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Quantification and comparison of functional phytochemicals in steamed and freeze-dried mature silkworm powders and freeze-dried mulberry leaves

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Abstract

Various health promoting effects of steamed and freeze-dried mature silkworm powder (SMSP) have been reported. However, it is not still clear which substances in SMSP are responsible for those health promoting effects yet. In this study, we examined and compared the quantities of phytochemicals in SMSP and freeze-dried mulberry leave powder (FMLP). To investigate the optimal solvent for extracting phytochemicals from SMSP and FMLP, we used four different solvents. Among them, 80% ethanol extracts from SMSP and FMLP contained the highest amount of five flavonoids examined. In addition, FMLP had high contents of flavonoids compared with those of SMSP. The amounts of rutin, isoquercetin, astragalin, quercetin, and kaempferol in FMLP were 5.078 ± 0.187 mg/g, 5.162 ± 0.083 mg/g, 2.989 ± 0.061 mg/g, 3.317 ± 0.236 mg/g, and 2.243 ± 0.237 mg/g, respectively, while the amounts of rutin, isoquercetin, astragalin, quercetin, and kaempferol in SMSP were 0.171 ± 0.024 mg/g, 0.252 ± 0.032 mg/g, 0.374 ± 0.031 mg/g, 0.645 ± 0.063 mg/g, and 0.0512 ± 0.047 mg/g, respectively. Taken together, SMSP could be a source for providing various and readily absorbable flavonoids

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Introduction

The sericulture is an industry with a long history of over 5,000 years (Cherry, 1989). Recently, the sericulture industry in Korea is focusing not only on the production of cocoons that make natural silk fabrics, but also on the production of various kinds of byproducts having a variety of health promoting properties (Ji *et al*., 2015; Ji *et al*., 2016a; Nguyen *et al.,* 2016). The various health promotion effects of silkworm and mulberry products have been known to the public for hundreds of years and were described in many ancient oriental medical literatures in Eastern Asian countries. Well known examples are including hypoglycemic effects of freeze-dried the 3rd day of fifth instar larvae (FDSP) (Ryu *et al*., 2013), sexual function improvement

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effects of male pupal extracts (Oh *et al*., 2012), and cognitive and memory improvement effects of fibroins, one of major components of silk proteins (Kim *et al*., 2005). Those functional materials responsible for health improvement effects of silkworm larvae and pupae were present in mature silkworms that had enlarged silk glands with various functional materials (Ji *et al*., 2016b; Ji *et al*., 2016c). However, if silk glands were not treated properly, they were too hard to chew and eat (Ji *et al*., 2015). In previous study, we found if mature silkworms were steamed for 130 min followed by freeze-drying at -50^{o}C, they could be easily chew to eat (Ji *et al*., 2015; Ji *et al*., 2017).

Recent studies in health improvement effects of steamed and freeze-dried mature silkworm powder (SMSP) have shown that there is an increased healthspan, enhanced resistance to Parkinson's disease (Ji *et al*., 2016a; Nguyen *et al*., 2016), improvement in liver detoxification ability (Cho *et al*., 2016), and skin whitening effects (Kim *et al*., 2017).

It has not yet been determined which substances are responsible for reported health promoting effects that appear when ingesting SMSP. However, recent proximate analysis of SMSP showed that it has high contents of proteins and amino acids compared with FDSP or freeze-dried mature silkworm powder (FMSP). In addition, the ratios of omega 3 vs. omega 6 unsaturated fatty acids and potassium vs sodium were very high, supporting possible involvement of fatty acids and minerals in health promoting effects of SMSPs (Ji *et al*., 2016b; Ji *et al*., 2016c). In addition, SMSP must have certain amounts of phytochemicals originated from the mulberry leaf. Since phytochemicals such as flavonoids, carotenoids, or phenolic compounds in mulberry leaves were shown to act as scavengers of free radical species generated during normal development or respirations, the kinds and quantity of various phytochemicals in mulberry leaves were investigated (Arabshahi-Delouee and Urooj, 2007; Iqbal *et al*., 2012). However, it is not still clear how much phytochemicals SMSP has. Thus, in this study, we extracted phytochemicals using four different solvents and then quantity of five flavonoids in FDML and SMSP were determined by Triple Quadrupole Liquid Chromatorygraphy-Mass spectrometry (Finnigan TSQ Quantum Ultra EMR). Our results suggested that flavonoids were most efficiently extracted by 80% ethanol. Five flavonoids were present from 2.243 ± 0.263 mg/g to 5.162 ± 0.083 mg/g in FMSP, while five flavonoids were present from 0.171 ± 0.024 mg/g to 0.645 ± 0.063 mg/g in SMSP.

