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Anti-hypercholesterolemic activity of standardized fermented *Allium cepa* L. var *aggregatum* extract: *In vitro* and *in vivo* studies

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ABSTRACT

Introduction: *Allium cepa* extract has been reported to have anti-hypercholesterolemic activity in rats. This study was conducted to investigate the effects of standardized fermented *A. cepa* L. var *aggregatum* extract on cholesterol levels and HMG-CoA reductase enzyme.
Methods: The fermented *A. cepa* extract was standardized by the presence of quercetin using a validated high performance liquid chromatography (HPLC) method. The activity of the extract on HMG-CoA reductase was determined using HMG-CoA Assay kits, then measured by Nano spectrophotometry. *In vivo* study was conducted in hypercholesterolemic rats. The extract was administered orally at doses of 100, 200, and 300 mg/kg body weight (bw) to rats for 21 days and the cholesterol levels were measured every week.
Results: All doses of fermented *A. cepa* extract and its marker compound, quercetin, ameliorated the levels of high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) as compared to those of negative control ($P<0.05$). Of all the doses, fermented *A. cepa* extract at the dose of 200 mg/kg bw displayed the highest reduction on LDL-C levels. In addition, the extract at the dose of 200 mg/kg bw showed the strongest enhancement in HDL-C levels. The fermented *A. cepa* extract and quercetin also inhibited the HMG-CoA reductase enzyme with inhibitory activity of 61.78%.
Conclusion: The ethanol extract of fermented *A. cepa* shows anti-hypercholesterolemic activity. The strong anti-hypercholesterolemic activity of the extract might be due to the high amounts of quercetin, although other constituents may also contribute.

Implication for health policy/practice/research/medical education:
The current study reported the scientific evidence of fermented extract of *Allium cepa* L. var *aggregatum* as anti-hypercholesterolemic agent. The result indicates the potency of the extract to prevent cardiovascular problem. The strong anti-hypercholesterolemic effect might be due to fermentation method that has increased the amount of quercetin. Hence, the extract might be developed as an effective anti-hypercholesterolemic agent.

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Introduction

Dyslipidemia is one of the risk factors that leads to coronary heart disease (1). The presence of atherosclerosis may cause cardiovascular disease. Subsequently, it affects total death and disability-adjusted life years in Europe. The primary clinical effects are ischemic stroke, coronary artery disease, and peripheral arterial disease (2). More than three-fourths of deaths in developing countries with low to middle-class societies occurred ~~by~~ because of cardiovascular disease (3).

The high death rate caused by heart disease is reduced by limiting cholesterol through inhibiting cholesterol absorption, bile acid sequestrant, regulation of apoC-III genes, mobilization of fatty acids, and cholesteryl

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ester transfer protein (4). The secondary metabolites that have a major role in reducing cholesterol levels are flavonoids (such as quercetin) and organosulphur compounds (such as alliin, allyl propyl disulfide, diallyl disulfide, dimethyl disulfide, S-methyl-cysteine sulfoxide, and S-propyl-cysteine sulfoxide) (5). Those compounds reduce cholesterol by inhibiting the activity of HMG-CoA reductase, which plays a role in the synthesis of mevalonate, subsequently affecting the low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels (6).

Allium cepa has been found to be rich of quercetin and organosulphur compounds. It has been used in folk medicine for the treatment of cardiovascular diseases, diabetes, bacterial infections, high lipidemia, and cancer (7). A previous study reported that the anti-hypercholesterolemic effect of *A. cepa* was at the dose of 100 mg/kg (8).

In an effort to search for more effective drugs, fermentation has become an alternative to enhance the active secondary metabolite levels in natural resources. The fermentation process increases the levels of nutrition subsequently enhances the benefit and economic values of the medicinal plants (9,10). The current study was carried out to evaluate the anti-hypercholesterolemia effects of fermented *A. cepa* var *aggregatum* extracts on HMG-CoA reductase activity and LDL-C and HDL-C levels.

