

Lipid-modulating effects of aqueous extract of *Rubus occidentalis* in hepatocarcinoma HepG2 cells

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To cite this article:

Young-Sook Moon, Ju-Hee Han, Tae-Bum Lee, Ji-Wung Kwon, Cheol-Hee Choi. Lipid-Modulating Effects of Aqueous Extract of *Rubus Occidentalis* in Hepatocarcinoma HepG2 Cells. *American Journal of BioScience*. Vol. 2, No. 2, 2014, pp. 64-69.

doi: 10.11648/j.ajbio.20140202.18

Abstract: Little knowledge exists on the lipid-modulating effect of *Rubus occidentalis* (RO). The present study investigated the molecular mechanisms of lipid-modulating effects of aqueous extract of RO (ROW) in hepatocarcinoma HepG2 cells. ROW decreased apolipoprotein B100 (ApoB100)/apolipoprotein A-1 (ApoA-1) ratio. ROW increased the expression of LDL-receptor (LDL-R). ROW decreased the gene expression of sterol regulatory element-binding protein 1c (SREBP-1c) as well as fatty acid synthase in a concentration-dependent manner. ROW not only down-regulated gene expression of SREBP-2 and HMG-CoA synthase mRNA expression, but also inhibited HMG-CoA reductase activity in a concentration-dependent manner ($IC_{50} = 240.6 \mu\text{g/ml}$). These results indicate that ROW decreases the level of bad cholesterol and increases good cholesterol levels by lowering the gene expression of transcription factors, SREBP-1c and SREBP-2, and by inhibiting the expression and/or activity of their downstream enzymes such as fatty acid synthase and HMG-CoA synthase and reductase.

Keywords: *Rubus Occidentalis*, Apolipoprotein B100, Apolipoprotein A-1, LDL-Receptor, Sterol Regulatory Element-Binding Protein (SREBP), HMG-CoA Reductase

1. Introduction

Rubus, which belongs to the Rosaceae family, is composed of 600–800 species all over the world [1]. Since raspberries contain a high number of phytochemicals with biological activities, its fruit has been used as an ingredient in traditional medicine [2,3]. *Rubus coreanus* (*R. coreanus* called Bokbunja in Korea) is a native raspberry in Korea. In fact, the majority of fruits grown for producing the consumable drink is *Rubus occidentalis* (*R. occidentalis*, black raspberry), which is native to North America. Raspberry anthocyanins added to drinking water, not as whole berries, prevented the development of dyslipidemia and obesity in mice [4]. On the contrary, black raspberry anthocyanins do not alter serum cholesterol and triglycerides levels and development of obesity in the obesity mouse model [5]. Thus, the lipid-modulating effect of *R. occidentalis* is little known and remains controversial. Therefore, the present study was performed to elucidate the molecular mechanisms by which *R. occidentalis* modulates

lipid metabolism using HepG2.

2. Materials and Methods

2.1. Materials

Preliminary study showed that polyphenol compounds of unripe fruits of *R. occidentalis* collected from Gochang (Jeollabuk-Do) area in South Korea was 3 times more than those of ripe one. Thereafter, the unripe fruits of *R. occidentalis* were extracted twice with 100°C tap water for future commercial production instead of distilled water using a reflux condenser. The extracts were filtered and concentrated. The concentrates were lyophilized in a freeze-dryer. Ellagic acid was used as marker compound to develop suitable identification test for raw materials.

2.2. Cell Culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen),

supplemented with 10% heat-inactivated fetal bovine serum and 100 µg/ml antibiotics in 5% CO₂ incubator at 37°C.

