

Effects of Gelam Honey on Oxidative Stress in Lung Cancer Cells

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ABSTRAK

Madu gelam telah didapati mempunyai kesan anti-oksidatif, anti-kanser dan anti-inflamasi terhadap banyak jenis kanser. Tujuan kajian ini adalah untuk menentukan kesan madu gelam terhadap aras tekanan oksidatif sel kanser paru manusia. IC₅₀ madu gelam ditentukan dengan merawat sel A549 dengan dos madu yang berbeza (50-200 mg/ml). Sel dibahagikan kepada 4 kumpulan dan diaruh tekanan oksidatif dengan menggunakan hidrogen peroksida (H₂O₂) mengikut kumpulan tertentu: kawalan, H₂O₂, madu gelam, H₂O₂ + madu gelam. Selepas 24 jam rawatan, biopetanda tekanan oksidatif seperti malondialdehid (MDA) dan protein karbonil ditentukan. Aruhan tekanan oksidatif meningkatkan aras MDA (p<0.05) tetapi tidak memberi kesan kepada protein karbonil. Rawatan madu gelam didapati menurunkan aras MDA (p<0.05) dalam sel A549 yang diaruh dengan H₂O₂ tetapi tidak memberi kesan kepada protein karbonil. Rawatan madu gelam sahaja tidak mempunyai kesan terhadap aras MDA dan protein karbonil. Kesimpulannya, madu gelam menurunkan peroksidasi lipid tetapi bukan oksidasi protein dalam sel paru manusia teraruh tekanan oksidatif.

Kata kunci: gelam, madu, kanser paru, malondialdehid, protein, stress

ABSTRACT

Gelam honey was reported to exhibit anti-oxidative, anti-tumour and anti-inflammatory properties against many types of cancer. The main aim of the present study was to evaluate the effect of Gelam honey on the oxidative stress level of human lung cancer cells. IC₅₀ of Gelam honey was identified by treating A549

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cells with different doses of honey (50-200 mg/ml). The cells were divided into four groups and induced with stress using hydrogen peroxide (H₂O₂) accordingly: control, H₂O₂, Gelam honey, H₂O₂ + Gelam honey. After 24 hrs of treatment, oxidative stress markers such as malondialdehyde (MDA) and protein carbonyl, were determined. Induction of oxidative stress significantly increased the levels of MDA ($p < 0.05$) but had no effect on protein carbonyl levels. Treatment with Gelam honey was found to reduce MDA levels ($p < 0.05$) in A549 cells exposed to H₂O₂ but had no effect on the level of protein carbonyl. Interestingly, Gelam honey treatment alone had no effects on the levels of MDA and protein carbonyl. In conclusion, Gelam honey reduces lipid peroxidation but not protein oxidation in human lung cancer cells subjected to oxidative stress.

Keywords: gelam, honey, lung cancer, malondialdehyde, protein, stress

INTRODUCTION

Lung cancer is a major cause of morbidity and mortality in the world (Ferlay et al. 2010; Ridge et al. 2013). Lung cancer initiates when cells in the lung grow extensively forming a tumour, lesion or nodule. It is divided into two main classifications, small cell lung cancer and non-small cell lung cancer (NSCLC). The NSCLCs include squamous cell carcinomas, adenocarcinomas, and large cell carcinomas. Squamous cell carcinoma is commonly found in men and is correlated with history of smoking. According to the National Cancer Registry Report 2007-2011, lung cancer was reported to be the third most common cancer in Malaysia affecting men predominantly (Azizah et al. 2016).

Generally, oxidative stress was implicated to be a causative factor for cancer development. Long term exposure to oxidative stress causes oxidative modifications to macromolecules such as DNA, lipid

and protein resulting in damages of cell structure and function (Poyton et al. 2009; Hussain et al. 2003). High levels of reactive oxygen species (ROS) produced by metabolic pathways also stimulate mutations and oncogenes formation leading to development of cancer cells (Fang et al. 2009; Trachootham et al. 2009; Waris & Ahsan 2006). On the other hand, few cancer treatment such as chemotherapy and radiotherapy utilises ROS for cancer elimination. Increased oxidative stress induced by these treatments may also cause further DNA damage and mutations leading to the development of drug-resistance in tumour cells. Hence, proper evaluation of anticancer strategies aimed to modulate levels of ROS are warranted (Gorrini et al. 2013).

