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Effect of extraction conditions on radical scavenging and cholesterol metabolism regulating capacity of silkworm larvae

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Abstract

High blood cholesterol levels and oxidized cholesterol are risk factors for cardiovascular disease, which displays high annual incidence. Although studies on sericulture products, including pupae, silk protein, and blood lymph, as hypocholesterolemic substances have been reported, insufficient research in this field has been focused on silkworm larvae. Six larval extracts (Low temperature distilled water, LW; hot temperature distilled water, HW; and 30-100% ethanol, E30-E100) were prepared, and their effects on cholesterol metabolism were examined. LW most potently reduced the risk of cholesterol-related disorders. Polyphenols were highly represented in LW, corresponding with its increased antioxidant potency. The cholesterol biosynthesis enzyme, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) was strongly inhibited by LW. Hepatocytes over-expressed LDL receptor (LDLR) after LW stimulation, promoting cholesterol elimination from plasma. LW also increased ATP binding cassette transporter 1 (ABCA1) gene expression, upregulating HDL biogenesis. In conclusion, LW exhibited strong antioxidant activity, suppressed cholesterol biosynthesis, improved LDL uptake from plasma, and upregulated HDL biosynthesis. In aggregate, these activities could reduce blood cholesterol levels and prevent cardiovascular disease.

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Introduction

Silkworms are edible insects, whose use as a functional food additive has been approved by the Ministry of Food and Drug Safety, Korea (Jo *et al*., 2019). The anti-diabetic effect of silkworm intake is well established and correlates with presence of 1-deoxynojirimycin (DNJ) compounds (Jo *et al*., 2020; Ryu *et al*., 2013). In addition, polyphenols such as rutin are found in silkworms (0.06-0.66%), and have antioxidant activity (Ju *et al*., 2015b). DNJ and rutin are produced in mulberry leaves (Ju *et al*., 2015a; Kim *et al*., 2017), and may accumulate in silkworms as a result of feeding. Intake of silkworms thus simultaneously supplies nutrients produced by silkworms and mulberry leaves.

Cholesterol is an essential component of cell membranes and is a precursor of steroid hormones (Hu *et al*., 2010). Elevated levels of blood cholesterol can induce hypercholesterolemia, atherosclerosis, and myocardial infarction (Dayton *et al*., 1970). Therefore, it is important to maintain an appropriate level of cholesterol. In modern society, significant demand exists for functional food and drug research due to increasing incidence of

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cardiovascular disease and stroke. Recent studies have demonstrated hypocholesterolemic effects of sericulture products, including pupa oil and hemolymph, through regulation of cholesterol synthesis resulting in reduced LDL-form cholesterol (Luo *et al*., 2020; Nam *et al*., 2018). To date, studies of cholesterol regulatory effects of silkworm larvae products remain elusive.

In this study, silkworm larvae (3^{rd} day of 5^{th} instar) extracts were prepared under six different conditions. The effects of each extract on polyphenol content, antioxidant efficacy, and marker genes in cholesterol metabolism were examined. The results of this study can be applied to cholesterol metabolism studies using silkworm larvae, and to extract manufacturing process development.

Materials and methods

Silkworm larvae extraction

Silkworm larvae (3^{rd} day of 5^{th} instar) powder was purchased from Uljinfarm (Uljin, Korea) and extracted for 2 h using distilled water at 100°C (HW), distilled water at 25°C (LW), and fermented ethanol concentrations ranging from 30-100% (E30, E50, E70, and E100) (Fig. 1). Extraction yields ranged from 8.8 to 27.7%, with a decreasing trend with increasing ethanol concentration. Extracts were freeze-dried and re-dissolved at 10 mg/mL concentration for further analyses.