2.3. Determination of ApoB100 and ApoA-1

The secretion of ApoB100 and ApoA-1 was determined according to the already reported protocol [6]. Briefly, HepG2 cells (1.5×10^5) were seeded on 24 wells and cultured for 2 days. After washing with PBS, they were incubated in 1% BSA in FBS-free DMEM media with drugs for 4 days. Cultured media were harvested and centrifuged ($15000 \times g$, 5 min), and then the supernatants were used for ApoB100 and ApoA-1 detection using the ELISA kit (ApoB100: ABIN415075, antibodies-online, Inc., USA; ApoA-1: 3710-1HP-10, Mabtech, Inc., USA) with µQuant ELISA Reader (Bio-Tek Instruments, Inc., Vermont). Each data was normalized with protein quantification.

2.4. Determination of Triglyceride and Cholesterol

HepG2 cells (2×10^6) were seeded on 100 mm dish and grown until confluence and then media were exchanged with FBS-free DMEM for 12 h. The amount of triglycerides was determined using the colorimetric assay kit (Cayman chemical, Michigan, USA) with µQuant ELISA Reader. Cholesterol levels were determined using the total cholesterol assay kit (Cell Biolabs, Inc., California, USA) using a luminescence spectrophotometer (Perkin Elmer, Massachusetts, USA).

2.5. 3-Hydroxy-3-Methylglutaryl-CoA (HMG-CoA) Reductase Activity Assay

HMG-CoA reductase activity was determined using the HMG-CoA reductase assay kit (CS1090, Sigma, MO, USA). Enzymatic reaction was performed in the presence or absence of drugs for 10 minutes at 37°C in a 96-well plate. The inhibition potential of HMG-CoA reductase was expressed as IC₅₀ (50% inhibition concentration) by detecting O.D. change of NADPH at 340 nm using a VersaMax Absorbance Microplate Reader (Molecular Devices, CA, USA). Pravastatin in the kit was used to validate the assay system, and atorvastatin (PZ0001, Sigma Aldrich, MO, USA) (2.5 µM) was used as a positive control.

2.6. RNA Purification and cDNA Preparation

After 24 hours of culture with different doses of ROW in 6 wells, HepG2 cells were harvested and total RNA was purified using TRI reagent (RNAiso Plus, Takara, Japan). For cDNA synthesis, 1 µg of total RNA was used in 20 µl reactions with moloney murine leukemia virus reverse transcriptase (Promega, Wisconsin, USA) and oligodT primer. Reverse transcription was performed using an Autorisierter Thermocycler (Eppendorf AG, Hamburg, Germany).

2.7. Real-Time PCR

Real-time qPCR was performed using SYBR Green Master Mix (Takara, Japan). Gene expression was analyzed using the Light Cycler 2.0 Instrument (Roche, Mannheim, Germany) and normalized by β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Each analysis reaction was performed in duplicate. Primers for each target gene are presented in Table 1.

Table 1. Primer sequences, size of amplicon and annealing temperatures

Gene	Primer sequence
ApoA-1	S 5'-TGTGTACGTGGATGTGCTCAAAGA-3' AS 5'-GTCACGCTGTCCAGTTGTCA-3'
ApoB100	S 5'-TCGCCTGCCAAACTGCTTC-3' AS 5'-CATTGGTGCCTGTGTTCCATTC-3'
FAS	S 5'-CAGCAGTTCACGGACATGGAG-3' AS 5'-CGGCACGCAGCTTGTAGTAGA-3'
HMG-CoA reductase	S 5'-CCAGCTTGTGTGCTCTTGGTATTA-3' AS 5'-GGCTGAGCTGCCAAATTGGA-3'
HMG-CoA synthase	S 5'-GTATGCCCTGGTAGTTGCAGGAG-3' AS 5'-TGTTGCATATGTGTCCACGAA-3'
LDL-R	S 5'-CAATGTCTCACCAAGCTCTG-3' AS 5'-TCTGTCTCGAGGGGTAGCTG-3'
SREBP-1c	S 5'-TTGCGCAAGGCCATCGACTACATT-3' AS 5'-ACAAGGGGCTGCTCTGGAAAG GTG-3'
SREBP-2	S 5'-CTATGGAGCAGCCTCAACGTCA-3' AS 5'-CCGTAGCGACAGTAGCAGGTCA-3'

ApoA-1, apolipoprotein A1; ApoB100, apolipoprotein B100; FAS, fatty acid synthase; LDLR, low-density lipoprotein receptor; SREBP-1c, sterol regulatory element-binding protein 1c.