Previous studies proved that Gelam honey contains high contents of phenols, mineral, protein, free amino acid, enzymes, and vitamins. These compounds provide different biological properties including antioxidant, anticancer, anti-diabetic, anti-inflammatory and improved

wound healing (Putri Shuhaili et al. 2016; Hussein et al. 2011) free radicals scavenging activities (Hussein et al. 2011). Phenolic compounds in honey such as caffeic acid, chrysin, phenyl ester, galangin, kaempferol, antioxidants, amino acids, and protein mediate antiproliferative effect towards cancer cells by inducing antioxidant effects, stimulation of TNF- α , induction of apoptosis and inhibition of lipoprotein oxidation (Tonks et al. 2001). Previous research reported that Gelam honey from Malaysia provided anti-cancer properties by inhibiting of cell proliferation and DNA damage as well as inducing of apoptosis of human osteosarcoma and cervical cancer cells (Tahir et al. 2015). Gelam honey has been proposed to reduce oxidative damage through modulation of the endogenous antioxidant enzyme activities (Sahhugi et al. 2014). Since, Gelam honey has been shown to possess both anti-cancer and antioxidants properties, the purpose of this study is to determine whether Gelam honey can attenuate or augment the oxidative damages in stress induced lung cancer cells.

MATERIALS & METHODS

A549 CELL LINE CULTURE

In this study, 3 different passages of A549 lung cancer cell were used. A cryovial of A549 stock was thawed in 37°C water bath. Under a sterile condition, the content of the vial was transferred into a 20 ml cell culture medium and was centrifuged for

5 mins at 200 rcf. The supernatant was removed and the pellet was resuspended in a 20 ml of culture medium and was transferred into a cell culture flask. The cells were kept in an incubator at 37°C with 5% CO₂. On the next day, the confluence of cells was inspected using a microscope. The cells with approximately 70% confluence were transferred into 2 new cell culture flasks. The cells were allowed to grow to certain passages at least three times. Later, trypsin-EDTA solution was used to detach the cells from the flask for the treatment with Gelam honey. A549 lung cancer cell lines were divided into 4 groups and treated accordingly: control; H₂O₂; Gelam honey; H₂O₂ and Gelam honey. After 24 hrs of treatment, 2 ml of PBS solution was added into each petri dish. The cells were scraped off the petri dish using a cell scrapper for further analysis.

GELAM HONEY TREATMENT

Gelam honey was obtained from the Department of Agriculture, Batu Pahat, Johor, Malaysia. Gelam honey stock of 200 mg/ml concentration was prepared with distilled water. Using MTS assay, a series of Gelam honey concentrations ranging from 25 mg/ml, 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml were used to treat A549 lung cancer cell lines for 24 hrs in culture flasks. Then, the treated cells were transferred into a 96-well plate for absorbent reading using multi-well spectrophotometer (ELISA reader). A graph of cell survival versus Gelam honey concentrations was plotted. The

concentration of honey that resulted in 50% of cell survival was considered as the IC₅₀.

INDUCTION OF OXIDATIVE STRESS

Cells were treated with 350 µg/ml H₂O₂ as reported previously (Yusof & Ismail 2014). Briefly, the cells were cultured in DMEM media containing 350 µg/ml H₂O₂ for 24 hrs. Subsequently, cells were transferred into DMEM media containing the IC₅₀ concentration of Gelam honey. After 24 hrs, the cells were harvested and kept at -80°C for the following analysis.