Materials and Methods

Chemicals and instruments

The chemicals used (Hexpharm Jaya, Indonesia) were Quercetin (Sigma Aldrich, USA), HMG-CoA Reductase Assay Kit (Abcam with catalog number ab 204701; Abcam PLC., United Kingdom), atherogenic feed (cow fat, used cooking oil, quail egg yolks), and Glory Cholesterol Kit (Linear Chemical, SLU). The instruments used in this study were Intelligent Automatic Fermentation machine, SpectroStar Nano (BMG LabTech, Germany), Spectrophotometer Microlab 300 (ELITechGroup, France), and HPLC Agilent Technologies (Agilent Technologies Inc., United States).

Plant materials

Plant materials of *A. cepa* var *aggregatum* (onion) were collected from Medan-North Sumatera, Indonesia. The plant was identified by Herbarium Medanense (MEDA) Universitas Sumatera Utara, Indonesia with the herbarium number of 4470/MEDA/2019.

Fermentation and extraction of *Allium cepa* L. var *aggregatum*

Onions were cleaned from dust and other impurities. They were arranged in Automatic Fermentation Machine. The fermentation was carried out for 15 days at 50-80°C. The fermented onions were extracted with ethanol 96%, filtered, and evaporated.

Standardization of the fermented *Allium cepa* L. var *aggregatum* extract by HPLC

HPLC analysis was conducted using C-18 column (Fortis Technologies; 100 × 4.6 mm UniverSil HS 5 µm) with eluted isocratically of mobile phase consisted of acetonitrile: water (40: 60) (11). The *A. cepa* L. var *aggregatum* and Quercetin, as reference standard, were dissolved in methanol at the concentrations of 4, 2, and 0.1 mg/mL, respectively. Subsequently, the fermented extract and quercetin were filtered using PTFE (polytetrafluoroethylene) membrane (0.22 µm) and injected into the HPLC system at a wavelength of 370 nm for 7 minutes with a flow rate of 0.5 mL/min and DAD as a detector. Identification of quercetin in *A. cepa* L. var *aggregatum* was determined by comparing the retention time of the peak onion fermented extract and quercetin. The method was validated by measurement of linearity (indicated by the correlation coefficient (r^2)), precision (by intra-assay and inter-assay validation), limit of detection (LOD) (calculated by 3.3 × (RSD/S)), and limit of quantification (LOQ) (calculated by 10 × (RSD/S)), where RSD refers to relative standard deviation and S refers to slope of calibration curves. The validated method was then used to quantify the Quercetin content in *A. cepa* L. var *aggregatum* by plotting calibration curves of Quercetin standard at five concentrations (100, 50, 25, 12.5, and 6.25 µg/mL).

In vitro HMG-CoA reductase assay

The HMG-CoA reductase inhibitory activity of the extract was performed using a colorimetric Assay Kit from Abcam with catalog number ab204701. The procedure was carried out by following the instructions from the manufacturer. Inhibitor was prepared at 10 ppm. The reaction mix consisting of mixture HMG-CoA:NADPH: HMG-CoA reductase buffer assay (12:4:174) was prepared (190 µL). A total of 5 µL HMG-CoA reductase enzymes with 2 µL inhibitors and 3 µL buffer were mixed in a 96 well flat bottom microplate. The addition of 190 µL of reaction mix indicated the start of enzymatic reaction. Then the absorbance was measured at 340 nm every 2 minutes for 14 minutes at 37°C.

In vivo anti-hypercholesterolemic assay

Animals used in this study were two months old male Wistar rats. All treatments for animals and procedures of this study were evaluated by the animal research committee (No. 0446/KEPH-FMIPA/2019) from Animal Research Ethics Committees/AREC of Biology Departement, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia. Thirty rats were used in this research, which were divided into 6 treatment groups and 5 rats in each group. They were negative control (vehicle only), positive control (commercial tablet containing atorvastatin at a dose of 1.8 mg/kg bw), quercetin at a dose of 15 mg/kg bw, and treatment

groups (extract of fermented *A. cepa* var *aggregatum* at 100, 200, and 300 mg/kg doses). Animals were given an atherogenic feed (mixture of cow fat 10%, used cooking oil 20%, quail egg yolks 20% and 50% of water equal 120 mL) until hypercholesterolemia occured. The extract *of A. cepa* var *aggregatum* was given for 21 days orally after hypercholesterolemic condition. Rats were fasted for about 15 hours before blood sampling. Then, the blood was taken from the rat's tail (2 mL) and transferred into an Eppendorf tube. The cholesterol levels were measured using the spectrophotometer Microlab 300.