Total polyphenol and flavonoid contents

To determine total polyphenol concentration, 10 μL of sample was added to 200 μL of 2% Na_2CO_3 and incubated for 3 min. Ten microliters of 50% Folin-Ciocalteu's reagent was added, and reactions were incubated for another 3 min. The absorbance of each mixture at 750 nm was determined using a spectrophotometer (Multiskan GO, Thermo, MA, USA). Gallic acid (0.1%) was used as a standard, and data are represented as IC_{50} values. Flavonoid quantification was performed as follows: samples (20 μL) were diluted in 100 μL of DW. Next, 6 μL of 5% $NaNO_2$ was added, and reactions were incubated for 6 min at room temperature. Thereafter, 12 μL of 10% $AlCl_3 \cdot 6H_2O$ solution was added and incubated for another 5 min., then 40 μL of 1 M NaOH was added, and the mixture was incubated for 10 min. Absorbance was read at 510 nm using a spectrophotometer (Thermo Fisher Scientific). 0.1% (+)-catechin was used as a standard (Lee *et al*., 2021).

Radical scavenging assay

For the DPPH (1, 1-diphenyl-2-picryl hydrazyl) assay, 0.2 mM reagent (Sigma-Aldrich, MO, USA) was prepared in ethanol. Samples (10 μL) were transferred to 96 well plates, and DPPH solution (200 μL) was added. After 10 min of incubation, absorbances were measured at 520 nm using a spectrophotometer. A radical solution for 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was prepared by addition of 7 mM ABTS (Sigma-Aldrich) and 2.5 mM potassium persulfate (Sigma-Aldrich) and allowing reactions to proceed for 24 h in the absence of light. Reagents were diluted prior to each assay. For the radical scavenging assay, 10 μL of each sample was transferred to a 96 well plate, and ABTS working solution (200 μL) was added. After 10 min of incubation, absorbance at 520

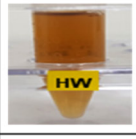
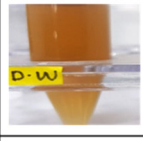
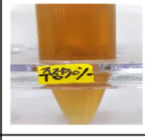
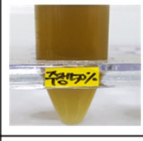
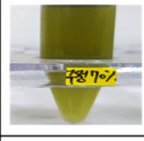
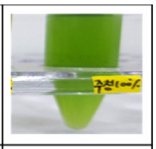
Extract						
Solvent	DW	DW	Fermented EtOH 30%	Fermented EtOH 50%	Fermented EtOH 70%	Fermented EtOH 100%
Temperature (℃)	100	25	25	25	25	25
Abb.	HW	LW	E30	E50	E70	E100

Fig. 1. *Extraction temperature and solvents*
Samples were extracted at 25°C or 100°C using distilled water (DW), or 30%, 50%, 70%, or 100% ethanol. Abbreviations are DW extract at 100°C (HW), DW extract at 25°C (LW), 30% ethanol extract at 25°C (E30), 50% ethanol at 25°C (E50), 70% fermented ethanol at 25°C (E70), and absolute ethanol at 25°C (E100).

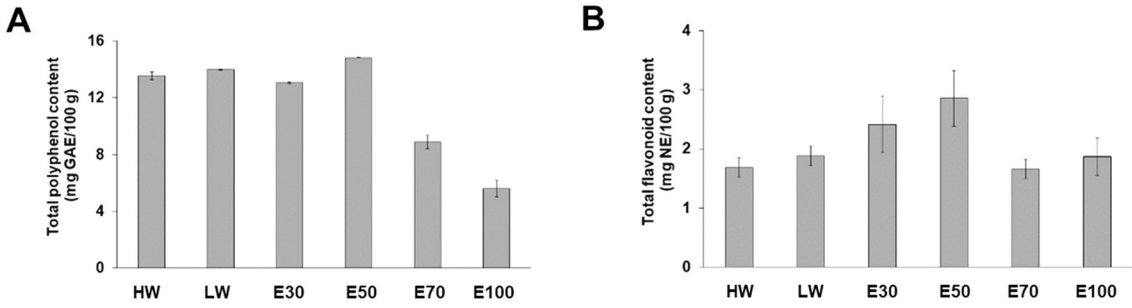


Fig. 2. *Antioxidant compound contents of silkworm larvae*
Total polyphenol (A) and flavonoid (B) contents in six types of silkworm larvae extracts were quantified. Gallic acid and naringin were used as standard compounds. Abbreviations are gallic acid equivalent (GAE) and naringin equivalent (NE). Data are expressed as means ± SD.

nm was read in a spectrophotometer (Thermo) (Bae *et al*., 2016).