Materials and Methods

Steamed and freeze-dried mature silkworm powder (SMSP) and freeze-dried mulberry leaf powder (FMLP) production protocol.

The white mulberry, *Morus alba* L used in this study were grown at the National Institute of Agricultural Science, Wanju-gun, Jellabook-do, Korea. The mulberry silkworm, *Bombyx mori* white jade strain (also known as "Baeokjam") used in this study was raised on white mulberry leaves. Freeze-dried mulberry leaf powder (FMLP) was prepared by lyophilizing mulberry leaves at 50°C (FDT-8612, Operon Ltd., Kimpo, Korea) and sequentially grounded twice using a hammer mill (HM001, Korean Pulverizing Machinery Co. Ltd., Incheon, Korea) and a disk mill (Disk Mill01, Korean Pulverizing Machinery Co. Ltd.).

Steamed and freeze-dried silkworm powder (SMSP) was generated according to previous reports (Ji et al., 2015). Mature silkworms were steamed at 100°C for 130 min using an electric pressure-free cooking machine (Kum Seong Ltd., Boocheon, Korea), freeze-dried at -50°C (FDT-8612, Operon Ltd.), and then pulverized to generate SMSP as describe above.

Phytochemical extraction using four different solvents

We used 80% and 100% ethanol (EtOH, DaeJung Chemical & Metal Co., Seoul, Korea) and 80% and 100% methanol (MeOH, DaeJung Chemical & Metal Co.) for extracting phytochemicals from FDML and SMSP. One g of FDML or SMSP was mixed with 10 ml of a solvent and then agitated for 1 hr at 25^{o}C using an orbital shaker. After spinning down at 3,000 x g for 15 min at room temperature, supernatants were obtained and filtered with 0.45 μm syringe filters (Sartorius AG, Gottingen, German) and then lyophilized at -50^{o}C with a Freeze dryer 8 (Labconco, Kansas City, MO, USA). Five biological replicates were generated for each solvent and sample.

Triple Quadrupole Liquid chromatography-Mass spectrometry

The amounts of five flavonoids including rutin, quercetin, isoquercetin, astragalin, and kaempferol in extracts were

Table 1. The gradient of the mobile phase for Triple quadrupole LC-Mass spectrometry.

Time	0.1% formic acid	Acetonitrile
0	90	10
10	10	90
13	10	90
14	90	10
10	90	10

quantified by using a Finnigan TSQ Quantum ultra EMR (Thermo Fisher Scientific, Waltham, MA, USA) equipped with Unison US-C18 (2 x 150 mm, 5 μm, Imtakt, Walnut, PA, USA). Analytical standards of five flavonoids (Sigma-Aldrich, St. Louis, MO, USA) were dissolved and diluted with MeOH. Dried extracts were dissolved in 200 μl of MeOH, filtered with a 0.45 μm pore size syringe filter, and then diluted x 100 for FDML and x 10 for SMSP with MeOH before analyzing. A mobile phase for LC was mixed with 0.1 % formic acid and Acetonitrile (Sigma-Aldrich). Serial diluted standards were used to generate standard curves for each flavonoid. The gradient of mobile phase was in Table 1. Ten μl of each sample was injected and flow rate was 0.2 ml/min. Analysis of variance (ANOVA) and t-test was performed using Excel program (Microsoft, Richmond, WA, USA).

Results

Rutin in FMLP and SMSP

Rutin, also known as rutinoside, quercetin-3-O-rutinoside and sophorin, is one of abundant flavonoids reported from extracts of mulberry leaves (Katsube *et al*., 2006). Since the extraction efficiency of flavonoids were variable dependent on extracting solvents, we used four different solvents to compare extracting efficiency for rutin from FDML and SMSP (Fig. 1). By performing Triple Quadrupole LC-Mass spectrometry, the amounts of rutin in 4 different extracts from FMLP and SMSP (Fig. 1). The extracted amounts of rutin from FMLP were variable among 4 solvents. When 80% EtOH were used as a solvent, the extracted amount of rutin was the highest (5.077 ± 0.189 mg/g, $p < 0.005$) compared with other solvents (100% EtOH = 0.76 ± 0.026 mg/g, 80% MeOH = 4.213 ± 0.158 mg/g, 100% MeOH = 4.084 ± 0.088 mg/g, Fig. 1B). In contrast, the amounts of rutin in four extracts from SMSP were less than 3.4% of those of FMLP (Fig. 1B and C). The amount of rutin in 80% EtOH extract was the highest (0.171 ± 0.024 mg/g, $p < 0.005$) compared with other solvents (100% EtOH = 0.0103 ± 0.0001 mg/g; 80% MeOH = 0.060 ± 0.002 mg/g, 100% MeOH = 0.045 ± 0.088 mg/g, Fig. 1C). These results suggested that 80% EtOH is the most suitable solvent for extracting rutin from FMLP and SMSP.

