Original Article

In Vitro Cell Culture Study of Anticholesterol and Antioxidant Activities of *Myrmecodia platytyrae (MyP)* Extract in WRL-68 Cells

M. K. Nik Hasan, I. S. Kamarazaman**, I. Abdul Wahab*, H. H. Mizaton*, M.A. Rasadah***

Abstract

In this cell culture study, the experiments were done in order to explain the efficacy of *Myrmecodia platytyrae* (*MyP*) water extract to reduce cholesterol production and its antioxidant effect. The experiment was performed by measuring total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol levels in the cytosol of the treated WRL-68 cells. In addition, lipid peroxidation was determined by evaluating the concentration of malondialdehyde (MDA) in the cell. The antioxidant effect was also determined by observing superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities in the treated cell. The results showed that treatment with *MyP* can decrease TG and LDL concentrations. It was also showed that 400 µg/ml *MyP* increased HDL concentration. Besides, the levels of MDA were gradually decreased when *MyP* concentration was increased. Treatment of *MyP* can also increase SOD and CAT. However, GPx concentration did not increase after *MyP* treatment. It was concluded that *MyP* could reduce cholesterol levels and increase SOD and CAT activities in a concentration-dependent manner.

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Keywords: *Anticholesterol, antioxidant, antioxidant enzymes, lipid peroxidation Myrmecodia platytyrae*

Introduction

Excessive cholesterol will increase the possibility of lipid peroxidation, which leads to atherogenesis.[1] Cholesterol itself is not risky for human's health.[2] Human's body needs cholesterol for normal body regulation and hormone production. However, oxidized cholesterol could be harmful to the blood vessels and endothelial cells.[3] Recently there have been many reports showing that herbal extracts can reduce cholesterol production in liver cells.[4] According to Farooqui *et al*., 2016[5] herbal extract also might help reduce the oxidation process on cell membrane lipid bilayer. Thus, the anticholesterol and antioxidant effects from herbal treatment have high potential to avoid hypercholesterolemia-related diseases such as atherosclerosis and stroke.

In this study, *Myrmecodia platytyrae* (*MyP*) was investigated for its roles in reducing cholesterol and its possibility to act as an antioxidant. This plant can be found in Southeast Asian countries such as Indonesia, Vietnam, Thailand, and Malaysia.[6,7] Generally, *Myrmecodia* tuber contains alkaloid, phenolic, and terpenoid compounds.[8] Moreover, Sanjaya *et al*., 2014,[9] stated that *Myrmecodia* tuber has proven to be rich in bioactive constituents, such as flavonoids, tocopherols, tannins, and a variety of essential minerals.

Previous studies showed that *MyP* water extract contains flavonoids which might help reduce reactive oxygen species (ROS) or free radical attack.[10] The flavonoids from the extract might directly or indirectly decrease the destructive effect of ROS on the endothelial cells. Thus, our experiments were designed to investigate the *MyP* water extract effects on the cell. Moreover, the results from this study can lead to a better understanding on the neutralization of ROS by the *MyP* water extract and reducing its harmful effect on the lipids.

Materials and Methods

Preparation of 10% *MyP* water extract

Ten percent *MyP* water extract was prepared by adding 100 g *MyP* powder into a beaker containing 1,000 ml distilled water and boiling at 100°C for 15 minutes. The solution was filtered and the supernatant was concentrated by using rotary evaporator at 50°C and freeze-dried. The 10% extract powder of *MyP* water extract was stored at -80°C until use.

Cell culture

Human hepatocytic cells (WRL-68) were maintained in RPMI-1640 media with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. The culture media was kept at 37°C in a water bath for 5 minutes. The cells were allowed to proliferate in 25-cm^2 tissue culture flasks. Then the cells were subcultured in 75-cm^2 flasks once they had reached 70-85% confluence.

*Faculty of Pharmacy, Universiti Teknologi MARA (UITM), Bandar Puncak Alam, Selangor, 42300, Malaysia;
**Natural Product Division, Forest Research Institute Malaysia, 52109 Kepong, Selangor Darul Ehsan, Malaysia.