3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) activity

This enzyme assay was performed according to manufacturer's instructions (Sigma-Aldrich). Briefly, samples and a positive control (pravastatin) were added (1 μL) to wells in a 96-well plate. A mixture of assay buffer, NADPH substrate solution, and HMGCR was added to each well. Enzyme activity was measured every 30 s for 10 min at 340 nm (Multiskan GO) (Lee *et al*., 2015).

Real-time PCR

HepG2 cells were stimulated with silkworm larvae extract (1 mg/mL) for 24 h. Cells were washed twice with PBS, and RNA was extracted using a PureLink RNA Mini Kit (Thermo, MA, USA). Complementary DNA (cDNA) was synthesized from 2 μg of RNA according to manufacturer's instructions (amfiRivertII cDNA Synthesis Master Mix, GenDEPOT, TX, USA). Transcription levels of sterol regulatory element binding protein 2 (SREBP2), LDL receptor (LDLR), and ATP binding cassette transporter 1 (ABCA1) were determined using a TOPreal qPCR 2X PreMiX kit (Enzynomics, Daejeon, Korea) (Park *et al*., 2014).

Results and discussion

Polyphenol composition of silkworm larvae extracts

HW, LW, E30, and E50 extracts yielded similar total polyphenol content (in the range of 13.05-14.83), with decreased concentrations in E70 and E100 (60% and 38%, respectively, compared to that of E50) (Fig. 2). Flavonoid content was highest in E50, and lowest in E70. Overall, E50 extracts displayed the highest polyphenol content, including flavonoids.

Insects obtain accumulating amounts of polyphenols by eating plants, such as mulberry leaves, which are a unique feed source for silkworms. Polyphenols, including flavonoids, are a predominant group of plant secondary metabolites that play roles in UV protection, defense against pathogens, and pigment production (Hassanpour *et al*., 2011). Additionally, polyphenols have been synthesized in insects via enzyme-mediated pathways (Nino *et al*., 2021). Phenoloxidase is the key enzyme in non-dietary polyphenol synthesis, which is critical to hardening and stabilizing the insect cuticle. In addition, phenoloxidase is an essential enzyme in silkworm larvae for modulating hormones required in the molting process (Wang *et al*., 2013). Therefore, silkworm larvae contain polyphenols acquired through dual pathways. Polyphenol content was determined by water-ethanol ratio, and polarity of the phenolic compounds.

Radical scavenging effect of silkworm larvae extracts

DPPH and ABTS radical scavenging assays were performed (Fig. 3). LW yielded the lowest IC_{50} values in both radical scavenging assays (7.85 mg/mL for DPPH, and 26.53 mg/mLfor ABTS). These IC_{50} values increased with increasing ethanol. Values for E100 were 62.14 and 105.02 mg/mL in DPPH and ABTS assays, respectively.

Radicals are generated by endogenous mechanisms, or by exposure to exogenous factors including UV radiation, pollution, alcohol intake, poor nutrition, and stress (Poljšak *et al*., 2012). Free radicals alter lipids, proteins, and DNA, and are implicated in age-related diseases (Lobo *et al*., 2010). In addition, cholesterol is oxidized by a free-radical-mediated mechanism, producing oxysterols. The atherogenic action of oxysterols

