IJIE Int. J. Indust. Entomol. 45(1) 1-11 (2022)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2022.45.1.1

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

In vitro screening of anti-skin aging and antioxidant properties of aqueous/solvent extracts from distinctive stages of silkworm (*Bombyx mori* L.) pupae

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Abstract

Silkworm pupae, a by-product of the silk industry are known to be valuable resource of nutrients for humans as well as animals besides encompassing diverse bioactive constituents. However, there is a paucity of knowledge on their role in amelioration of oxidative stress and anti-skin aging properties. In the present study, we evaluated the inhibitory effect of aqueous as well as ethanolic (30% and 70%) extracts from distinct stages of male and female silkworm pupae belonging to two silkworm varieties on skin aging-related enzymes. The activities of collagenase, elastase and tyrosinase were effectively inhibited by 70% ethanolic silkworm pupal extracts (SPE), followed by 30% with aqueous extracts exhibiting meager inhibitory potential. SPE were also investigated for their antioxidant activity in oxidative-stressed murine fibroblasts (L929). The intracellular ROS and lipid peroxidation induced by *tert*-butyl hydroperoxide (*t*-BHP) in fibroblasts was better attenuated by pre treatment with ethanolic (30%) and aqueous extracts, respectively. The safety of the extracts was determined by studying their effect on fibroblast cell viability and it was found that none of the extracts were cytotoxic. Our findings indicate the potential utility of SPE as anti-aging components in cosmeceuticals.

Int. J. Indust. Entomol. 45(1), 1-11 (2022)

Received : 3 Jun 2022
Revised : 14 Jul 2022
Accepted : 24 Aug 2022

Keywords:
Silkworm pupae,
Anti-skin aging,
Skin whitening,
Antioxidant activity,
Cosmeceuticals

Introduction

Sericulture or silk farming is an allied activity of agriculture that involves the process of rearing silkworms, a monophagous lepidopteran insect deriving its nutrition from mulberry leaves, for the production of natural fiber, silk (Singh and Jayasomu, 2002). Farming fraternity across sixty countries of the world practice sericulture and the global annual production in 2020 is 91,765 metric tons according to the International Sericulture Commission (URL: https://inserco.org/en/statistics: accessed on 25 May 2022). This mammoth production is often accompanied with generation of a substantial quantum of by-products posing disposal challenges. The most notable by-product of the silk manufacturing industry is silkworm pupae. It is anticipated that every kg of raw silk production involves generation of approximately 2 kg of dry pupae as by-product (Tangsanthatkun *et al*.,

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2022; Reddy *et al*., 2021).

Silkworm pupae have long been consumed by humans, mostly in Asian countries which include China, India, Japan, Korea and Thailand, as well as in African and Latin American countries (Sadat *et al*., 2022; Wu *et al*., 2021). Silkworm pupae majorly comprise proteins rich in all essential amino acids followed by fats with high content of unsaturated fatty acids and carbohydrates. They also possess vitamins (thiamine, riboflavin, niacin and tocopherol) and minerals (calcium, sodium and iron) in trace amounts apart from anti-nutritional factors derived from mulberry including alkaloids, flavonoids, phytate, saponin and oxalate in proportions that are safe for human consumption. Consumption of 100 g of silkworm pupae provides about 230 kcal of energy in humans which is far more than chicken, egg white, milk and shrimp and is thus regarded as a balanced nutritional constituent (Wu *et al*., 2021).

Studies on health promotion aspects employing silkworm pupae and its numerous bioactive constituents have been conducted across the world for the past several decades, in addition to defining the value of the same as dietary constituents (Kumar *et al*., 2015). In general, silkworm pupae are subjected to a variety of extraction processes, and their biological and therapeutic capabilities have been studied *in vitro* and *in vivo*, with the following being the most important biomedical functionalities of silkworm pupae. A study found that 30% ethanolic SPE significantly lowered the intracellular ROS levels in *t*-BHP treated HepG2 cells (Lee *et al*., 2021a). Chitosan isolated from silkworm pupae was found to possess antibacterial and antifungal properties (Battampara *et al*., 2020). Diverse peptides of silkworm pupae were reported to exhibit anti-tumour (Li *et al*., 2018), anti diabetic (Lee *et al*., 2012) as well as immunomodulatory activities (Li *et al*., 2020; Li *et al*., 2019). The cardiovascular and hepato-protective properties of silkworm pupal oil were also demonstrated (Kim *et al*., 2020; Long *et al*., 2019).