2.8. Western Blot Analysis

After incubation with test sample, cells were washed with PBS and lysed in 50 mM Tris-Cl (pH 7.4), 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The Bradford method was used to determine the concentration of proteins [7]. Western blotting analysis was performed using a slight modification of the method described previously [8]. Membranes were then blocked with 5% skim milk in Tris-buffered saline (TBS; 25 mM Tris pH 7.4, 136 mM NaCl, 2.6 mM KCl) containing 0.1% Tween-20 and incubated with primary rabbit polyclonal antibodies overnight at 4 °C for anti-SREBP-1c (Novus Biologicals, Littleton, CO, USA), anti-SREBP-2 (Abcam, Cambridge, UK), anti-LDLR (Abcam, Cambridge, UK), anti-GAPDH (Santa Cruz, CA, USA) and then with horseradish peroxidase-conjugated secondary antibody. Thereafter, membranes were stained using the detection reagent of the ECL detection kit (Amersham, Piscataway, NJ, USA).

3. Results and Discussion

3.1. Effects of ROW on Secretion and Expression of ApoB100 and ApoA-1

Recently, ApoB100 (a major component of LDL) and

ApoA-1 (a major HDL-c component) involved in lipid clearance are believed to be an accurate measure for predicting cardiovascular diseases compared to parameters such as lipoproteins including LDL and HDL used previously [9, 10]. The higher the ApoB100/ApoA-1 ratio, more atherogenic the cells and vice versa. In this study, HepG2 cells were treated with ROW in the presence of Na-oleate (0.75 mM) to enhance lipid secretion for 4 days. The amount of secreted ApoB100 and ApoA-1 was regulated by protein. We found that ApoB100 secretion was decreased by ROW whereas ApoA-1 secretion was increased by ROW, as compared to the control, resulting in a decrease of ApoB100/ApoA-1 ratio by 0.41(88.1%) and 0.34 (71.9%) as compared with the control ratio (0.48) after it was treated with 10 and 50 µg/ml of ROW, respectively. This result helped in indicating that ROW contains substances with hypolipidemic property.

To determine the effect of ROW on the mRNA expression of ApoA-1 and ApoB100, HepG2 cells (3×10^5) were treated with various concentrations of ROW for 24 hours. ROW (5–50 µg/ml) increased the mRNA expression of ApoA-1, but decreased the mRNA expression of ApoB100 as atorvastatin did, which has been shown to decrease ApoB100/ApoA-1 ratio (Figure 1). In fact, statins are known as the most effective lipid-lowering drugs because of their involvement in lipid removal mechanisms such as inhibiting the secretion of proatherogenic lipoprotein from liver and intestine [11]. But statin-induced side effects such as hepatotoxicity and myopathy pose great concern [12]. Therefore, ROW lacking adverse effects may be an alternative to overcome these problems.

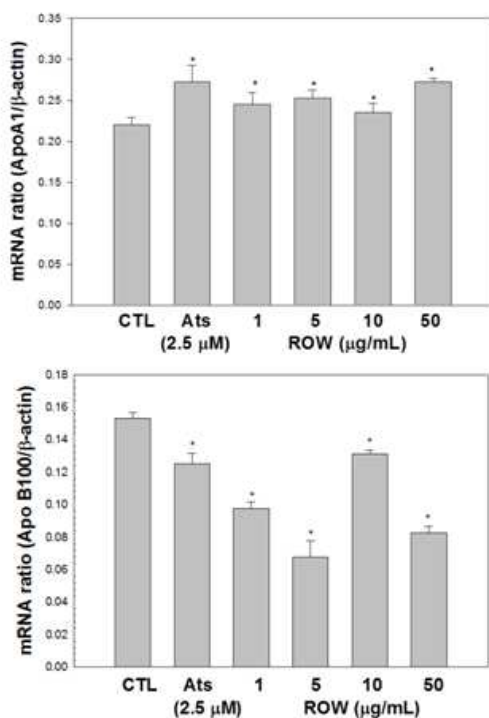


Figure 1. Effect of ROW on mRNA expression of ApoA-1 and ApoB100 in HepG2 cells. Ats, atorvastatin; ROW, *R. occidentalis* water extracts. *, $P < 0.05$ vs CTL ($n = 3$).