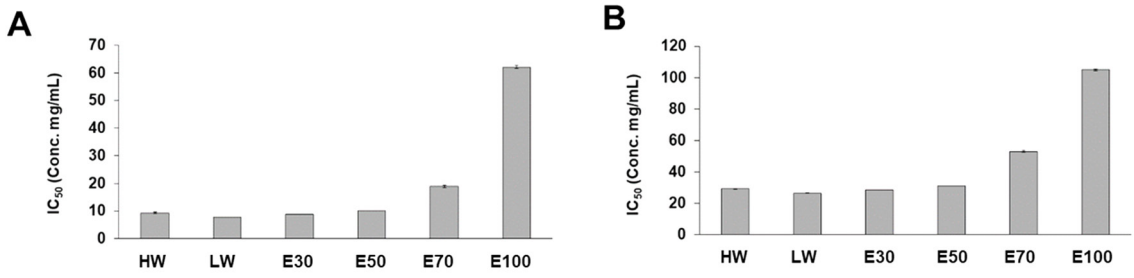


Fig. 3. *Antioxidant activity of silkworm larvae*
DPPH (A) and ABTS (B) radical scavenging effects in six types of silkworm larvae extracts were examined. Antioxidant activity was expressed as an IC_{50} against each radical. Abbreviations are 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and inhibitory concentration 50 (IC_{50}). Data are expressed as means ± SD.

has been reported in previous studies. Therefore, preventing elevated levels of radicals is critical to ameliorating aging and preventing atherogenic-related diseases (Valenzuela *et al*., 2003). Silkworm larvae extracts in water or 30-50% ethanol, which contain high levels of polyphenols, effectively neutralized DPPH and ABTS radicals (Fig. 2 and 3). Polyphenols are antioxidants that neutralize radicals, forming stable products and preventing further oxidation reactions (Shahidi *et al*., 2015). High levels of polyphenols in silkworm larvae extracts may contribute to radical neutralization, exert antioxidant activity, and protect blood vessels from oxysterols.

Inhibitory effect of silkworm larvae extracts on HMGCR

Cholesterol biosynthetic enzyme HMGCR activity was assayed (Fig. 4). Statins, which inhibit HMGCR, reduced HMGCR activity by 22%. In addition, HW, LW, and E30 reduced HMGCR activity by 17%, 11%, and 17%, respectively, revealing more potent enzyme inhibitory efficacy than that of statins.

HMGCR is a key enzyme for reducing plasma cholesterol levels, thereby preventing cardiovascular diseases (Sharpe and Brown, 2013). Cholesterol is synthesized via the mevalonate pathway, and HMGCR is involved in the rate-limiting step of converting HMG-CoA to mevalonic acid. Silkworm larvae extracts, especially the HW, LW, and E30 groups, inhibited HMGCR, more effectively than a statin (positive control). High polyphenol content is expected to inhibit HMGCR. However, E50, which has the highest flavonoid and polyphenol content, was less inhibitory than HW, LW, and E30. Previous studies have reported that HMGCR inhibition depends on polyphenol type (Cho *et al*., 2006; Ademosun *et al*., 2015; Son *et al*., 2017).

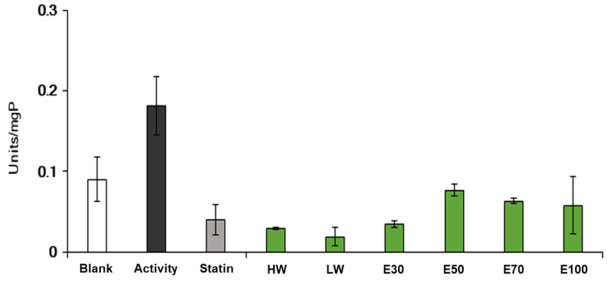


Fig. 4. *Inhibitory effect of silkworm larvae on HMGCR*
HMGCR is a rate-limiting enzyme in cholesterol biosynthesis. Enzyme activity was measured after addition of six types of silkworm larvae extracts. The blank group indicates background activity without enzyme. The activity group contained HMGCR enzyme and substrate. Statins are positive controls for HMGCR inhibition. Data are expressed as means ± SD.

Since different polyphenols display widely varying solvent affinities, we postulate that hydrophilic polyphenols in silkworm larvae may inhibit HMGCR more potently than hydrophilic compounds. In addition, water-soluble polysaccharides and proteins have been reported to inhibit HMGCR (Gil-Ramírez *et al*., 2016; Drotningsvik *et al*., 2018). Identification of the active component(s) of silkworm larvae extracts remains to be achieved.