Results

Fermentation and extraction of *Allium cepa* L. var *aggregatum*

The fermentation method in this study was spontaneous fermentation. Spontaneous fermentation is a fermentation process that occurs naturally without an additive that supports the fermentation (12). The fermented onion had a soft texture, brown to black in color, sweet, and savory in taste, and it produced an alcohol smell.

The alteration of colour in the fermented onion occurred due to the Maillard reaction. Maillard reaction is a condensation reaction between free amino acids or peptides with carbonyl groups on reducing sugars, and triggers in formation of Amadori compounds. These compounds are a precursor for the production of aroma, taste, and color (13-15). These results were in agreement with a previous study that the Maillard reaction occurred due to an amino acid reaction to reducing sugars causing changes in color, taste, and texture in food. This study also indicated that thiosulfonate levels significantly decreased 30-fold when compared with fresh onions; hence the smell and taste of fermented onions changed (16). Sour taste arose due to the reduction of pH during the fermentation process. This study was supported by a previous study that reported the pH of fermented garlic decreased from 6.33 to 3.74 during the fermentation process at 70˚C (17).

In addition, according to Shin et al, an increase in fermented garlic acid caused a decrease of black garlic pH from 6.12 to 3.90 (18). Futhermore, carbohydrate contents in onions became an important factor that determined the sweet taste in fermented onions. Carbohydrate compounds were converted into simple sugars during the fermentation process; hence sugar levels were increased and gave a sweet taste. In a study, the sugar levels of fermented garlic increased during the fermentation process (19). Fresh onions were hard before and then soft after fermentation. That was due to the water content of fresh onions, which decreased at high temperatures. This result was supported by previous research, which stated that the texture of fermented garlic was softer and more elastic when the water content stood between 40%-50%. Meanwhile, if the water level of fermented garlic was 35%-40%, the fermented garlic became drier and elasticity was poor. Moreover, if the water content was below 30%, the texture of fermented garlic would turn harder and the elasticity would be low (20,21).

Standardization of the fermented *Allium cepa* L. var *aggregatum* extract by HPLC

The chromatograms of the reversed phase HPLC column of the ethanol extract of fermented *A. cepa* L. var *aggregatum* showed a major peak for quercetin, corresponding to retention time at 5.350 minutes, respectively. The peak was identified by comparing it with HPLC of the reference standard of quercetin at 5.350 minutes (Figure 1). *A. cepa* var *aggregatum* before fermentation contained quercetin with amount of 5.6619 µg/mL. After fermentation, the amount of quercetin increased to 11.6340 µg/mL. Calibration curves plotted were linear with a correlation coefficient (r^2) of 0.9994 µg/mL. LOD and LOQ were found to be 0.0122 and 0.0372 µg/mL, respectively. The precision validation presenting the intraday and interday assay of retention time and peak are shown in Table 1.

In vitro HMG-CoA reductase assay

The effect of extract on inhibition of HMG-CoA reductase was determined (Table 2). The inhibition of *A. cepa* var *aggregatum,* atorvastatin, and quercetin were calculated on the enzyme activity of of HMG-CoA reductase. Based on Table 2, it was shown that *A. cepa var aggregatum*

