000 x g for 10 minutes at 4^{o}C. Pellet obtained were washed in 1 ml ethanol/ethyl acetate mixture for 2 times and the final protein pellets were resuspended in 500 µl guanidine hydrochloride. Each tube was centrifuged again to remove any leftover debris. Finally, 220 µl of supernatant from each sample and control tube were measured at a wavelength of 370 nm.

2.8 Cytochrome oxidase activity in cardiac mitochondria tissues

Cytochrome oxidase activity in cardiac mitochondria tissues was measured using cytochrome oxidase activity colorimetric assay kit (Biovision, USA). Firstly, the efficiency of cytochrome c solution reduction should be tested. The absorbance of reduced cytochrome c must be in between 0.2-0.6. Samples, 10 µl s were added to a designated sample well. While, control well contained equal volume of enzyme dilution buffer. The reaction was initiated by adding 120 µl diluted cytochrome c to all wells. The absorbance was measured at 550 nm on the kinetic program over a period of 45 minutes at 5-minute interval. The rate of reaction was calculated by measuring the changes in OD/min using the maximum linear rate. One unit of cytochrome oxidase was defined as the amount of enzyme required to oxidize 1 µmole reduced cytochrome c per minute.

2.9 Statistical analyses

Data were reported as mean ± SEM. Differences between the experimental groups were analyzed using one-way ANOVA. Differences were considered to be statistically significant for $p<0.05$. All statistical analyses were carried out using SPSS for Windows version 20.0.

3. Results

3.1 Antioxidant enzyme activities

The activity of antioxidant enzymes, MnSOD and GPx wereshowed no changes in the young control compared to aged control group (Fig. 1). Gelam honey supplementation significantly increased the antioxidant enzyme activity (MnSOD, GPx) in the young groupcompared to controls, but no changes were observed in the aged group.

3.2 Level of cardiacmalondialdehyde and protein carbonyl

Malondialdehyde (MDA) level was increased in the young group compared to aged group. Gelam honey supplementation reduces the MDA level in the young group, but no changes were observed in the aged group when compared to their respective control group (Fig. 2A). The pattern of changes in MDA is also shown in protein carbonylwhen comparing young and aged group and also with the gelam honey supplementation, however there are no significant changes observed (Fig. 2B).

3.3 Cytochrome oxidaseactivity

The activity of cytochrome oxidase was increased in the aged groupcompared to young group.Gelam honey supplementation increased activity of cytochrome oxidase in young group, but no changes were observed in the aged group (Fig. 3).

Figure 1: The effects of gelam honey supplementation on antioxidant enzymes activities

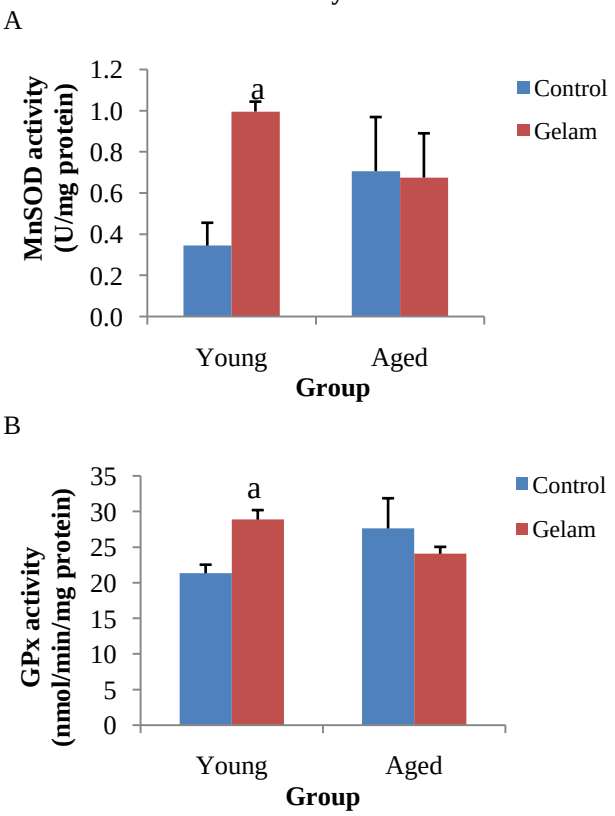
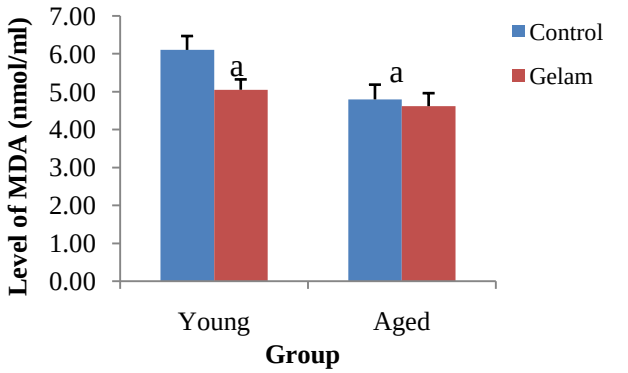