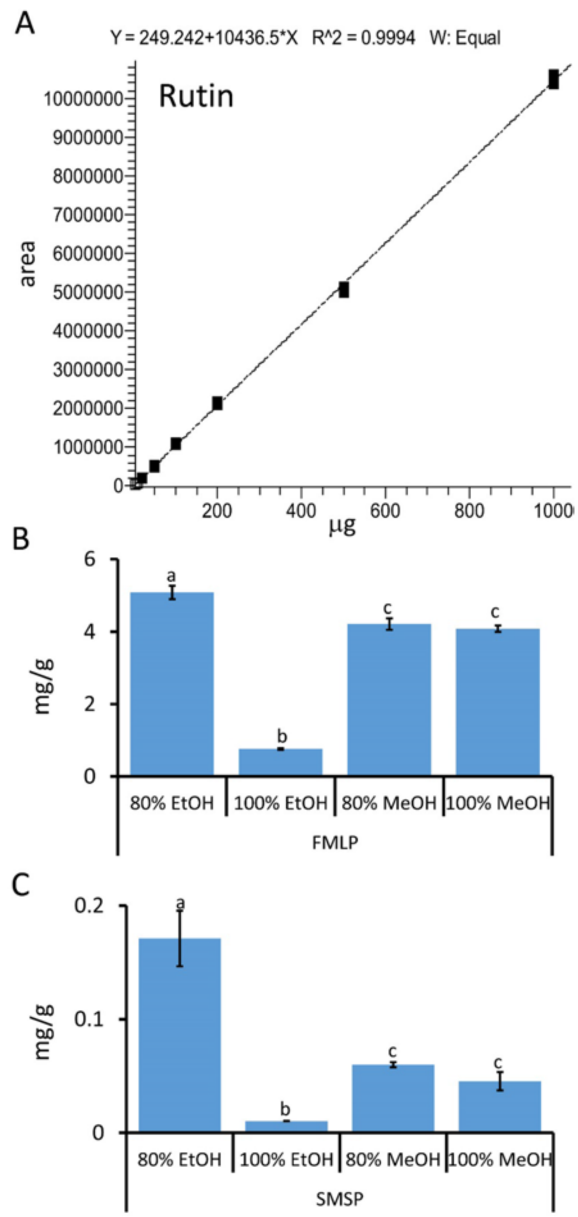

Fig. 1. The standard curve and quantification results of rutin in FDML and SMSP

Isoquercetin in FMLP and SMSP

Isoquercetin, a 3-O-glucoside of quercetin, is abundantly present in mulberry leaves (Katsube *et al.*, 2006). The extraction efficiency among four solvents was compared to identify the most suitable extracting solvents for FMLP and SMSP (Fig. 2). The extracted amounts of isoquercetin from FMLP and SMSP were variable among four solvents. 80% EtOH extracts for FMSP contained the highest amount of isoquercetin (5.162 ± 0.083 mg/g, $p < 0.005$) compared with other solvents (100% EtOH = 0.996 ± 0.027 mg/g, 80% MeOH = 4.429 ± 0.074 mg/g, 100% MeOH = 4.529 ± 0.151 mg/g, Fig. 2B). Similar to those of rutin, the amount of isoquercetin extracted from SMSP was less than 4.9% of those of FMLP (Fig. 2B and C). The amount of extracted isoquercetin in 80% EtOH was the highest (0.252 ± 0.032 mg/g, $p < 0.0005$) compare with other solvents (100% EtOH = 0.0237 ± 0.0004 mg/g, 80% MeOH = 0.115 ± 0.004 mg/g, 100% MeOH = 0.077 ± 0.010 mg/g, Figure 2C). These results suggested that 80% EtOH is the best solvent for extracting isoquercetin from samples.