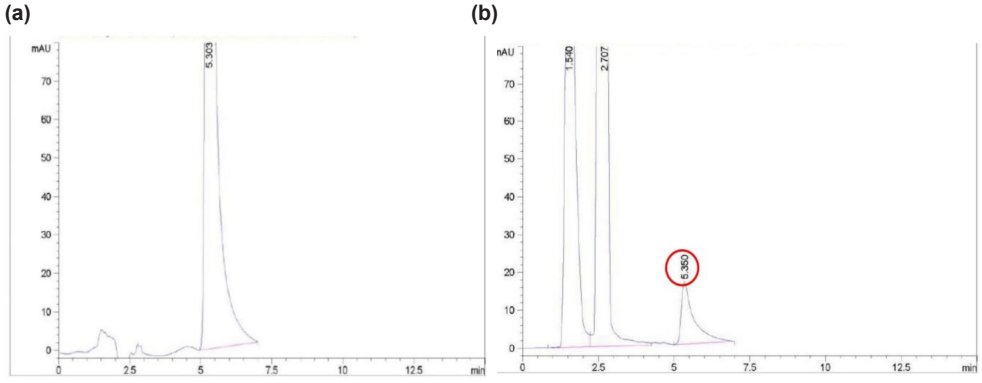

Figure 1. Representative of HPLC chromatogram. (a) Quercetin and (b) fermented *A. cepa* L. var *aggregatum* extract.

Table 1. Precision of intraday and interday of quercetin

Concentration (µg/mL)	RT	Intraday[a] (RSD%)	Peak	Intraday (RSD%)	RT	Interday[b] (RSD%)	Peak	Interday (RSD%)
6.25	5.40	0.04	219.233	0.42	5.35	1.70	216.667	2.69
25	5.37	0.04	1105.867	0.45	5.35	0.03	1178.667	5.92
100	5.31	0.12	5248.633	0.27	5.25	2.07	5318.833	0.89

[a] Intraday repetitions for each concentration were analyzed on the same day; [b]Interday repetitions for each concentration. RSD: Relative standard deviation; RT: retention time.

could inhibit HMG-CoA reductase enzymes at 61,78%. However, *A. cepa var aggregatum* inhibitory activity was still lower than atorvastatin by in vitro assay.

This result was supported by a previous study, which showed that administration of *A. cepa* var *aggregatum* in male Sprague-Dawley rats at dose of 200 mg/kg bw for 45 days improved hyperlipidemia by inhibiting the activity of HMG-CoA reductase. Furthermore, quercetin-rich onion contributed for HMG-CoA reductase inhibition activity. Quercetin inhibited HMG-CoA reductase activity at 81.72% (15). In this study, quercetin inhibited HMG-CoA reductase activity at 72.56%.

In vivo anti-hypercholesterolemic assay

LDL-C levels of rats after administration of the fermented *A. cepa* var *aggregatum* and its marker compound, quercetin decreased significantly. In contrast, the extract and quercetin increased the HDL-C as shown in Table 3. All doses were able to reduce LDL-C levels. *A. cepa* var *aggregatum* at the dose of 200 mg/kg bw showed the highest activity on reducing LDL-C levels (66.81%).

In agreement with the result of LDL-C levels, the fermented *A. cepa* var *aggregatum* at the dose of 200 mg/kg bw showed the strongest activity in increasing the HDL-C levels, comparable to those of positive control, atorvastatin.

Table 2. The effect of fermented *A. cepa var aggregatum* extract on HMG-CoA reductase activity

HMG-CoA Reductase Inhibitor	Mean ± SEM	Inhibition (%)
Atorvastatin	0.0040 ± 0.01	98.92
Quercetin	0.1012 ± 0.04	72.56
A. cepa var *aggregatum*	0.1409 ± 0.00	61.78

SEM, standard error of the mean.

Discussion

Cholesterol is synthesized in endoplasmic reticulum from acetate through the mevalonate pathway, which is mediated by HMG-CoA reductase. Gene that regulates cholesterol synthesis and uptake of sterol regulatory element-binding protein (SREBP), specifically SREBP2 and 1a, are present in LDL-C and HMG-CoA reductase receptors (22). The activity of fermented *A. cepa* var *aggregatum* extract on HMG-CoA reductase is one of several mechanisms to reduce cholesterol. Administration of *Allium cepa* extract reduces cholesterol by enhancing the up-regulation of LXRα and CYP7A1 (23). Moreover, quercetin-rich onion improved fat metabolism and increased the number of skeletal muscle mitochondrial as a consequence of the increase in energy expenditure (24). The increase of fat metabolism would contribute to high cholesterol deposits in artery walls and a decrease in HDL-C (25). One of the hypocholesterolemic mechanisms of *Allium cepa* was related to flavonoid and organosulphur content by antioxidant activities. Antioxidants are important for scavenging free radicals that damage the structure and function of cells (26). A previous study showed that *Allium cepa* L. inhibited LDL oxidation. Oxidized LDL is the pathogenesis of atherosclerosis by macrophage uptake, resulting in forming foam cells and endothelial cells, which will be accumulated by cholesterol (7). Furthermore, the existence of platelet adhesion, monocytes, and neutrophils on endothelium leads to atherosclerotic (27).