Corresponding author: M. K. Nik Hasan, Ph.D.
E-mail: mohdkamal@frim.gov.my

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Determination of *MyP* water extract lethal concentration (LC_{50}) on WRL-68

Assays were performed by seeding 1×10^6 cells per well in a 96-well plate for 24 hours. A complete medium was added to each well. Then the cells were exposed to a range of *MyP* water extract concentrations (0.1, 1, … µg/ml to 1 g/ml). After that the 96-well plate was incubated for another 24 hours. Viable cells were detected using MTT assay. This experiment was performed in triplicates.

MTT assay

The assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was based on a modified method by Moodley *et al*., 2014.[11] The concentration of MTT solution was 5 mg/ml. The solution was filtered by using a 0.2 µm syringe filter for sterilization. After 24 hours of incubation, 20 µl of MTT solution were added to each cell-containing well. Then the plate was incubated in a CO_2 incubator for four hours. The plate was transferred to a plate reader and the absorbance was read at 570 nm. Eventually, the percent of cell viability was calculated as follows:

$$\text{Percentage of viability (\%)} = \frac{\text{Absorbance of treated cells} \times 100}{\text{Absorbance of untreated cells}}$$

Determination of optimal oleate and *MyP* concentrations for lipid lowering study

Cells were grown in 6-well plates at a concentration of 1×10^6 cells/well, followed by incubation in a CO_2 incubator at 37°C for 48 hours or until the cells were 80% confluent. Oleate and *MyP* water extract concentration selection experiments were done in order to determine the best concentration for subsequent experiments.

Oleate concentration determination to increase lipid in WRL-68 cells Oleate was used as an inducer to increase low-density lipoprotein cholesterol (LDL) formation in the cells, as reported by Lin *et al*., 2011.[12] WRL-68 cells were exposed to 10 µl of oleic acid at a concentration ranging from 50 to 250 µM. Then the plate was incubated for 24 hours. After that the cells were scraped, transferred into centrifuge tubes, centrifuged at 1,000 rpm at 4°C, and washed three times with PBS. The pellets containing cells were lysed by using a lysis buffer and centrifuged at 4,000 rpm, 4°C, to discard the cell membrane. The concentrations of low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triglyceride (TG), and total cholesterol (TC) were measured with commercial kits (Elabscience Biotechnology, Wuhan, China). All experiments were performed in triplicates.

Lipid lowering effect of MyP water extract on oleate-induced WRL-68 cells Cells were grown in 6-well plates, followed by incubation in a CO_2 incubator at 37°C for 48 hours. Briefly, the normal control was WRL-68 without any treatment. Negative control was oleate-induced WRL-68 cells. Positive control was oleate-induced WRL-68 plus simvastatin. Treatment groups were treated with the selected effective *MyP* water extract concentrations for 24 hours with complete medium. Afterwards, the treatment groups were exposed to 10 µl oleate and then incubated. The concentrations of LDL, HDL, TG, and TC were measured as described above. All experiments were performed in triplicates.

Antioxidant study: Determination of H_2O_2 lethal concentration (LC_{50}) on WRL-68

The toxicity assay of H_2O_2 on WRL-68 was carried out as described earlier, except that the *MyP* water extract was replaced with H_2O_2 at concentrations of 0.1, 1, 10, 100, and 1,000 µg/ml. The cell viability was detected using the MTT assay. This experiment was performed in triplicates.

Effective concentration of *MyP* water extract in preventing H_2O_2-oxidation of WRL-68 cells

The purpose of this experiment was to determine the effective concentration of *MyP* water extract that can prevent WRL-68 oxidation. Cells were plated as above and subjected to additions of selected *MyP* water extract concentrations and incubation for 30 minutes. Then H_2O_2 at 40 µM was added to each cell-containing well and the cells were incubated. Viable cells were detected using MTT assay which was performed in triplicates.

Sample preparation for lipid peroxidation study

This experiment was carried out after the effective concentration of *MyP* water extract had been identified. Cells were grown in 6-well plates. Then they were treated with *MyP* water extract at 400, 200, and 100 µg/ml for 24 hours with complete media. Following the incubation period, the cells were exposed to H_2O_2. Positive control was WRL-68 without any treatment. Negative control was WRL-68 plus 40 µM H_2O_2. Then the plate was incubated. All experiments were performed in triplicates.

Determination of lipid peroxidation

Lipid peroxidation on WRL-68, as reflected by malondialdehyde (MDA) levels, was determined by thiobarbituric acid reactive substances (TBARS) assay as described by Ohkawa, *et al.*, 1979,[13] with slight modifications. The cell lysate was used for the determination of lipid peroxidation.