Figure 1 show the effects of gelam honey supplementation on MnSOD (A) and GPx(B) of young and aged groups. The results are expressed as mean ± SEM. a indicates significant difference compared to young control group ($p<0.05$).

Figure 2: The effects of gelam honey supplementation on MDA level and protein carbonyl

A



B

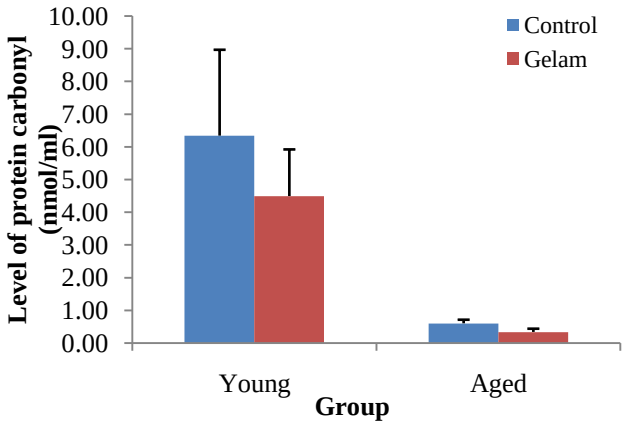
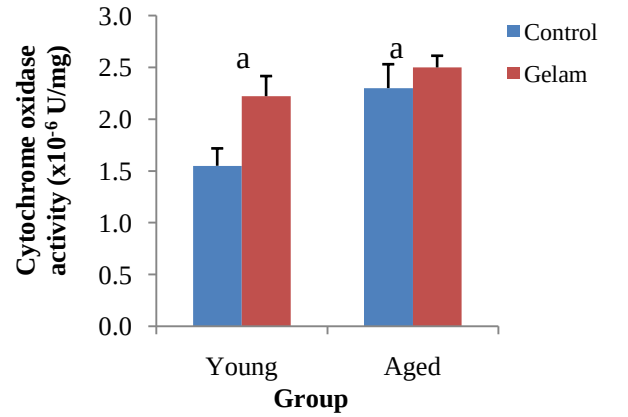


Figure 2 show the effects of gelam honey supplementation on malondialdehyde level (A) and protein carbonyl content (B) in the heart young and aged groups. The results are expressed as mean ± SEM. a indicates significant difference compared to young control group ($p<0.05$).

Figure 3: The effect of gelam honey supplementation on cytochrome oxidase activity



The results are expressed as mean ± SEM. a indicates significant difference compared to young control group ($p<0.05$).