3.2. Effect of ROW on LDL Receptor (LDL-R) Expression

Since excess of cholesterol is very toxic, it is regulated very precisely to maintain appropriate blood concentrations [13]. Generally, when intracellular cholesterol increases, it leads to a reduction of the synthesis of LDL-R and HMG-CoA reductase, resulting in a decreased cholesterol input from plasma and then reduced endogenous cholesterol synthesis. Thus, cholesterol homeostasis is exquisitely regulated by the dual regulatory feedback system such as LDL-R and HMG-CoA reductase [14]. The genetic defects of LDL-R are observed in familial hypercholesterolemia patients [14]. In this study, we found that the expression of LDL-R mRNA in HepG2 cells was decreased by the addition of less than 10 µg/mL ROW for 24 hour but increased significantly with the addition of 50 µg/ml ROW (Fig. 2A). Western blot analysis demonstrated that ROW increased LDL-R protein production but atorvastatin resulted in no change (Fig. 2B). These results are in agreement with the previous reports that drugs and plant-derived components having hypolipidemic effects upregulate LDL-R and decrease lipid accumulation [15,16].

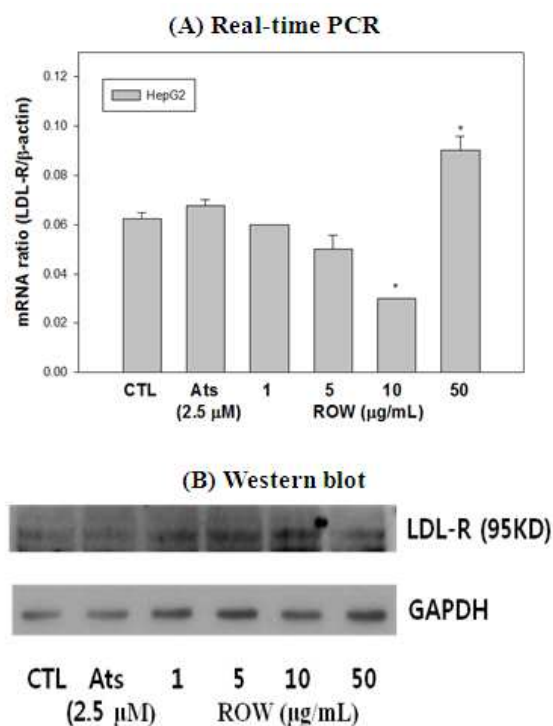


Figure 2. Effect of ROW on gene expression of LDL-R. $P < 0.05$ vs CTL ($n = 3$).

3.3. Effect of ROW on Synthesis of Cholesterol and Triglyceride

Regulation of lipogenic gene expression is mediated by transcription factors such as SREBPs [17, 18]. SREBP-1c selectively induces lipogenic genes mediating fatty acid and triglyceride metabolism [19], whereas SREBP-2 is selectively involved in cholesterol metabolism [20, 21].

ROW significantly inhibited the expression of SREBP-1/2 mRNA as well as the expression of protein in a concentration-dependent manner (Figure 3). But the addition of 50 $\mu\text{g/ml}$ of ROW showed variable effects on SREBP-1/2 mRNA expression, which may be due to concentration-dependent effects of many hydrophilic compounds.