Antioxidant enzymes study

The preparation of cells followed a similar procedure as before. Then cells were resuspended and sonicated for one minute to obtain cell lysate, which was transferred into falcon tubes and centrifuged at 4°C and 15,000 rpm for 15 minutes. After that the cell lysate was aliquoted and protein measurement was done. Then the activity of enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were assayed by using commercial kits (USCN Life Sciences, Houston, Texas, USA).

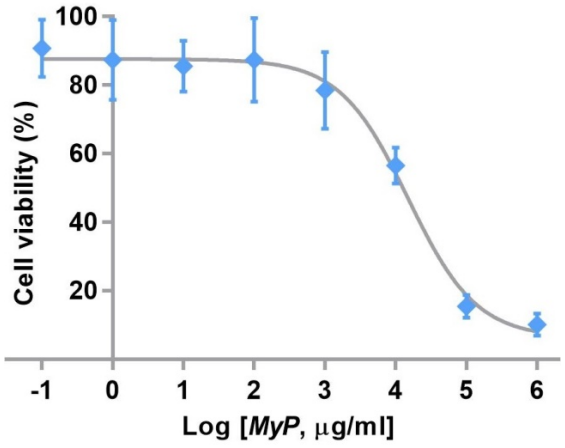

Figure 1 Determination of *MyP* water extract lethal concentration (LC_{50}) on WRL-68. Percentage of WRL-68 cell viability after treated with different concentrations of *MyP* water extract. Mean ± SD, n = 3; LC_{50} = 14.94 ± 0.001 mg/ml (obtained by fitting data with the dose-inhibition equation).

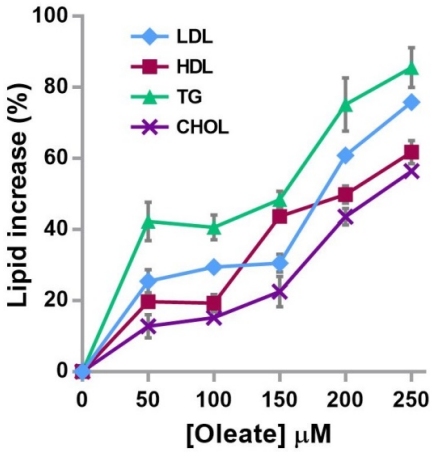

Figure 2 Oleate concentration determination to increase lipid in WRL-68. Percentage of lipid increase in WRL-68 cells after treated with different oleate concentrations. Mean ± SD, n = 3.

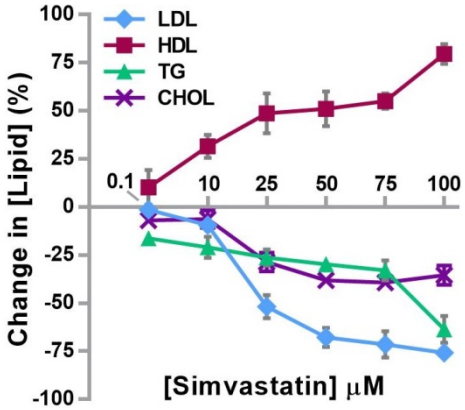

Figure 3 Simvastatin concentration determination for lipid lowering effect. Percentage of lipid in WRL-68 cells treated with simvastatin compared to normal cells. Mean ± SD, n = 3.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The differences between groups were analyzed using one-way analysis of variance (SPSS, version 15) and *P* value <0.05 was considered significant.

Results

The percentage of WRL-68 cell viability after treated with *MyP* water extract at different concentrations was determined in order to observe the toxicity of the extract. The result showed that LC_{50} was more than 10 mg/ml (Figure 1), and therefore *MyP* water extract was considered not toxic to WRL-68 cells. In addition, the percentage of lipid rising in WRL-68 cell after treated with different oleate concentrations was measured to find the optimum oleate concentration that can increase lipid in the cells. The resulting graph showed that 220 µM oleate could increase total cholesterol by 50% in the WRL-68 cells (Figure 2).