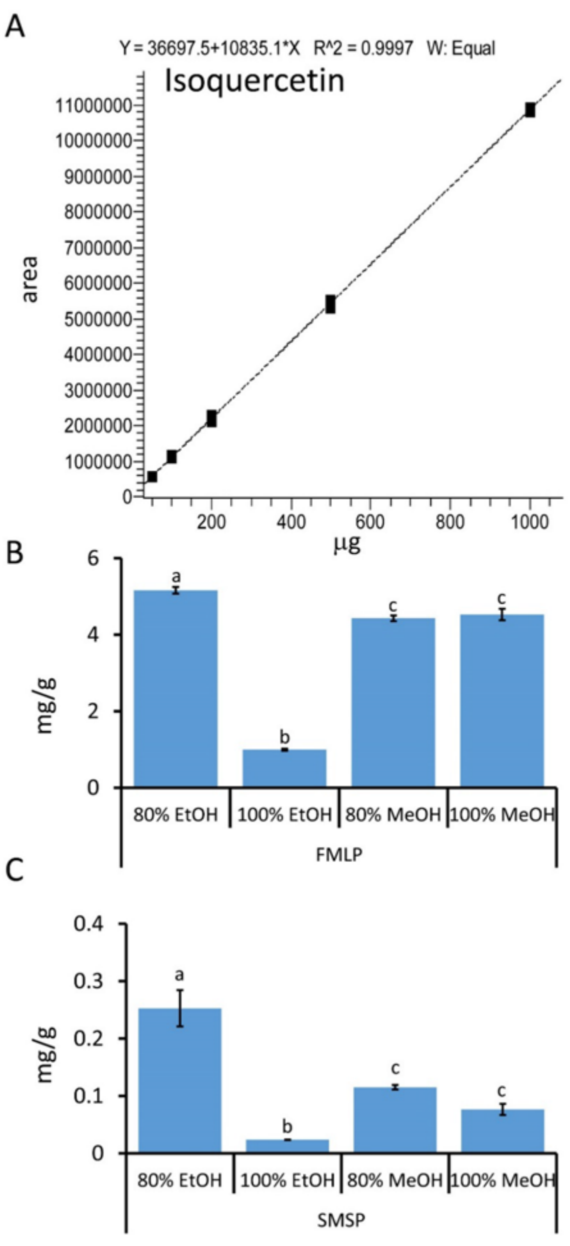

Fig. 2. The standard curve and quantification results of isoquercetin in FDML and SMSP.

Astragalin in FMLP and SMSP

Astragalin, 3-O-glucosied of kaempferol, is known to abundantly present in mulberry leaves (Katsube *et al.*, 2006). The comparison of four extraction solvents revealed that astragalin in FMLP was extracted with similar efficiencies to the three solvents. (80% EtOH = 2.989 ± 0.061 mg/g, 80% MeOH = 2.641 ± 0.083 mg/g, 100% MeOH = 2.637 ± 0.053 mg/g, $p > 0.05$, Fig. 3B). The amount of extracted astragalin was the lowest in 100% EtOH (0.544 ± 0.016 mg/g, $p < 0.005$). The amounts of astragalin extracted to 80% EtOH and MeOH to SMSP were similar (80% EtOH = 0.373 ± 0.031 mg/g, 80% MeOH = 0.313 ± 0.013 mg/g, $p > 0.05$), but significantly more than those in other solvents (100% EtOH = 0.035 ± 0.001 mg/g, 100% MeOH = 0.123 ± 0.004 mg/g, $p < 0.005$, Fig. 3C). The amounts of extracted astragalin for SMSP was less than 13% of those FMLP. These results suggested that aqueous EtOH and MeOH could be used to extracts astragalin from FMLP and SMSP.

