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


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 (GPxs), 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, GPxs 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.


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

Introduction

Excessive cholesterol will increase the possibility of lipid peroxidation, which leads to atherosclerosis.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., 20165 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., 20149 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% CO2. The culture media was kept at 37°C in a water bath for 5 minutes. The cells were allowed to proliferate in 25-cm2 tissue culture flasks. Then the cells were subcultured in 75-cm2 flasks once they had reached 70-85% confluence.
Determination of MyP water extract lethal concentration (LC50) 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 CO2 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:

Percentage of viability (%) = Absorbance of treated cells × 100 / 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 CO2 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 conifuge 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 CO2 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 H2O2 lethal concentration (LC50) on WRL-68
The toxicity assay of H2O2 on WRL-68 was carried out as described earlier, except that the MyP water extract was replaced with H2O2 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 H2O2-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 H2O2 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 H2O2. Positive control was WRL-68 without any treatment. Negative control was WRL-68 plus 40 µM H2O2. 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 Okawara, 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).
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 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 1](image1.png)

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](image2.png)

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](image3.png)

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.

![Figure 4](image4.png)

Figure 4 Lipid-lowering effect of $MyP$ water extract on oleate-induced WRL 68 cells. Mean ± SD, n = 3; different super-scribed 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.
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\textsubscript{2}O\textsubscript{2}. Figure 5 indicated that the LC\textsubscript{50} value of the H\textsubscript{2}O\textsubscript{2} was approximately 40 μM. Cells were exposed to 40 μM H\textsubscript{2}O\textsubscript{2} and the effect of MyP water extract on H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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. 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.
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 LC50 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.7 In this cell culture study, WRL-68 normal liver cell was induced to increase malonate 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 oxidation25 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, 201132 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, H2O2 was used as a free radical inducer to attack the cell membrane of WRL-68. H2O2 is often used as an experimental source of oxygen-derived free radicals.33 The free radical produces by H2O2 was already known for its damaging effect on cell DNA, as reported by Ward et al., 1987.34 Meanwhile, Wu et al., 199635 reported that the pathway taken by H2O2 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 H2O2 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 H2O2 was increased. The highly hydroxyl reactive species produced by H2O2 damaged biologically relevant

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

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>17.5±1.87a</td>
<td>19.4±2.07a</td>
<td>7.7±2.08a</td>
</tr>
<tr>
<td>Negative control</td>
<td>15.75±0.36b</td>
<td>10.62±0.98b</td>
<td>15.33±1.56b</td>
</tr>
<tr>
<td>Positive control</td>
<td>22.28±2.52c</td>
<td>21.44±0.95c</td>
<td>7.71±0.62c</td>
</tr>
<tr>
<td>400 μg/ml MyP</td>
<td>16.51±3.41a</td>
<td>19.35±1.99a</td>
<td>6.55±0.75a</td>
</tr>
<tr>
<td>200 μg/ml MyP</td>
<td>15.95±0.22a</td>
<td>19.16±1.23a</td>
<td>6.12±0.78a</td>
</tr>
<tr>
<td>100 μg/ml MyP</td>
<td>11.29±1.65a</td>
<td>18.27±2.53a</td>
<td>6.36±0.80a</td>
</tr>
</tbody>
</table>

Normal control received no treatment; Negative control, 40 μM H2O2; Positive control, 40 μM H2O2 + 60 μM simvastatin. Data are mean ± SD in Umg 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.
molecules, such as DNA, proteins, carbohydrates, and lipids and caused cell death. Lobo et al., 2010, reported that all free radicals attack important macromolecules leading to cell damage and homeostatic disruption. The result showed that the LC50 of H2O2 was around 40 μM. Nevertheless, the result of H2O2 concentration from this study cannot be compared with other previous results because the lethality effect of H2O2 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 H2O2-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 H2O2 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•). This lipid peroxidation generates a number of degradation products, and results in the formation of 4-hydroxy-2-nonenal (HNE) in addition to MDA, which are carcinogenic in nature. 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. However, these antioxidant defense mechanisms become weaker during chronic fatigue and other disease conditions. SOD dismutases superoxide radicals to form H2O2 and O2. 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 the breakdown of to form water and O2. 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 H2O2 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.

References

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