Next, cells were induced with 220 µM oleate to increase lipid production. Percentage of lipid change in WRL-68 cell treated with simvastatin was used as a positive control to be compared with normal cells. Cells in the normal group were not induced with oleate. Cholesterol production was decreased to 40% after treated with 60 µM simvastatin as shown in Figure 3. The concentration of lipids (LDL, HDL, TG

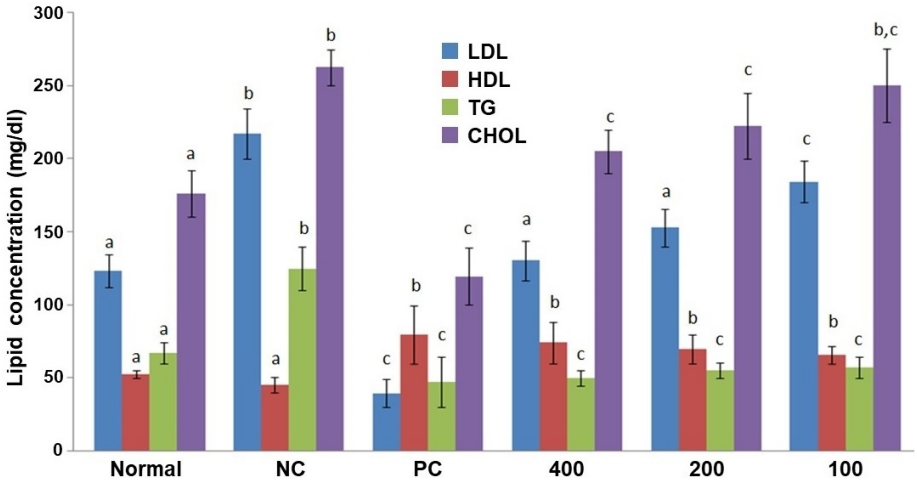

Figure 4 Lipid-lowering effect of *MyP* water extract on oleate-induced WRL 68 cells. Mean ± SD, n = 3; different superscripted letters on top of each bar indicate statistical differences ($P < 0.05$). Normal, no treatment; NC, negative control (220 µM oleate only); PC, positive control (oleate + simvastatin); 400, 200, 100, µg/ml *MyP* water extract treatment + oleate.

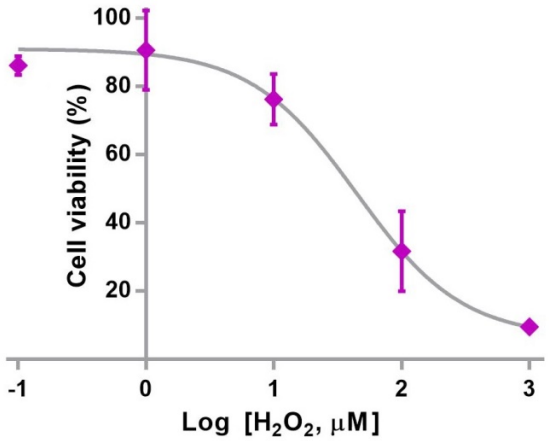

Figure 5 Determination of H_2O_2 lethal concentration (LC_{50}) on WRL-68 cells. MTT assay was performed to evaluate H_2O_2 cytotoxicity. Mean ± SD, n = 8. LC_{50} = 44.36 ± 1.39 µM (obtained by fitting data with the dose-inhibition equation).

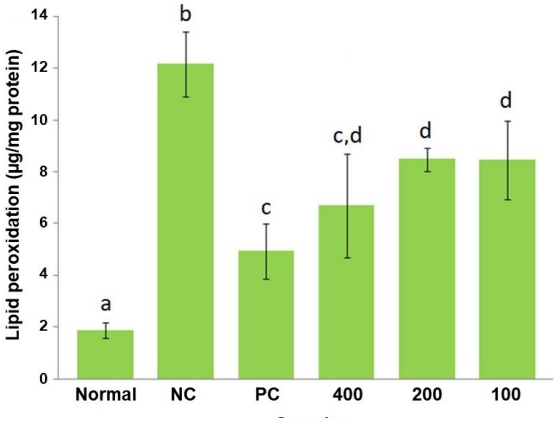

Figure 7 Determination of lipid peroxidation. Malondialdehyde levels, obtained by TBARS assay, represent lipid peroxidation in cell lysates. Mean ± SD, n = 3; different superscripted letters on top of each bar indicate statistical differences ($P < 0.05$). Normal, no treatment; NC, negative control (40 µM H_2O_2 only); PC, positive control (H_2O_2 + simvastatin); 400, 200, 100, µg/ml *MyP* water extract treatment + H_2O_2.