PROTEIN CARBONYL LEVEL MEASUREMENT

One ml of cell mixture and PBS solution was transferred from the petri dish into 2.0 Eppendorf tube. The solution was centrifuged at 1000 RCF for 10 mins at 4°C. Next, 2 ml of cold PBS was added to the pellet and sonicated on ice briefly. The mixture was centrifuged at 10000 rcf for 10 mins at 4°C. Then, 200 µl of supernatant was transferred into a 2 ml Eppendorf tube and later 800 µl of 2,4-dinitrophenylhydrazine (DNPH) was added into the tube. The mixture was incubated in the dark at room temperature for 1 hr and was vortexed briefly every 15 mins throughout the incubation period. One ml of Trichloroacetic acid (TCA) was added into the tube and vortexed briefly. The tubes were placed on ice for 5 mins before centrifugation at 10000 rcf for 10 mins at 4°C. The supernatant was removed from each

tube and 10 ml of 10% TCA was added into each pellet. The tubes were placed on ice for 5 mins before centrifugation again. The supernatant was removed and pellet was resuspended in 1 ml of Ethanol/ Ethyl Acetate Mixture in a 1:1 ratio, then the tubes were vortexed thoroughly before centrifugation again. These steps were repeated twice. After the final wash, the protein pellets were resuspended in 500 µl of Guanine Hydrochloride by vortexing. The mixture was centrifuged again. An amount of 220 µl of supernatant was transferred into a 96-well plate. The absorbent of supernatants were measured at 360 nm wavelength using multi-well spectrophotometer (ELISA reader).

MALONDIALDEHYDE (MDA) LEVEL MEASUREMENT

Using Oxiselect™ Thiobarbituric Acid Reactive Substance (TBARS) Assay Kit (MDA Quantification) (Cell Biolabs, Inc), a series of MDA standard was prepared by serial dilution method of the MDA standard ranging from 0 µM-125 µM. An amount of 100 µl of the SDS lysis solution was added into the samples and incubated at room temperature for 5 mins. Then, 250 µl of TBA reagent was added and incubated at 95°C for 45-60 mins before cooling to room temperature for 5 mins. Samples were centrifuged at 3000 rpm for 15 mins. Supernatants from the samples were used for spectrophotometric measurement. MDA standards and samples were transferred into a 96-well plate accordingly and the absorbances were measured using

spectrophotometer (ELISA reader) at 532 nm.

STATISTICAL ANALYSIS

Data was analyzed using SPSS package (version 21). Results obtained were expressed as mean ± SD. Statistical evaluation was assessed using analysis of variance (ANOVA) with the significance level of $p < 0.05$

RESULTS

Dose optimization of Gelam honey (IC_{50}) was done via MTS assay. After treating A549 cells with different doses of Gelam honey, IC_{50} of 123.75 mg/ml was obtained (Figure 1). Results showed that MDA levels significantly ($p < 0.05$) increased when cells were induced with H_2O_2 compared to the control (Figure 2). A549 cells treated with Gelam honey after exposure to H_2O_2 showed significant ($p < 0.05$) reduction in MDA levels compared to H_2O_2 treated cells. However, no significant differences in MDA levels were observed when the cells were treated with Gelam honey alone compared to the control. Gelam honey also had no significant effect on protein carbonyl levels with and without H_2O_2 induction (Figure 3). Similarly, protein carbonyl levels were not affected by exposure to H_2O_2 alone.

DISCUSSION

Imbalance in the level of oxidants and antioxidants that subsequently elevate the rate of oxidative stress has

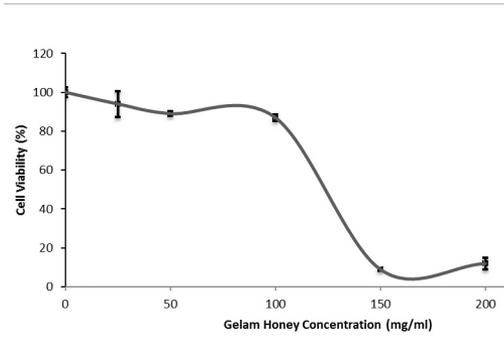


Figure 1: Effect of Gelam honey on the viability of A549 lung cancer cell lines. Cell viability was determined via MTS assay after 24-hour treatment with increasing concentrations of Gelam honey. The IC_{50} of Gelam honey is 123.75mg/ml.