Quercetin in FMLP and SMSP

Quercetin is a flavonoid found from wide range of plants and abundantly present in mulberry leaves (Katsube *et al.*, 2006). The extraction efficiency of quercetin to FMLP was similar among three solvents (80% EtOH = 3.316 ± 0.236 mg/g, 80% MeOH = 2.810 ± 0.142 mg/g, 100% MeOH = 2.947 ± 0.134 mg/g, $p > 0.05$, Fig. 4B). The amount of quercetin in 100% EtOH extract to FMLP was 1.475 ± 0.087 mg/g, $p < 0.005$), significantly lower than those of other extracts. The amount of quercetin in 80% EtOH extract to SMSP was the highest (0.645

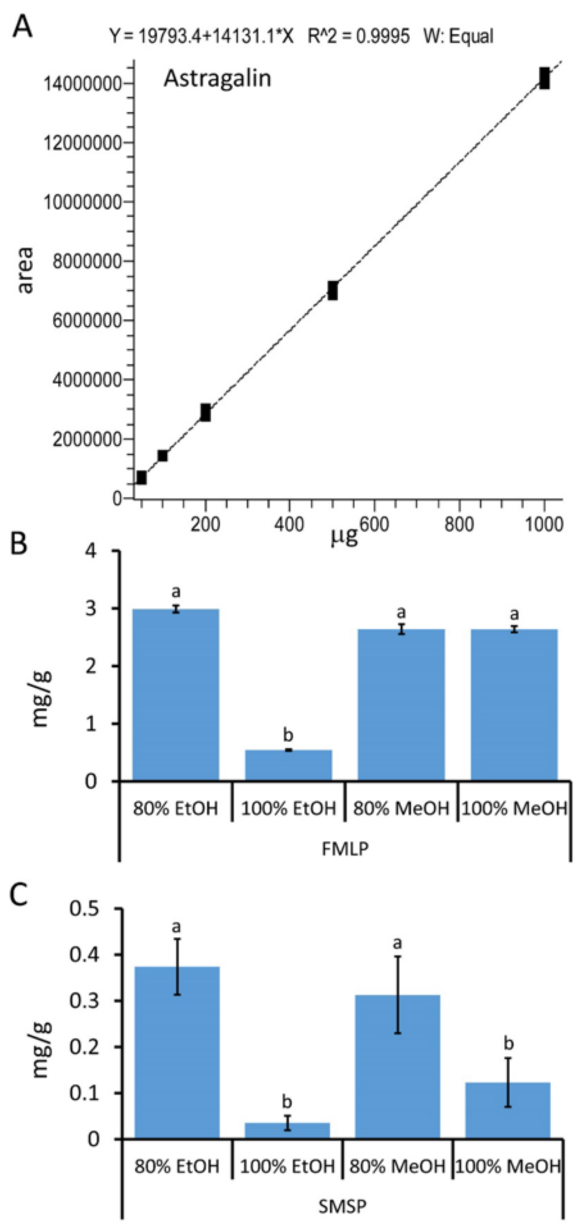

Fig. 3. The standard curve and quantification results of astragalin in FDML and SMSP.

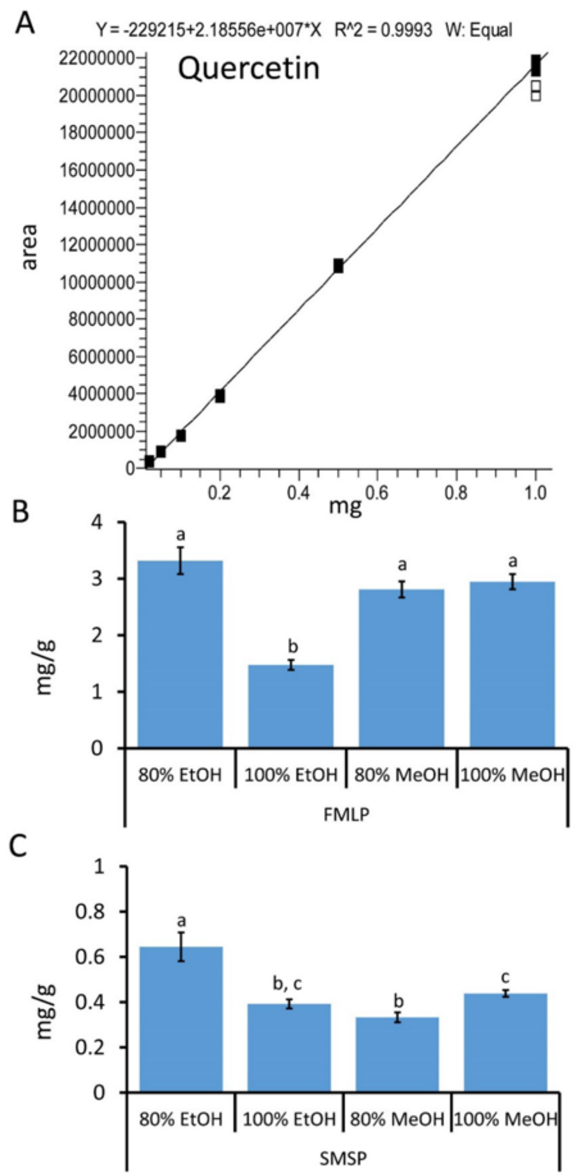

Fig. 4. The standard curve and quantification results of quercetin in FDML and SMSP.