and CHOL) in the WRL-68 cell after treated with *MyP* water extract was depicted in Figure 4. The result showed that treatment with 400 and 200 µg/ml *MyP* water extract significantly decreased LDL concentration compared to the negative control. However, LDL concentration increased when *MyP* water extract concentration was decreased to 100 µg/ml. On the other hand, HDL concentration significantly increased in *MyP* extract-treated cells compared to negative control and normal groups. Moreover, the cellular TG concentration was significantly decreased in positive control and all *MyP*-treated groups.

In the next experiment, MTT assay was performed to evaluate the cytotoxicity of H_2O_2. Figure 5 indicated that the LC_{50} value of the H_2O_2 was approximately 40 µM. Cells were exposed to 40 µM H_2O_2 and the effect of *MyP* water extract on H_2O_2-induced oxidative damage in WRL-68 cells was determined. Figure 6 showed that 400 µg/ml *MyP* water extract could increase cell viability in H_2O_2-treated cells. The extract efficacy was concentration dependent.

Determination of TBARS in cell lysate treated with *MyP* water extracts was shown in Figure 7. The result showed that 400, 200, and 100 µg/ml *MyP* water extract could decrease MDA concentration. Changes in enzymatic antioxidant activities in WRL-68 cells after treated with *MyP* water extract could be observed for SOD and CAT (Table 1). Treatment of *MyP* water extract did not increase GPx activity.

Discussion

The liver plays an important role in regulating circulating cholesterol levels by controlling cholesterol *de novo* synthesis, uptake, storage and conversion to bile acids.[14,15] Normal cholesterol metabolism ensures that the human body gets sufficient cholesterol from the endogenous and exogenous cholesterol sources. This experimental work used liver WRL-68 cell lines because of its ability to produce cholesterol and mimicking normal cholesterol metabolism.[16]

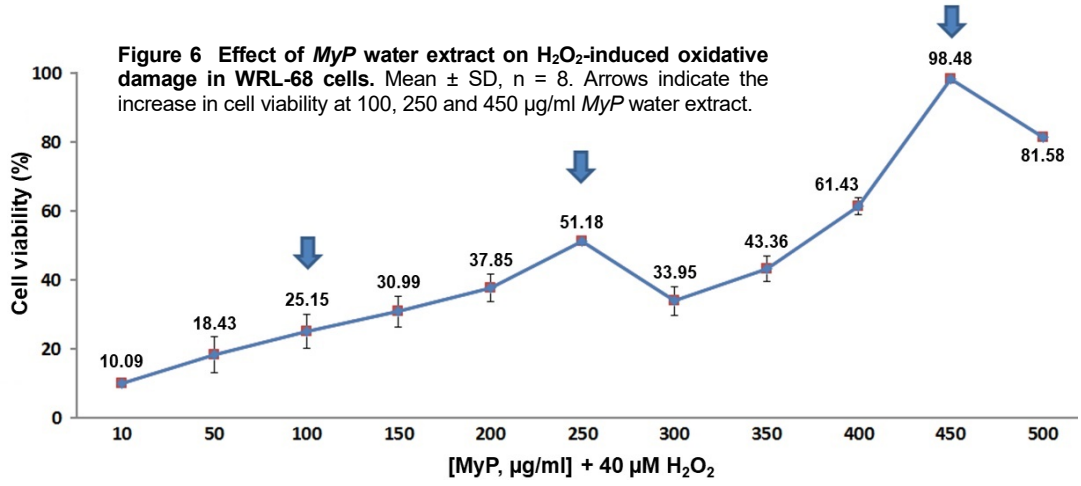

Figure 6 Effect of *MyP* water extract on H_2O_2-induced oxidative damage in WRL-68 cells. Mean ± SD, n = 8. Arrows indicate the increase in cell viability at 100, 250 and 450 µg/ml *MyP* water extract.

Table 1 Antioxidant enzyme study. Changes in enzymatic antioxidant activities in WRL-68 cell after treated with *MyP* water extract.