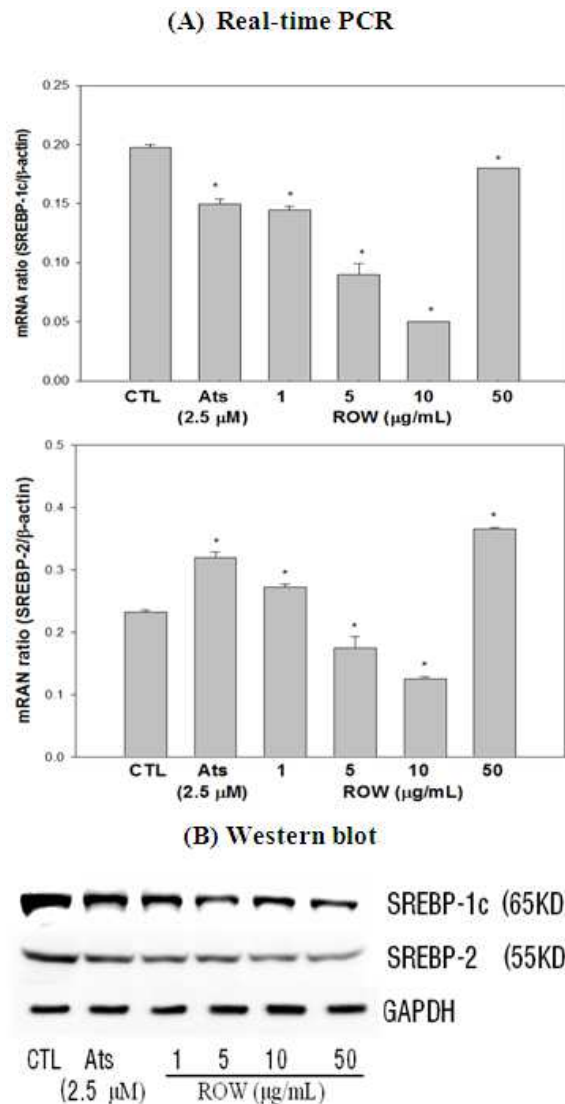


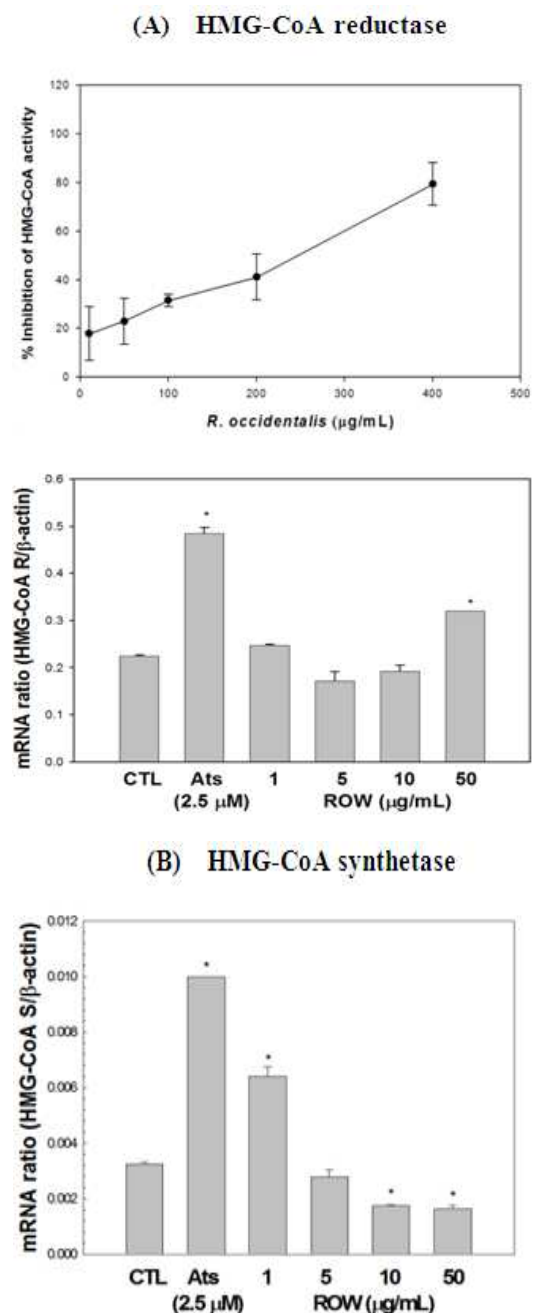
Figure 3. Effect of ROW on SREBP-2 and SREBP-1c gene expression in HepG2 cells. *, $P < 0.05$ vs CTL ($n = 3$).