Effect of silkworm larvae extracts on transcription of cholesterol metabolism genes

Transcription levels of SREBP2, LDLR, and ABCA1, which are known to be involved in cholesterol metabolism, were measured using real-time PCR (Fig. 5). SREBP2 and LDLR expression levels were increased by 2.5 and 2.1 folds in LW, respectively. By contrast, ABCA1 was increased in every silkworm larva treatment group (1.8-7.9 folds).

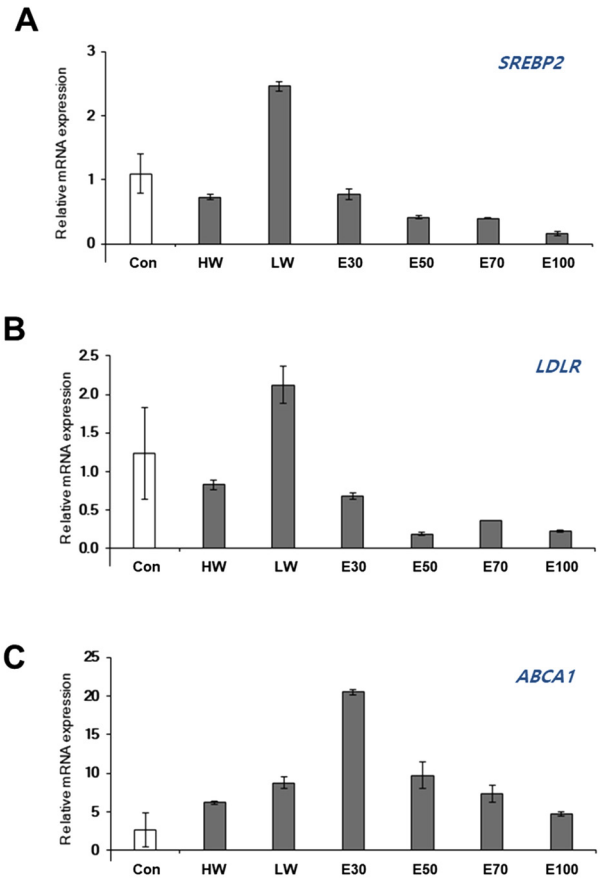


Fig. 5. *mRNA expression level in HepG2 cells after stimulation with six types of silkworm larvae extracts*
Major genes involved in cholesterol metabolism, including SREBP2 (A), LDLR (B), and ABCA1 (C), were compared using real-time PCR. SREBP2 regulates transcription of cholesterol metabolism genes including LDLR. LDLR removes plasma LDL and delivers it to intracellular site. ABCA1 contributes to generation of mature HDL. Fold changes were calculated using cycle threshold (Ct) values. Data are expressed as means ± SD.

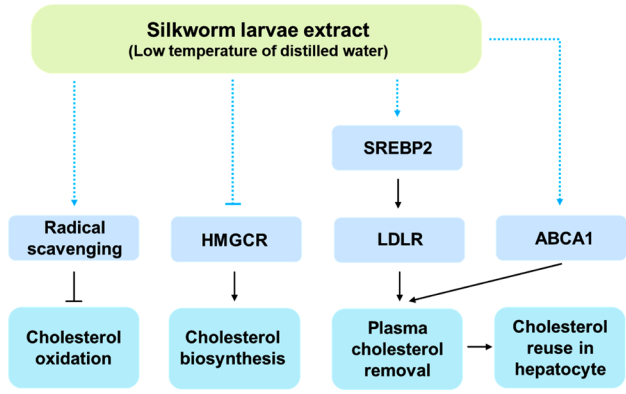


Fig. 6. *Effect of silkworm larvae on cholesterol metabolism*
Silkworm larvae extracted using low temperature DW showed the most effective regulation of cholesterol metabolism via three pathways. First, high antioxidant activity and polyphenol content reduce cholesterol oxidation. Second, the extract reduces HMGCR activity, suppressing cholesterol biosynthesis. Third, plasma cholesterol levels were reduced by up-regulation of LDLR and ABCA1.