Our previous studies have documented the nutritional composition of silkworm pupae belonging to different varieties (Baegokjam, BG and GoldenSilk, GS) of Korean origin according to the pupation time and sex wherein, it was observed that female pupae possessed higher levels of protein content and essential amino acids in comparison to males. The fatty acid composition and content varied amongst the silkworm varieties with male pupae having higher fat levels. The amount of fiber and minerals in each stage of pupal growth also differed (Lee *et al*., 2021b). Another study revealed that the antioxidant activity of aqueous and ethanolic SPE differed depending on pupation timing and sex. Extracts of silkworm pupae with shorter pupation duration exhibited stronger antioxidant activity (Lee *et al*., 2021a). The above studies clearly indicated that the biochemical composition as well as antioxidant properties of silkworm pupae varied across different sex, pupation period and varieties.

The present study was formulated with an objective to determine the efficacy of SPE in possessing skin-anti aging activities, their role in mitigation of ROS and lipid peroxidation in murine fibroblasts (L929) as well as to observe the variation of the above properties according to the extraction solvent (water, 30% and 70% ethanol), silkworm variety (BG, GS), pupation time (6-7 days: early stage, E and 11-13 days: late stage, L) and sex (male, M and female, F). Numerous natural products, most importantly extracts from different parts of diverse plant species were previously reported to exhibit skin anti-aging as well as antioxidant properties in view of the diverse bioactive components they possess (Yasin *et al*., 2017). In case of silkworm or its derived materials, silk cocoon extract (Kumar and Mandal, 2019), silk fibroin (Su *et al*., 2019), silk gland hydrolysates (Jung *et al*., 2013) and silk sericin (Kitisin *et al*., 2013; Jena *et al*., 2018; Kumar *et al*., 2018) were previously reported to possess the aforementioned properties. Since, silkworm pupae were not previously investigated for their role in inhibition of skin aging related enzymes, this study can provide valuable information on the possible use of these natural substances in cosmeceuticals.

Materials and Methods

Silkworm pupae and Extract preparation

Silkworm pupae of distinct pupation time (E, L) belonging to silkworm varieties, BG as well as GS were obtained from Uljin Silk Farm (Uljin, Korea). They were brought to the laboratory, segregated into males and females based on visual examination of the ventral surface of silkworm pupa's abdominal segment. The silkworm pupae were stored at -80°C overnight and then subjected to freeze drying in a lyophilizer (IlShinBioBase, Gyeonggi-do, Korea). Subsequently, they were coarsely ground in a domestic mixer grinder and 2 g each of the respective sample was dissolved in 40 mL of distilled water; 30 and 70% (v/v) of ethanol in water and subjected to overnight shaking at room temperature. Subsequently, the extracts were filtered using a Miracloth (Merck), centrifuged at 11200 × g for 10 min

and the resultant supernatant was lyophilized, powdered and refrigerated till use. For further experimentation, the samples were dissolved in the respective extraction medium to obtain a desired concentration (Lee *et al*., 2021a).

Determination of anti-collagenase activity of SPE

The ability of the SPE to inhibit collagenase activity was determined using a fluorometric collagenase inhibitor screening kit (Abcam, Cambridge, UK) following manufacturer's instructions in a 96 well plate. The SPE at concentration of 100 µg/mL were used for analysis. The test extracts (1 µL) were mixed with collagenase assay buffer (44 µL) and collagenase (5 µL). The samples were subjected to incubation for 15 min at room temperature followed by addition of 50 µL of reaction mix containing collagenase substrate in collagenase assay buffer to each well. The fluorescence was measured immediately at excitation/emission = 490/520 nm at 37°C using microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The ability of SPE to inhibit collagenase activity was calculated from the equation, % relative inhibition = (enzyme control - sample (test compound)/ enzyme control) × 100.