± 0.063 mg/g, $p < 0.005$) compared with other extracts (100% EtOH = 0.392 ± 0.020 mg/g, 80% MeOH = 0.333 ± 0.022 mg/g, 100% MeOH = 0.439 ± 0.015 mg/g, Fig. 4C). These results suggested that 80% EtOH is the best option for extracting quercetin from FMLP and SMSP.

Kaempferol in FMLP and SMSP

Kaempferol is a secondary metabolite found in mulberry leaves and other many plants (Katsube *et al*., 2006). The amounts of kaempferol detected from three extracts to FMLP (80% EtOH = 2.242 ± 0.37 mg/g, 80% MeOH = 1.796 ± 0.240 mg/g, 100% MeOH = 2.078 ± 0.271 mg/g, $p > 0.05$) were similar, but that of 100% EtOH (0.870 ± 0.100 mg/g, $p < 0.005$, Fig. 5B). The amounts of kaempferol in 80% EtOH extract to SMSP were the highest (0.512 ± 0.047, $p < 0.005$, Fig. 5B) compared with those of other extracts (100% EtOH = 0.370 ± 0.013 mg/g, 80% MeOH = 0.388 ± 0.019 mg/g, 100% MeOH = 0.366 ± 0.008 mg/g, Fig. 5C). These results suggested that 80% EtOH was the most suitable solvent for extracting kaempferol.

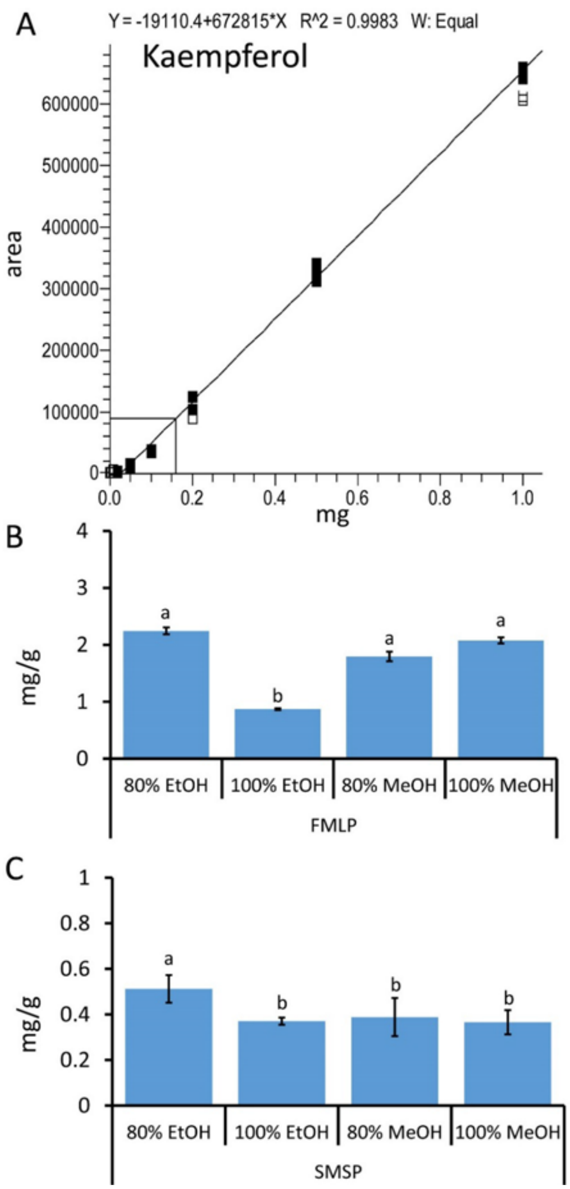

Fig. 5. The standard curve and quantification results of kaempferol in FDML and SMSP