4. Discussion

Mitochondria are the critical components that can regulate and induce aging based on mitochondrial theory of aging [13]. Aging is a universal phenomenon affecting all living organisms that are characterized by a progressive decline in physiological and body functions due to increase in oxidative damage by overproduction of ROS [14]**.** Mitochondrial theory of agingpostulates that electrons from electron transport chain produce ROS which lead to a cyclic increase in intracellular ROS levels and a decline in mitochondrial function [15]. Cytochrome oxidase (CcO) is the terminal component of complexes in the ETC. It functions is to transfer electrons from cytochrome c to an oxygen molecule and simultaneously create a proton gradient across the mitochondrial inner membrane that can provide the force to produce ATP. Thus, CcO exerts a crucial role in cellular energy transformation. This study showed that cytochrome oxidase activity decreased in the young control group compared to aged group.Low activity of cytochrome oxidase is one of the most frequent causes of mitochondrial defects and has been associated with a wide range of human disorders [16]. Deficiency of cytochrome oxidase also facilitates mitochondrial apoptosis in response to oxidative stress [17]. Low activity of CcO in the young group might be due to high levels of malondialdehyde (MDA). The CCO is vulnerable to oxidative stress as it can be inhibited by several oxidative species including MDA [18]. Interestingly, supplementation of gelam honey increased the activity of cytochrome oxidase in the young group. Thus, gelam honey might enhance mitochondrial function through aging.

The study showed that aging is associated with protein and lipid oxidation [19]. The protein carbonyl level is determined as an index of protein damage while malondialdehyde level is a marker of lipid peroxidation. According to the free radical theory of aging, the level of oxidative damage is high in aged individual compared to younger ones [2]. Contradictory finding was obtained which the level of MDA was reduced in aged group. Supplementation of gelam honey only decreased MDA level in the young group. It might due to the MDA level in aged group was low and supplementation might not give any changes. Low level of MDA is parallel with high activity of CcO in aged group. Another study also reported gelam honey supplementation able to reduce the MDA level in the young group [20,21]. While, Batumalaie *et al* [22] revealed that gelam honey and its flavonoids reduced MDA production in the cell culture.Thus, gelam honey has a capability to protect from oxidative damage.

Antioxidant enzymes are the first line of defense mechanism against oxidative damage [23]. Mitochondrial superoxide dismutase (MnSOD) which dominantly present

in the matrix [24] were responsible for dismutation of superoxide anions ($O^{2\cdot-}$) into hydrogen peroxide (H_2O_2) and into water by glutathione peroxidase (GPx) [25] and catalase during different acute stress conditions [26]. Supplementation of gelam honey seems to exert a protective role against oxidative damage as it increases theactivity of MnSOD and GPx in the young group.These results are parallel with anotherstudy which demonstrates that gelam honey modulates antioxidant activities in rat [20,21,27]. The high antioxidant capacity of gelam honey could be mediated through its relevant total phenolic content.

Studies by Chua *et al* [5] found that gelam honey has biochemical constituents such as total phenol, total flavonoid content, and total water-soluble vitamins (vitamin B_1, B_2, B_3, B_9, B_{12}, vitamin C) which act as antioxidant and free radical scavenging activitythat could maintain cells and organ to function normally. Phenolic reacts with reactive oxygen and reactive nitrogen species in a termination reaction where the radical is stabilized by delocalization, thus breaking the cycle of generation of new radicals as a phenolic hydroxyl group are good hydrogen donor [28]. Apart from that, flavonoid give protective effect by the involvement of the chelating metal ions (copper or iron) where it prevents the generation of the ROS [29]. A study has reported that gelam honey has radical scavenging activity of approximately 58% at a concentration of 60 mg/ml [30]. Several studies also had confirmed the contributions of phenolic compounds in combating free radicals [31]. These results might suggest gelam honey as dietary antioxidant against oxidative damage.

5. Conclusions

Gelam honey gives protection against oxidative stress by modulating antioxidant enzyme activity, reduced oxidative damage and enhance the activity of cytochrome oxidase which was more prominent in the young group. This action of honey is might be due to its phenolic content.

Competing interests: The authors declare that there are no competing interests.

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