Determination of anti-elastase activity of SPE

Neutrophil elastase inhibitor screening kit (Abcam) was used to evaluate the anti-elastase activity of the SPE (100 µg/mL). The experiment was performed in 96 well (black) plates. In brief, the test inhibitor wells comprised 25 µL of the extract and neutrophil elastase in assay buffer (50 µL). The mixture was incubated at 37°C for 5 min, subsequent to which substrate in assay buffer (25 µL) was added. The fluorescence was measured immediately at excitation/emission = 400/505 nm at 37°C in a microplate reader. The ability of the extracts to inhibit elastase activity was calculated from the equation, % relative activity = (ΔRFU test inhibitor/ ΔRFU enzyme control) × 100.

Determination of anti-tyrosinase activity of SPE

The ability of the SPE (100 µg/mL) to inhibit tyrosinase enzyme activity was determined using a colorimetric tyrosinase inhibitor screening assay kit (Abcam) following manufacturer's instructions in a 96 well plate. In brief, the test inhibitor wells comprised 20 µL of SPE and tyrosinase enzyme in assay buffer (50 µL). The mixture was incubated at 25°C for 10 min subsequent to which substrate in assay buffer and tyrosinase enhancer (30 µL) was added. The absorbance was measured using microplate reader at 510 nm. The ability of the extracts to inhibit tyrosinase activity was calculated from the equation, % relative inhibition = (slope of enzyme control-slope of sample/ slope of enzyme control) × 100.

Cytocompatibility studies in murine fibroblasts

Murine fibroblast cells (L929) from the Korean cell line bank, Seoul, Korea were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Caisson, North Logan, UT, USA) with 10% fetal bovine serum (Gendepot, Barker, TX, USA) and combination of antibiotics which included 1% penicillin and streptomycin (Caisson). The cells were seeded at 2×10^4 cells/well in a 96-well culture plate and incubated for 24 h. Subsequently, the cells were treated with SPE at 100 µg/ mL concentration. Upon incubation for 24 h, Ez-Cytox assay (DoGENBio Co., Ltd., Seoul, Korea) was performed as per manufacturer's instructions to evaluate cell viability. In brief, 20 µL of EZ-Cytox reagent was added to the wells and incubated for 1 h upon which the absorbance was measured using microplate reader at a wavelength of 450 nm. The results were expressed as a percentage of the untreated control.

Determination of intracellular ROS production

The effect of SPE on intracellular ROS generation was determined as illustrated earlier with minor modifications (Lee *et al*., 2021a; Iosageanu *et al*., 2021). L929 cells were cultured as described above. 3×10^4 cells/well were seeded in a 96-well plate (black) and cultured for 24 h before being treated with SPE at 100 µg/mL and further cultured for 24 h. Subsequently, 2', 7'-dichlorofluorescin diacetate (25 µM) was added upon which the cells were treated for 1 h with *t*-BHP (1 mM) to generate oxidative stress. The concentration of intracellular ROS was determined in a microplate reader at 485/530 nm (excitation/ emission) and the results were represented as percentages of those obtained in the control group.

Determination of lipid peroxidation by thiobarbituric acid reactive substances (TBARS) assay

Employing the same methodology as described above, the levels of lipid peroxidation in SPE-treated oxidatively stressed fibroblast cells (L929) were determined by measuring the malondialdehyde (MDA) generation using TBARS assay (Cayman Chemical, Michigan, USA). 3×10^4 cells/well were