The amounts of flavonoids in FMLP were similar to a previous report

We compared our flavonoid quantification data with those of Kim *et al.* (2014a; 2014b), Katsube *et al.* (2006), and Sánchez-Salcedo *et al.* (2015). Kim *et al.* (2014a; 2014b) showed that the amounts of rutin were variable among diverse mulberry species. Among them Chunggilppong used in this study contained 0.14% of total FDML which was only 1/3 of our quantification data (Fig. 1). The amounts of rutin, isoquercetin, and astragalin in white mulberry reported by Sánchez-Salcedo *et al.* (2015) were variable among clones. For examples, the amounts of rutins in four mulberry leave clones were ranged from 0.59 ± 0.04% to 1.21 ± 0.32%. The amounts of isoquercetin in four mulberry leave clones were ranged from 0.92 ± 0.10% to 1.53 ± 0.36%. The amounts of astragalin in four mulberry leave clones were ranged from 0.82 ± 0.05% to 1.39 ± 0.36% (Sánchez-Salcedo *et al.,* 2015). In addition, the amounts of rutin, isoquercetin, quercetin, and astraglin in FDML reported by Katsube *et al.* (2006) were 5.73 ± 0.86 mg/g, 1.94 ± 0.26 mg/g, 9.00 ± 1.46 mg/g, and 0.31 ± 0.05 mg/g, respectively. In contrast, the amounts of rutin, isoquecertin, quercetin, and astraglin obtained from this study were 5.08 ± 0.76 mg/g, 5.16 ± 0.08 mg/g, 3.32 ± 0.24 mg/g, and 2.98 ± 0.06 mg/g, respectively. The amounts of flavonoids quantified from mulberry leaves among clones and were different, suggesting that cultivation areas and growing seasons of the white mulberry might be contributed on differences in the amounts of flavonoids in the white mulberry leaves.

Discussion

Recent studies have shown that SMSP developed in 2015 (Ji *et al.,* 2015) has a wide variety of health promotion effects (Cho *et al*., 2016; Ji *et al*., 2016a; Nguyen *et al*., 2016; Kim *et al*., 2017). Although it is not still clear which substances in SMSP are responsible for health promoting effects, researches in nutrient composition studies in silkworm 3rd day of fifth instar larva, mature larvae, and pupae (Ji *et al*., 2016b; Ji *et al*., 2016c) suggested that various functional materials in them might contribute on wide range of health promoting effects. Among various functional substances, phytochemicals originated from the mulberry leaves must have important contributions, since previous studies in phytochemicals extracted from mulberry leaves have shown to involve with various health promoting effects in addition to act as natural anti-oxidants (Arabshahi-Delouee and Urooj, 2007; Aramwit *et al*., 2011; Gryn-Rynko *et al*., 2016). Thus, we hypothesized that the various health promoting effects of SMSP reported recently might be contributed by phytochemicals in SMSP. Because most of phytochemicals in silkworms were originated from mulberry leaves, we compared the amounts of five flavonoids in FMLP and SMSP (Figs. 1 ~ 5). As consistent with our expectations, SMSP contained certain amounts of five flavonoids, although the amounts of flavonoids in SMSP were consistently lower than those in FMLP. In addition, we also found that 80% EtOH was the most efficient solvents for extracting flavonoids from FMPL and SMSP, suggesting that bio-physical status of flavonoids in FMLP and SMSP were similar.

Five flavonoids investigated in this study were known to have certain health improvement effects in addition to free radical species scavenger activity. For examples, rutin was known to prevent blood clots, lower cholesterol, and reduces arthritis pains (Sattanathan *et al*., 2011). Quercetin and isoquercetin were known to reduce glucose, cholesterol and lipids in bloods and have anti-inflammatory effects (Panda and Kar, 2007). Astragalin were known to have anti-inflammatory and antioxidant effects (Choi *et al*., 2013). Kaempferol were known to have anti-cancer effects by regulating Mitogen-activated protein kinase 1 (Calderon-Montaño *et al*., 2011).

Although the amounts of five flavonoids in SMSP were only from 3.4% (rutin) to 19.4% (quercetin) of those in FMLP, flavonoids in SMSP might have certain advantages. Because SMSP does not require excipients, the actual amounts of flavonoids taken would not be lower. In addition, flavonoids in SMSPs were much easily absorbed because biophysical features of them were absorbed forms which formed complexes with other minerals or substances. Furthermore, some of flavonoids known to be toxic if high dose was treated. Taken together, SMSP contained variety of flavonoids including rutin, isoquercetin, quercetin, astragalin, and kaempferol examined in this study. Further studies in functional substances in SMSP will reveal how it can accomplish health promoting effects in animals and humans.

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