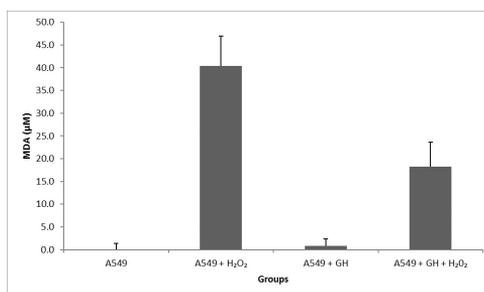


Figure 2: MDA levels in A549 cell with various treatments. a: significant ($P < 0.05$) compared to control, b: significant ($P < 0.05$) compared to A549 cells + H_2O_2 , c: significant ($P < 0.05$) compared to A549 cells + Gelam honey

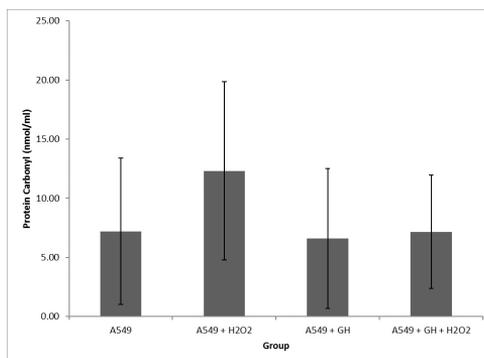


Figure 3: Protein carbonyl levels in A549 cell with various treatments.

been well recognized to play a role in the pathogenesis of lung cancer (Peddireddy et al. 2012). Continuous high concentration of oxidative stress causes damages to macromolecules such as lipids, proteins and nucleic acids. This results in accumulation of oxidative damages in molecules, cells, tissues, and organs (Back et al. 2012; Sakhugi et al. 2014). Subsequently, oxidized substances such MDA is formed. Parallel to this, increased oxidative stress and decreased anti-oxidant enzymes have been observed in lung cancer patients as the disease progresses (Gupta et al. 2010).

Schramm et al. (2003) reported that natural honey contains numerous antioxidant compounds that delay, prevent or remove oxidative damages (Erejuwa et al. 2012). Aljadi and Kamaruddin (2004), as well as Chua and co-workers (2012) have also found that the antioxidant properties of Gelam honey are correlated with its content of phenols, flavonoids and vitamins. The putative anti-oxidative activities of Gelam honey have also been confirmed by animal studies done previously (Lee et al. 2011; Erejuwa et al. 2012).

In the present study, we observed a significant ($p < 0.05$) decrease of MDA level, as a measure of lipid peroxidation, in A549 lung cancer cells treated with Gelam honey as compared to H_2O_2 induction alone. This result is similar with that obtained by Blasa et al. (2007) who reported that honey flavonoids prevent the production of MDA. The phenolic compounds in honey may have also caused the reduction of oxidative stress in this

study because phenolic compounds are capable of scavenging free radicals (Kishore et al. 2011). Interestingly, the level of MDA did not change in cells treated with Gelam honey alone. This finding was supported by a study done by Steinberg and Witztum (2000) which reported that supplementation of antioxidants was beneficial to subjects with high oxidative stress level only. Similarly, studies done by Lee et al. (2011) and Sakhugi et al. (2014) demonstrated that Gelam honey supplementation reduced plasma MDA in aged rats which hypothetically have higher oxidative stress level compared to young rats. Perhaps, this can be explained by the difference in anti-oxidative properties present in cells, where antioxidant capacity is suggested to be high and potent in cells not treated with H_2O_2 (Sakhugi et al. 2014). In other words, antioxidants have better therapeutic effects in oxidative stressed conditions.

Protein carbonyl level was measured as an index of protein oxidation. Overall, neither H_2O_2 induction nor Gelam honey supplementation showed significant effect on the level of protein carbonyl indicating that protein carbonyl in lung cancer cells was not affected by the changes in its oxidative environment. Enhanced capability to overcome oxidative protein carbonylation may be means of cancer cells to produce prosurvival in oxidative stress environment. This is because carbonylation of proteins in cells such as glucose-regulated protein-78, heat-shock protein 60, heat-shock protein cognate 71, phosphate disulphide isomerase, calreticulin,

beta-actin, tubulin-alpha-1-chain and enolase-alpha trigger the apoptosis of cells (Magi et al. 2004). Further analysis to validate these hypotheses were not performed in this study due to limitation of funds.

CONCLUSION

Gelam honey reduces lipid peroxidation in A549 lung cancer cells induced with oxidative stress but had no effect on the level of protein carbonyl.

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