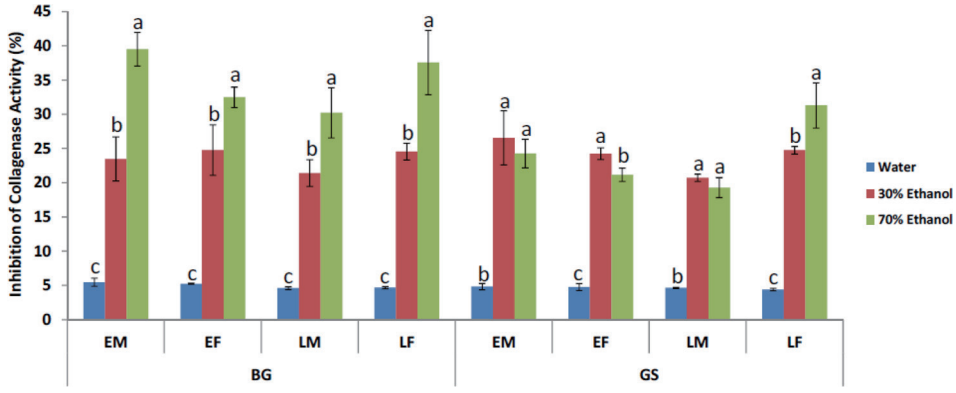

Fig. 1. Inhibition of collagenase activity by aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way analysis of variance (ANOVA). Different lowercase letters (a,b,c) above the columns indicate significant differences among the treatments (same sex and pupation time of a silkworm variety differing in extraction solvent) according to Duncan's multiple range test ($P<0.05$). Same letter indicates no significant difference ($P > 0.05$) whereas different letters indicate a significant difference ($P < 0.05$).

seeded and cultured for 24 h before being treated with SPE at 100 μg/mL and further cultured for 24 h. Subsequently, the cells were treated with *t*-BHP (1 mM) for 1 h to generate oxidative stress. The cells were then lysed, supernatants collected and TBARS assay was performed as follows. To, 100 μL of the test sample or standard MDA in a 5 mL vial, same amount of SDS solution was added. Subsequently, 4 mL of color reagent (thiobarbituric acid, acetic acid and sodium hydroxide-prepared as per the manufacturer instructions) was added and subjected to incubation in a water bath set at 95°C for 1 h. Then, the tubes were immediately placed on ice for 10 min and centrifuged at 1600 × g, 10 min at 4 °C. The supernatants (150 μL) thus obtained were transferred to a 96 well plate and the absorbance was read at 535 nm. The values of MDA in μM was determined following the equation (corrected absorbance)-(y-intercept)/slope. The results were represented as percentages of those obtained in the control group.

Statistical analysis

All of the results from the above mentioned assays carried out in triplicates were depicted as mean ± standard deviation. The data were analyzed by one way ANOVA. Significant differences among mean values were determined by adapting Duncan's multiple range test where as Dunnett's test was performed to compare mean values of multiple treatments and the control group mean using the software package SAS 7.1 (SAS Institute Inc., Cary, NC, USA). P values < 0.05 were regarded as significant.

Results

Anti-collagenase activity of SPE

Collagenase inhibitory activities were exhibited by both aqueous and ethanolic SPE. The collagenase activity inhibition percentages of 70%, 30% ethanolic and aqueous extracts of silkworm pupae differing in pupation time and sex belonging to BG varied from 30.21-39.51%, 21.41-24.76% and 4.58-5.45%, respectively. The same for GS ranged in between 19.28-31.29%, 20.71-26.54% and 4.39-4.83%. In case of SPE from BG, 70% ethanolic extracts were significantly superior in inhibiting collagenase activity when compared to 30% ethanolic and aqueous extracts. However, in case of GS, the above trend was only true with LF extracts (Fig. 1). The collagenase inhibitory activity by 30% ethanolic extracts regardless of pupation time, sex or silkworm variety didn't differ significantly which was otherwise with 70% ethanolic and aqueous extracts.