3.4. Effect of ROW on HMG-CoA Reductase Activity

Statins such as atorvastatin are well-known inhibitors of HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis [22]. In this study, we examined if ROW inhibits the activity of HMG-CoA reductase. As shown in Figure 4(A), ROW inhibits HMG-CoA reductase in a concentration-dependent manner but not mRNA expression of HMG-CoA reductase. But the IC_{50} value (240.6 $\mu\text{g/ml}$) of ROW for HMG-CoA reductase is higher than the nanomolar IC_{50} of statins [23] and 50 $\mu\text{g/ml}$ of garlic water extract [24]. HMG-CoA reductase activity was found to be

inhibited by 84% with the addition of 400 $\mu\text{g/ml}$ of ROW. What compounds the inhibition of HMG-CoA reductase activity in ROW remains to be determined.

In addition to HMG-CoA reductase, HMG-CoA synthase known as a rate-limiting enzyme for cholesterol synthesis is also the target gene of SREBP-2 [25]. It was observed in this study that 10 and 50 $\mu\text{g/ml}$ of ROW down-regulated mRNA expression of HMG-CoA synthase (Figure 4B), resulting in decreased level of cholesterol as an end-product (Figure 4C). On the other hand, atorvastatin did not alter SREBP-2 mRNA expression and even increased mRNA expression of these target enzymes: HMG-CoA synthetase and reductase. It was already reported that atorvastatin increased the expression of mRNA of HMG-CoA reductase in HepG2 cells as a feedback regulation [26].



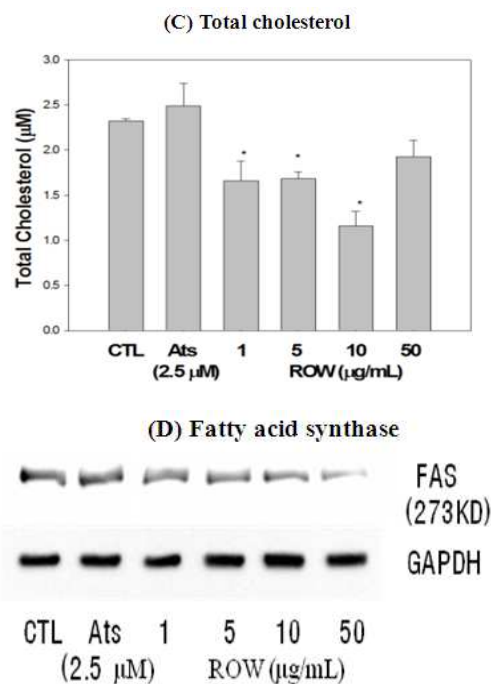


Figure 4. Effect of ROW on activity and mRNA expression of HMG-CoA reductase (A), mRNA expression of HMG-CoA synthase (B), total cholesterol level (C) and fatty acid synthase (D). *, $P < 0.05$ vs CTL ($n = 3$).