Cholesterol in the bloodstream can be removed by LDLR, which is expressed in cell membranes. SREBP2, a transcription factor that regulates cholesterol metabolism, activates LDLR expression (Madison, 2016). In this study, LW induced SREBP2 expression. As a result, LDLR was also highly expressed. ABCA1 plays a critical role in mature HDL formation. Plasma HDL accumulates cholesterol from LDL, then returns it to the liver, where it can be reused or converted to bile acid (Marques *et al*., 2018). Silkworm larvae extracts increased ABCA1 levels compared to the control, which may improve plasma cholesterol levels. In conclusion, silkworm larvae extract regulated cholesterol metabolic genes to an extent that depended on the extraction solvent used. LDLR levels were only induced by LW through enhancement of SREBP2 expression, while ABCA1, which could elevate HDL levels, was increased in every silkworm-treated group. Animal studies are required to assess the *in vivo* hypocholesterolemic effects of silkworm larvae.

Conclusion

Six types of silkworm larvae extract were prepared, and their effects on antioxidant properties and cholesterol metabolism were examined. Total polyphenols were effectively extracted using DW, 30% ethanol, and 50% ethanol. High polyphenol content extracts revealed low DPPH and ABTS radical scavenging IC_{50}. In our HMGCR activity assay, LW exerted the strongest inhibitory effect compared to the other groups. Likewise, LW stimulation increased LDLR expression by upregulating SREBP2 and ABCA1, thereby reducing plasma cholesterol levels (Fig. 6). In conclusion, polyphenol-rich extracts such as LW may reduce plasma cholesterol levels by regulating HMGCR, SREBP2, LDLR, and ABCA1. Further animal studies using silkworm larvae on cholesterol metabolism are required.

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Reference

Ademosun AO, Oboh G, Passamonti S, Tramer F, Ziberna L., Boligon AA, *et al.* (2015) Phenolics from grapefruit peels inhibit HMG-CoA reductase and angiotensin-I converting enzyme and show antioxidative properties in endothelial EA. Hy 926 cells. Food Sci Hum Wellness 4, 80-85.

Bae SM, Jo YY, Lee KG, Kim HB, Kweon H (2016) Antioxidant activity of silkworm powder treated with protease. Int J Indust Entomol 33, 78-84.

Cho SH, Park YY, Yoon JY, Choi SW, Ha TY (2006) The effect of polyphenols from safflower seed on HMG-CoA reductase (HMGR) activity, LDL oxidation and apo A1 secretion. Korean J Food Sci Technol 38, 279-283.

Dayton S, Chapman JM, Pearce ML, Popjak GJ (1970) Cholesterol, atherosclerosis, ischemic heart disease, and stroke. Ann Intern Med 72, 97-109.

Drotningsvik A, Vikøren LA, Mjøs SA, Oterhals Å, Pampanin D, Flesland O, *et al.* (2018) Water-soluble fish protein intake led to lower serum and liver cholesterol concentrations in obese zucker fa/fa rats. Mar Drugs 16, 149.

Gil-Ramírez A, Caz V, Smiderle FR, Martin-Hernandez R, Largo C, Tabernero M, *et al.* (2016) Water-soluble compounds from Lentinula edodes influencing the HMG-CoA reductase activity and the expression of genes involved in the cholesterol metabolism. J Agric Food Chem 64, 1910-1920.

Hassanpour S, MaheriSis N, Eshratkhah B (2011) Plants and secondary metabolites (Tannins): A Review. Int J For Soil Eros 1, 47-53.

Hu J, Zhang Z, Shen WJ, Azhar S (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutr Metab 7, 1-25.

Jo YY, Kim SB, Eom TD, Park SW, Kim SR, Kim SW, *et al.* (2019) Sterilization effect and fatty acid composition of silkworm powder (*Bombyx mori* L.) by heat treatment. Int J Indust Entomol 39, 39-44.