Anti-elastase activity of SPE

Elastase enzyme activity was better inhibited by 70% ethanolic SPE, followed by 30% and aqueous extracts. The 70% and 30% ethanolic SPE of BG inhibited activity of elastase enzyme by 59.55-77.31% and 35.63-43.27%, respectively whereas the same in case of GS varied in between 44.80-64.97% and 22.14-37.46%, respectively. Aqueous extracts of silkworm pupae of BG and GS exhibited the least inhibition percentages ranging in

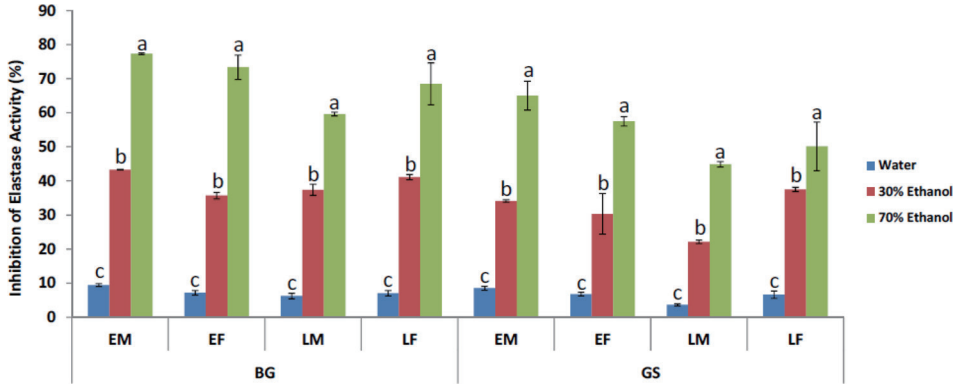

Fig. 2. Inhibition of elastase activity by aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way ANOVA. Different lowercase letters (a,b,c) above the columns indicate significant differences among the treatments (same sex and pupation time of a silkworm variety differing in extraction solvent) according to Duncan's multiple range test ($P<0.05$). Same letter indicates no significant difference ($P > 0.05$) whereas different letters indicate a significant difference ($P < 0.05$).

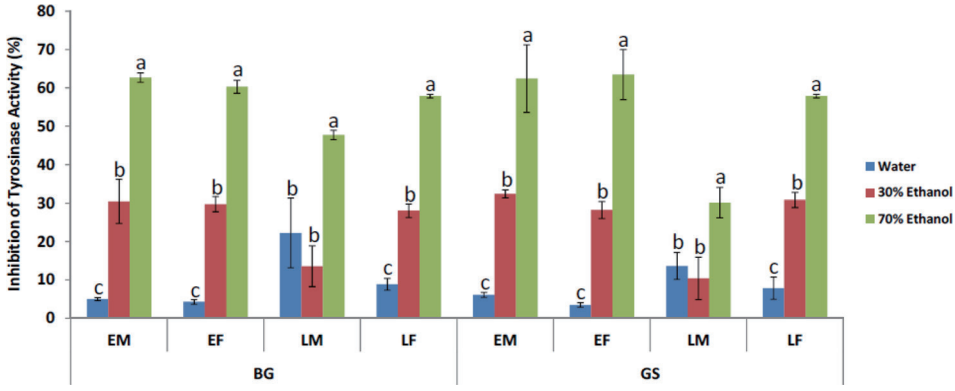

Fig. 3. Inhibition of tyrosinase activity by aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way ANOVA. Different lowercase letters (a,b,c) above the columns indicate significant differences among the treatments (same sex and pupation time of a silkworm variety differing in extraction solvent) according to Duncan's multiple range test ($P<0.05$). Same letter indicates no significant difference ($P > 0.05$) whereas different letters indicate a significant difference ($P < 0.05$).

between 3.64-9.44% (Fig. 2). The inhibition of elastase enzyme activity by 70% ethanolic, 30% ethanolic and aqueous extracts of silkworm pupae regardless of pupation time, sex and silkworm variety differed significantly.

Anti-tyrosinase activity of SPE

The 70% ethanolic SPE of both the silkworm varieties were way superior in inhibition of tyrosinase enzyme activity (BG: 47.73-62.66%; GS: 30.13-63.46%) when compared to 30% ethanolic (BG: 13.55-30.43%; GS: 10.42-32.37%) and aqueous (BG: 4.29-22.22%; GS: 3.46-13.65%) extracts. The above inhibition pattern (70% ethanolic > 30% ethanolic > aqueous extracts) is observed across EM, EF and LF extracts of both the silkworm varieties. However, with respect to LM, 70% ethanolic extracts were better and no significant differences were observed in inhibitory activities between 30% ethanolic and aqueous extracts. Among the ethanolic extracts of both the silkworm varieties, significant reduction in tyrosinase inhibitory activity was observed with LM when compared to EM, EF and LF whereas the trend reversed in case of aqueous extracts wherein LM exhibited better inhibition rates when compared to EM, EF and LF (Fig. 3).