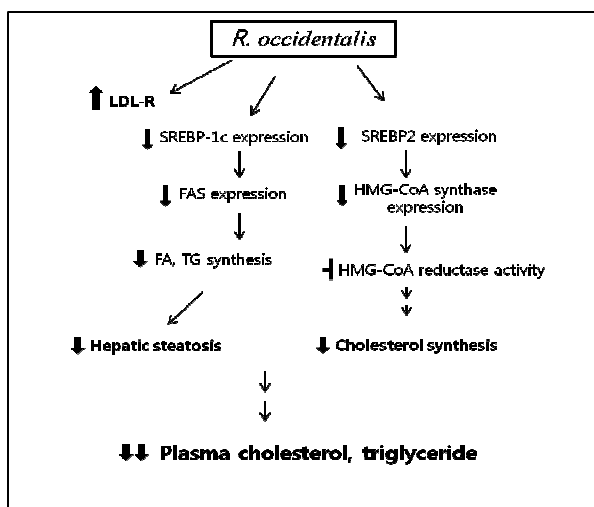


Fig 5. Lipid-modulating effects of ROW in HepG2 cells. ↓, inhibition.

Thus, ROW has been shown to down-regulate cholesterol synthesis in HepG2 cells by inhibiting SREBP, HMG-CoA synthase and HMG-CoA reductase, which in turn presumably increased expression of LDL-R as shown in Fig. 2. It was reported that polyphenols obtained from red wine displayed remarkable hypolipidemic effects through similar mechanisms [27, 28]. Although 10 μg/ml of ROW only decreased the level of triglycerides slightly (data not shown), it also decreased the expression of protein in SREBP-1c and downstream fatty acid synthase in a concentration-dependent manner in HepG2 cells (Fig. 4D).

Taken together, the lipid-modulating effects of ROW can

be summarized as in Fig. 5.

4. Conclusion

ROW decreases the level of bad cholesterol and increases good cholesterol levels by lowering the gene expression of transcription factors, SREBP-1c and SREBP-2, and by inhibiting the expression and/or activity of their downstream enzymes such as fatty acid synthase and HMG-CoA synthase and reductase. Therefore, it can be expected that ROW could show a beneficial effect on fatty liver disease via mechanism involving down-regulation of SREBP-1c. Together with down-regulated expression of cholesterol, ROW might decrease the secretion of the bad cholesterol such as VLDL, IDL and LDL from the liver, thereby exerting hypolipidemic effect.

Acknowledgments

This study was supported by the 2011 Region Agrifood Lead Cluster Promotion Project through Gochang Black Raspberry Research Institute and Gochang-gun, Korea.

References

- [1] Bushakra JM, Stephens MJ, Atmadjaja AN, Lewers KS, Symonds VV, Udall JA, et al. Construction of black (*Rubus occidentalis*) and red (*R. idaeus*) raspberry linkage maps and their comparison to the genomes of strawberry, apple, and peach. *Theor. Appl. Genet.* 2012; 125(2): 311-27.
- [2] Seeram NP. Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *J Agric Food Chem.* 2008; 56(3): 627-9.
- [3] Schreckinger ME, Lotton J, Lila MA, de Mejia EG. Berries from South America: a comprehensive review on chemistry, health potential, and commercialization. *J. Med. Food.* 2010; 13(2): 233-46.
- [4] Prior RL, Wu X, Gu L, Hager T, Hager A, Wilkes S, et al. Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet. *Mol. Nutr. Food Res.* 2009; 53(11): 1406-18.
- [5] Prior RL, Wilkes S, Rogers T, Khanal RC, Wu X, Hager TJ, et al. Dietary black raspberry anthocyanins do not alter development of obesity in mice fed an obesogenic high-fat diet. *J. Agric. Food Chem.* 2010; 58(7): 3977-83.
- [6] Itoh M, Abe Y, Iwama Y, Kimura F, Satoh M, Shoji M, et al. HPLC analysis of lipoproteins in culture medium of hepatoma cells: an in vitro system for screening antihyperlipidemic drugs. *Biotechnol. Lett.* 2009; 31(7): 953-7.
- [7] Schleicher E, Wieland OH. Evaluation of the Bradford method for protein determination in body fluids. *J Clin Chem Clin Biochem.* 1978; 16(9): 533-4.
- [8] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 1979; 76(9): 4350-4.