Onion contains 89% water, protein, vitamins B1, B2, C, potassium, selenium, polysaccharides, essential oils, as well as sulfur, phenolic, and flavonoid compounds. The flavonoid contents in onion include kaempferol, isorhamnetin, and quercetin (8).

Anti-hypercholesterolemic activity of onion may be related to flavonoid and sulfur compounds. Onion

Table 3. Rats lipid profile after treated by fermented *A. cepa* var *aggregatum* extract

Treatment	Dose	Percentage decrease in LDL-C	Percentage increase in HDL-C
Negative control	Carboxymethyl cellulose Na 0.5%	6.05 ± 0.82	-21.35 ± 1.62
Atorvastatin	1.8 mg/kg	71.30 ± 1.96*	11.66 ± 1.50*
Quercetin	15 mg/kg	41.38 ± 1.54*	10.42 ± 0.45*
A. cepa var *aggregatum* extract	100 mg/kg	47.41 ± 0.60*	19.07 ± 1.86*
A. cepa var *aggregatum* extract	200 mg/kg	66.81 ± 0.71*	19.04 ± 1.08*
A. cepa var *aggregatum* extract	300 mg/kg	53.28 ± 0.85*	9.80 ± 0.61*

Data are presented as mean ± SEM (n=5). *$P < 0.05$ compared to the negative control.
HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol

contains quercetin about 739-1000 mg/kg as free quercetin, quercetin glycosides, and oxidation products (28). An increase in flavonoid content of onion through fermentation was accompanied by an increase in quercetin content. Flavonoid compounds in onion have a role in overcoming cardiovascular disease, such as atherosclerosis (29). However, other compounds present in onion, such as sulfur compounds (S-allyl-cysteine and S-ethyl-cysteine) have activity on HMG-CoA reductase (30,31).

Quantitative analysis showed that fermented *A. cepa* var *aggregatum* extract contained quercetin. The amount of quercetin in fermented *A. cepa* var *aggregatum* extract was higher after fermentation. Increase in quercetin content in fermented *A. cepa* var *aggregatum* was in accordance with previous studies (32,33). This might be due to flavonoid pathway such as glycosylation, deglycosylation, ring termination, methylation, glucuronidation, and sulfoconjugation, which were facilitated by microorganisms (32). The strong anti-hypercholesterolemic activity of fermented *A. cepa* var *aggregatum* extract is due to high content of quercetin, although other constituents might also contribute.

Conclusion

The current study reported the scientific evidence of fermented extract of *Allium cepa* L. var *aggregatum* as anti-hypercholesterolemic agent. The result indicates the potency of the extract to prevent cardiovascular problem. The strong anti-hypercholesterolemic effect might be due to fermentation method that has increased the amount of quercetin. Hence, the extract might be developed as an effective anti-hypercholesterolemic agent. The standardized fermented *A. cepa* var *aggregatum* extract and its marker compound were able to ameliorate the HDL-C and LDL-C levels. They also inhibited the activity of HMG-CoA reductase.

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Authors' contributions

Y and ASR constructed the idea and hypothesis for research and/or manuscript, planned the methods to generate hypothesis or to reach the conclusion, prepared biological materials and reagents, took responsibility in logical interpretation and presentation of the results. PS, DA, and AD organized and supervised the course of the project or the article, planned the methods to generate hypothesis or to reach the conclusion, data management, and reports. All authors critically reviewed and approved the final manuscript for publication.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

All treatments for animals and procedures of this study were evaluated by the animal research committee No. 0446/KEPH-FMIPA/2019 from Animal Research Ethics Committees/AREC of Biology Departement, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia.

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