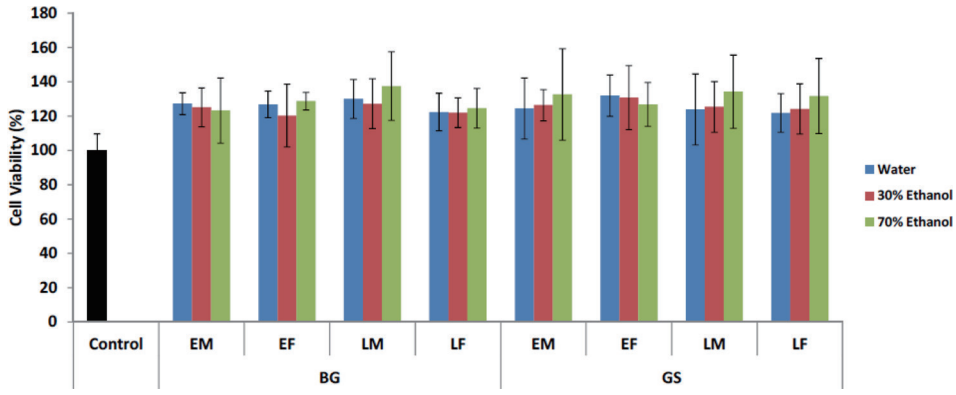

Fig. 4. Effect of aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae on viability of L929 cells. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way ANOVA (Dunnett's post hoc). There was no significant difference between the treatment groups and the control group.

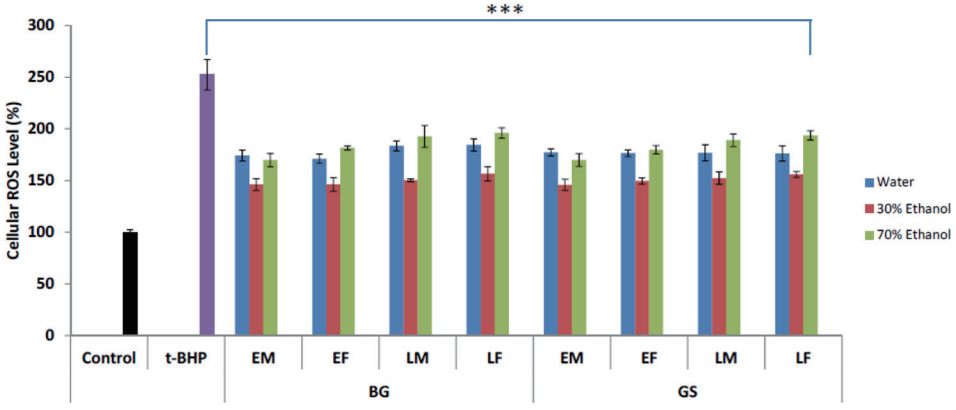

Fig. 5. Effect of aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae on cellular ROS levels in *t*-BHP stimulated L929 cells. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way ANOVA (Dunnett's post hoc). *** indicates a significant difference in comparison to *t*-BHP treated control at $p<0.001$.

Cytocompatibility of SPE in murine fibroblast cells (L929)

The SPE irrespective of silkworm variety, pupation time, sex and extraction solvent were compatible with fibroblasts, since none of the treatments exhibited any significant cytotoxic or cell proliferative effect on L929 cells (Fig. 4).