Jo YY, Kim S, Lee JH, Kweon H, Ju WT, Kim HB, *et al*. (2020) Characteristics of the 3 rd day of 5 th instar silkworm powder: effect of preparation method. Int J Indust Entomol 40, 22-27.

Ju WT, Kim HB, Kim KY, Sung GB, Kim YS (2015a) Screening of 1-deoxynojirimycin (DNJ) producing bacteria using mulberry leaf. Int J Indust Entomol, 31, 48-55.

Ju WT, Kim KY, Sung GB, Kim YS (2015b) Quantitative analysis of rutin content using silkworm genetic resources. Int J Indust Entomol 31, 56-61.

Kim HB, Kim JB, Ju WT, Kim SL, Lim JD (2017) Development of powder with increased rutin content from mulberry leaves for the application of food materials. Int J Indust Entomol 35, 77-82.

Lee JH, Lee SY, Kim B, Seo WD, Jia Y, Wu C, *et al.* (2015) Barley sprout extract containing policosanols and polyphenols regulate AMPK, SREBP2 and ACAT2 activity and cholesterol and glucose metabolism in vitro and in vivo. Food Res Int 72, 174-183.

Lee S, Koo B, Ju WT, Kim HB, Kweon H, Lee JH (2021) Effect of extraction conditions on chemical composition and antioxidant properties of mulberry fruit. Int J Indust Entomol, 42, 25-32.

Lobo V, Patil A, Phatak A, Chandra N (2010) Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 4, 118.

Luo Y, Wang L, Lv Y, Wu X, Hou C, Li J (2020) Regulation mechanism of silkworm pupa oil PUFAs on cholesterol metabolism in hepatic cell L□02. J Sci Food Agric 100, 1418-1425.

Madison BB (2016) Srebp2: A master regulator of sterol and fatty acid synthesis1. J Lipid Res 57, 333-335.

Marques LR, Diniz TA, Antunes BM, Rossi FE, Caperuto EC, Lira FS, *et al.* (2018) Reverse cholesterol transport: molecular mechanisms and the non-medical approach to enhance HDL cholesterol. Front Physiol 9, 526.

Nam YR, Ko YE, Ly SY (2018) Anti-obesity and LDL-cholesterol lowering effects of silkworm hemolymph in C57BL/6N mice fed high fat diet. J Nutr Health 51, 201-207.

Nino MC, Reddivari L, Osorio C, Kaplan I, Liceaga AM (2021) Insects as a source of phenolic compounds and potential health benefits. J Insect Food Feed 1-12.

Park YM, Kim KA, Kang MU, Park KH, Nho SK (2014) Screening of silkworm strains for efficient recombinant protein production by Autographa californica nucleopolyhedrosis virus (AcNPV). Int J Indust Entomol 28, 10-18.

Poljšak B, Dahmane R (2012) Free radicals and extrinsic skin aging. Dermatol Res pract 2012.

Ryu KS, Lee HS, Kim KY, Kim MJ, Sung GB, Ji SD, *et al*. (2013) 1-Deoxynojirimycin content and blood glucose-lowering effect of silkworm (Bombyx mori) extract powder. Int J Indust Entomol 27, 237-242.

Shahidi F, Ambigaipalan P (2015) Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. J Funct Foods 18, 820-897.

Sharpe LJ, Brown AJ (2013) Controlling cholesterol synthesis beyond 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). J Biol Chem 288, 18707-18715.

Son KH, Lee JY, Lee JS, Kang SS, Sohn HY, Kwon CS (2017) Screening of phenolic compounds with inhibitory activities against HMG-CoA reductase. J Life Sci 27, 325-333.

Valenzuela A, Sanhueza J, Nieto S (2003) Cholesterol oxidation: health hazard and the role of antioxidants in prevention. Biol Res 36, 291-302.

Wang MX, Lu Y, Cai ZZ, Liang S, Niu YS, Miao YG (2013) Phenol oxidase is a necessary enzyme for the silkworm molting which is regulated by molting hormone. Mol Biol Rep 40, 3549-3555.