- [9] Scanu AM. Lipoprotein(a) as a cardiovascular risk factor. *Trends Cardiovasc. Med.* 1991; 1(7): 294-9.
- [10] Heneghan HM, Huang H, Kashyap SR, Gornik HL, McCullough AJ, Schauer PR, et al. Reduced cardiovascular risk after bariatric surgery is linked to plasma ceramides, apolipoprotein-B100, and ApoB100/A1 ratio. *Surg. Obes. Relat. Dis.* 2013; 9: 100-107.
- [11] Goldstein JL, Brown MS. The LDL receptor. *Arterioscler. Thromb. Vasc. Biol.* 2009; 29(4): 431-8.
- [12] Jacobson TA. Statin safety: lessons from new drug applications for marketed statins. *Am. J. Cardiol.* 2006; 97(8A): 44C-51C.
- [13] Klopotek A, Hirche F, Eder K. PPAR gamma ligand troglitazone lowers cholesterol synthesis in HepG2 and Caco-2 cells via a reduced concentration of nuclear SREBP-2. *Exp. Biol. Med.* 2006; 231(8): 1365-72.
- [14] Yuan G, Wang J, Hegele RA. Heterozygous familial hypercholesterolemia: an underrecognized cause of early cardiovascular disease. *CMAJ.* 2006; 174(8): 1124-9.
- [15] Vijayakumar MV, Pandey V, Mishra GC, Bhat MK. Hypolipidemic effect of fenugreek seeds is mediated through inhibition of fat accumulation and upregulation of LDL receptor. *Obesity.* 2010; 18(4): 667-74.
- [16] Scharnagl H, Marz W. New lipid-lowering agents acting on LDL receptors. *Curr. Top. Med. Chem.* 2005; 5(3): 233-42.
- [17] Kim HJ, Miyazaki M, Man WC, Ntambi JM. Sterol regulatory element-binding proteins (SREBPs) as regulators of lipid metabolism: polyunsaturated fatty acids oppose cholesterol-mediated induction of SREBP-1 maturation. *Ann. NY Acad. Sciences.* 2002; 967: 34-42.
- [18] Yamamoto T, Shimano H, Inoue N, Nakagawa Y, Matsuzaka T, Takahashi A, et al. Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of liver X receptor in the liver. *J. Biol. Chem.* 2007; 282(16): 11687-95.
- [19] Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie.* 2004; 86(11): 839-48.
- [20] Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* 2002; 109(9): 1125-31.
- [21] Jeon TI, Osborne TF. SREBPs: metabolic integrators in physiology and metabolism. *Trends Endo. Metabol.* 2012; 23(2): 65-72.
- [22] Kajinami K, Mabuchi H, Saito Y. NK-104: a novel synthetic HMG-CoA reductase inhibitor. *Expert Opin. Investig. Drugs.* 2000; 9(11): 2653-61.
- [23] Parker RA, Clark RW, Sit SY, Lanier TL, Grosso RA, Wright JJ. Selective inhibition of cholesterol synthesis in liver versus extrahepatic tissues by HMG-CoA reductase inhibitors. *J. Lipid Res.* 1990; 31(7): 1271-82.
- [24] Gebhardt R. Inhibition of cholesterol biosynthesis by a water-soluble garlic extract in primary cultures of rat hepatocytes. *Arzneimittelforschung.* 1991; 41(8): 800-4.
- [25] Sakakura Y, Shimano H, Sone H, Takahashi A, Inoue N, Toyoshima H, et al. Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis. *Biochem. Biophys. Res. Commun.* 2001; 286(1): 176-83.
- [26] Conde K, Roy S, Freake HC, Newton RS, Fernandez ML. Atorvastatin and simvastatin have distinct effects on hydroxy methylglutaryl-CoA reductase activity and mRNA abundance in the guinea pig. *Lipids.* 1999; 34(12): 1327-32.
- [27] Pal S, Ho N, Santos C, Dubois P, Mamo J, Croft K, et al. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *J. Nutr.* 2003; 133(3): 700-6.
- [28] Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science.* 1986; 232(4746): 34-47.