Effect of SPE on intracellular ROS production in fibroblast cells

The potential of SPE in attenuation of intracellular ROS in *t*-BHP induced oxidatively stressed murine fibroblast cells (L929) was evaluated in the present study. The cellular ROS levels augmented by ~2.5 times in *t*-BHP treated fibroblasts when compared to untreated counterparts. Our results indicate that the extent of ROS production declined significantly ($p<0.001$) in L929 cells pretreated with SPE (Fig. 5). The best reduction in ROS levels was brought about by 30% ethanolic extracts (BG: 37.92-42.01%; GS: 38.06-42.12%) followed by aqueous (BG: 26.81-32.06%; GS: 29.66-30.14%) and 70% ethanolic extracts (BG: 22.15-32.56%; GS: 23.12-32.59%). The reduction in intracellular ROS levels by 30% ethanolic and aqueous extracts of silkworm pupae regardless of pupation time, sex and silkworm variety didn't differ significantly which was otherwise with 70% ethanolic extracts.

Effect of SPE on lipid peroxidation in fibroblast cells

The intracellular MDA levels increased by a momentous 5.26

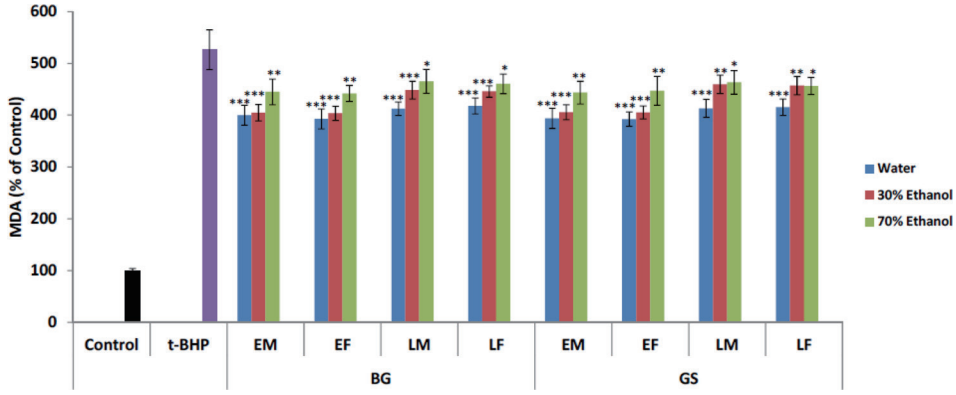

Fig. 6. Effect of aqueous, 30% ethanol and 70% ethanol extracts of silkworm pupae on lipid peroxidation malondialdehyde (MDA) levels in *t*-BHP stimulated L929 cells. Pupation time of 6-7 days: early stage (E), 11-13 days: late stage (L); Sex: male (M), female (F); Silkworm varieties: Baegokjam (BG), GoldenSilk (GS). Error bars represent standard deviation from measurements in triplicate. Data were analyzed by one way ANOVA (Dunnett's post hoc). *, ** and *** indicates a significant difference in comparison to *t*-BHP treated control at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

fold in *t*-BHP stressed cells and the same were significantly reduced by pretreatment with SPE (Fig. 6). The aqueous extracts were superior in reducing MDA levels (BG: 20.40-25.12%; GS: 20.78-25.20%) followed by 30% (BG: 14.60-23.03%; GS: 12.40-22.74%) and 70% ethanolic extracts (BG: 11.20-15.71%; GS: 11.60-15.47%). The reduction in MDA levels by 70% ethanolic, 30% ethanolic and aqueous extracts of silkworm pupae regardless of pupation time, sex and silkworm variety didn't differ significantly.

Discussion

Skin aging is an intricate biological mechanism induced by a combination of intrinsic (genetical factors, hormonal changes, metabolic processes) and extrinsic (environmental exposure, ultraviolet radiation from sunlight, xenobiotic stress) factors (Gilaberte *et al*., 2016; Ganceviciene *et al*., 2012). UV radiation from sunlight is a key stimulant that triggers excessive production of ROS resulting in oxidative stress as well as augmenting the levels of matrix metalloproteinases (MMPs) in skin cells. MMPs, a diverse group of zinc containing endopeptidases eventually mediate disintegration of extracellular matrix components, most notably collagen and elastin by collagenase and elastase, respectively (Jiratchayamaethasakul *et al*., 2020). Apart from the above, chronic exposure to UV radiation is also involved in aberrant melanogenesis by influencing the process through which melanocytes synthesize the pigment melanin in melanosomes regulated through tyrosinase (Hsu *et al*., 2020). All the above cause skin to age prematurely, altering its structural and physiological features, resulting in a lot of changes in its appearance. Dullness, roughness, wrinkles, lack of elasticity, and discolouration or irregular pigmentation are all signs of aging skin (Ganceviciene *et al*., 2012; Pittayapruek *et al*., 2016). As a result, the above mentioned enzymes (collagenase, elastase and tyrosinase) have become the primary focus of greater part of skin anti-aging research efforts across the world (Pittayapruek *et al*., 2016; Qian *et al*., 2020).