Group	SOD	CAT	GPx
Normal control	17.53±1.87[a]	19.41±2.07[a]	7.72±0.88[a]
Negative control	15.75±0.36[a]	10.62±0.98[b]	15.33±1.56[b]
Positive control	22.28±2.52[b]	21.44±0.95[a]	7.71±0.62[a]
400 µg/ml *MyP*	16.51±3.41[c]	19.35±1.99[a]	6.55±0.75[c]
200 µg/ml *MyP*	15.95±0.22[c]	19.16±1.23[a]	6.12±0.78[a]
100 µg/ml *MyP*	11.29±1.65[a]	18.27±2.53[a]	6.36±0.80[a]

Normal control received no treatment; Negative control, 40 µM H_2O_2; Positive control, 40 µM H_2O_2 + 60 µM simvastatin. Data are mean ± SD in U/mg protein. Results with different letters in a column are significantly different (one-way ANOVA followed by Tukey's multiple comparison test; $P < 0.05$; n = 3). SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

The growth of the WRL-68 cells in the presence of various concentrations of *MyP* water extract was examined. Under the experimental conditions, *MyP* water extract did not exhibit growth inhibitory effects on WRL-68 cells over a 24 h period. The LC_{50} for WRL-68 cells was more than 10 mg/ml for the *MyP* water extract as shown in Figure 1. This finding was in agreement with the study by Mizaton *et al*., 2010.[17]

The imbalance of cholesterol metabolism could occur with many factors, such as free radical attack, sedentary lifestyles, and genetic, which cause hypercholesterolemia. Hypercholesterolemia is an excessive level of cholesterol in the blood and this condition increases the risk of heart disease.[5] In this cell culture study, WRL-68 normal liver cell was induced to increase mevalonate pathway for endogenous cholesterol production. In order to develop hypercholesterolemic cells, oleate was used to boost up the cholesterol production in the WRL-68 cells. According to Cohen *et al*., 2015,[18] oleate increases lipid metabolism in cultured cell and enhances formation of lipid droplet. As shown in Figure 2, it can be seen that 220 µM oleate increased the production of total cholesterol. This concentration was higher compared to previous reports.[18,19] Besides that, the mixture of oleate and palmitate (2:1) can be used to induce the hepatic lipid accumulation.[20] The graph also showed higher LDL when oleate concentration was increased. The subsequent experiments were done by using the concentration of oleate that was determined from Figure 2. The optimized concentration of oleate ensured the formation of lipid group in the cells. Figure 3 exhibited that treatment with 60 µM simvastatin can reduce 50% of cholesterol production compared with untreated normal cells, and this concentration was used as a positive control in the next experiment. Meanwhile, *MyP* water extract concentrations used in this study were 100, 200 and 400 µg/ml. These concentrations were selected in consideration of the efficacy to reduce cholesterol production and the lack of cell toxicity.

Cholesterol is transported by lipoproteins which are classified based on density.[21] Each lipoprotein has their own function and total cholesterol is the sum of all the lipoproteins, such as HDL, VLDL, and LDL.[22] Figure 4 showed that *MyP* water extract can reduce the production of TC, LDL and TG in a dose-dependent manner. Interestingly, the results also showed that *MyP* water extract treatment can enhance HDL production which is the most important molecule for reverse cholesterol transport (RCT).[23] The function of HDL is very supportive since it appears to have cardioprotective properties because of its involvement in RCT processes.[24] Research has reported that HDL is able to impede LDL oxidation[25] and can also transfer cholesterol from the macrophage back to the plasma in the process of RCT.[24] The concept of RCT suggested that excess cholesterol in peripheral tissues was transported by HDL back to the liver for excretion.[26]

Hypercholesterolemia leads to hepatic steatosis, atherosclerosis and other metabolic pathologies. The diseases are related to excessive lipid accumulation in cells.[27] It was reported that dry extract of *Myrmecodia* contain flavonoids, such as kaempferol, luteolin, rutin, quercetin, and apigenin. Oil red O staining showed that the number of intracellular lipid droplets decreased in adipocytes treated with kaempferol.[28] It was also reported that luteolin inhibited intracellular TG accumulation in a dose-dependent manner by inhibiting the transactivation of peroxisome proliferator-activated receptor gamma (PPARγ) without cytotoxicity effect.[29] Besides that, RT-PCR results demonstrated that mRNA expression of adipogenic transcription factors, such as PPARγ and CCAAT/enhancer binding protein-alpha (C/EBPα) in 3T3-L1 cells were remarkably downregulated by rutin treatment.[30] Quercetin at a concentration of 10 µM is reported to have the ability to inhibit adipogenesis and reduce fat accumulation in mature adipocytes.[31] Ono and Fujimori, 2011[32] reported that apigenin activates 5'-adenosine monophosphate-activated protein kinase (AMPK) in a dose-dependent manner, leading to decreased expression of adipogenic and lipolytic genes, thus suppressing adipogenesis in 3T3-L1 cells.

In the experiment, H_2O_2 was used as a free radical inducer to attack the cell membrane of WRL-68. H_2O_2 is often used as an experimental source of oxygen-derived free radicals.[33] The free radical produces by H_2O_2 was already known for its damaging effect on cell DNA, as reported by Ward *et al*., 1987.[34] Meanwhile, Wu *et al*., 1996, [33] reported that the pathway taken by H_2O_2 to kill normal cells is through inhibition of the glycolytic pathway, with hydrolysis of intracellular ATP and the resultant intracellular acidification. Current research shows that the uncontrolled production of H_2O_2 can be fatal to the cell and causes oxidative injury.[35] Figure 5 showed that the number of viable cells in the 96-well plate was decreased when the concentration of H_2O_2 was increased. The highly hydroxyl reactive species produced by H_2O_2 damaged biologically relevant

molecules, such as DNA, proteins, carbohydrates, and lipids and caused cell death. Lobo *et al*., 2010,[36] reported that all free radicals attack important macromolecules leading to cell damage and homeostatic disruption. The result showed that the LC_{50} of H_2O_2 was around 40 µM. Nevertheless, the result of H_2O_2 concentration from this study cannot be compared with other previous results because the lethality effect of H_2O_2 depends on its half-life. The next experiment was executed to find the best concentration of *MyP* water extract that can increase cell viability above 50%.

The protective effect of *MyP* extract on H_2O_2-induced WRL-68 cells was showed in Figure 6 with generally increasing percentage of cell viability from 10 to 500 µg/ml, particularly at 250 and 450 µg/ml. Presumably, this is because the hydroxyl group from H_2O_2 was neutralized by the active compounds in *MyP* which acted as antioxidants, reducing the oxidative reaction on cell membranes. Hydroxyl radicals can attract an electron from the polyunsaturated fatty acids in the cell membrane to form carbon-centered lipid radicals, which further interact with molecular oxygen to produce lipid peroxyl radical ($LOO^{\bullet}$).[37,38] This lipid peroxidation generates a number of degradation products,[39] and results in the formation of 4-hydroxy-2-nonenal (HNE) in addition to MDA, which are carcinogenic in nature.[38] Thus, higher *MyP* water extract concentrations should reduce lipid peroxidation. Figure 7 showed that this is the case: *MyP* water extract could significantly inhibit lipid peroxidation and lower MDA concentration.

Liver antioxidant enzymes, such as SOD, GPx, and CAT are produced by hepatocytes for antioxidative protection from free radicals.[40] However, these antioxidant defense mechanisms become weaker during chronic fatigue and other disease conditions. SOD dismutases superoxide radicals to form H_2O_2 and O_2.[41] The SOD activities in 200 µg/ml and 400 µg/ml *MyP* water extract-treated groups also showed statistically significant improvement in comparison to the negative control. Meanwhile, CAT catalyzes H_2O_2 breakdown of to form water and O_2.[41] In the oleate-treated group, CAT activity was significantly lower compared to the normal control group. However, pretreatment with *MyP* water extract for 24 h from 100 to 400 µg/ml *MyP* water extract completely prevented a decrease in CAT activity. A dose-dependent manner was found in CAT activity between low (100 µg/ml) and high (400 µg/ml) *MyP* water extract treatment. Furthermore, GPx is an enzyme responsible for reducing H_2O_2 or organic hydroperoxides to water and alcohol, respectively. However, the administration of *MyP* water extract did not increase GPx.

Conclusion

It was concluded that *MyP* water extract might increase HDL and decrease LDL in liver cell. This shows that *MyP* treatment can control the cholesterol production. Besides that, *MyP* water extract also has antioxidant potential which increased the antioxidative enzymes SOD and CAT. The extract also can decrease the MDA production, indicating that it can acts as a good antioxidant.

Acknowledgments

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Conflict of Interest

None to declare.

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