Many natural products have been explored for their role in inhibition of the aforementioned enzymes and in this study, we assessed extracts of silkworm pupae for their ability to do so, since they are highly underexplored. Our findings revealed some fascinating insights. Collagenase, elastase and tyrosinase enzyme activities were inhibited by aqueous as well as ethanolic (30% and 70%) extracts from silkworm pupa, regardless of silkworm variety, pupation times, or sex. However, with a few exceptions, the inhibitory effects varied significantly depending on the extraction solvent with 70% ethanolic SPE outperforming 30% ethanolic and aqueous extracts. Alleviation of ROS and MDA levels in fibroblasts by SPE was also observed. Our preliminary studies also indicated that 100% ethanol extracts of silkworm pupae exhibited meager anti-skin aging effects and amelioration of ROS activities.

The bioactive constituents in contributing to the aforementioned activities were not assessed in the present study. However, reports from the earlier studies indicated that

silkworm pupae encompass diverse bioactive components which include carbohydrates (chitin, chitosan, silkrose); fatty acids (α-linolenic acid, docosahexanoic acid, eicosapentanoic acid, linolenic acid, myristic acid, oleic acid, palmitic acid, stearic acid); polyphenols (catechin, epicatechin, kaempferol, luteolin, myricetin, naringenin, quercetin, rutin, trans-resveratol), proteins and selenium rich peptides (Sadat *et al*., 2022).

The fatty acids mentioned above except for α-linolenic acid, docosahexanoic acid and eicosapentanoic acid were earlier reported to be potential inhibitors of collagenase and elastase activities (Rennert and Melzig, 2002). Also, reduction in melanogenesis in murine melanoma cell (B16F10) cultures via acceleration of proteolytic degradation of tyrosinase by linoleic acid was reported (Ando *et al*., 1999). Seed oils from *Torreya grandis* Fort. ex Lindl. and five Amazonian native flora oils comprising oleic acid in major amounts were also found to inhibit tyrosinase enzyme activity (Cui *et al*., 2018; Teixeira *et al*., 2012). The above scientific evidence explains that fatty acids of SPE may have played a prominent role in inhibiting collagenase, elastase and tyrosinase activities. Our previous studies also indicated abundance of palmitic acid and oleic acid in male pupae; stearic acid, linoleic acid, and linolenic acid in female pupae with levels varying across developmental stages and silkworm varieties (Lee *et al*., 2021b).

Among the other bioactive constituents of SPE, phenolics were emphasized to possess strong antioxidant potential primarily owing to the presence of phenolic hydroxyl groups (Działo *et al*., 2016; Bosch *et al*., 2015). Moreover, they also confront ROS via enhancing cellular antioxidant activity by modulating Nrf2-mediated pathway or by regulating mircoRNAs as well as through regulation of endogenous antioxidant and oxidase enzyme production (Luo *et al*., 2021). These components of SPE might have played a major role in attenuation of ROS and MDA levels in *t*-BHP induced fibroblasts. Reduction of ROS levels on the other hand, may be a beneficial strategy for preventing photoaging-induced cellular damage too. Our previous studies have indicated that aqueous and ethanolic SPE comprised polyphenols. It was also observed that 30% ethanolic extracts exhibited superior DPPH and ABTS radical scavenging activities over aqueous, 50%, 70%, 100% ethanol and hexane extracts. However, the same differed across the silkworm varieties, developmental stages and sex (Lee *et al*., 2021a). Our results from the present study corroborates with the aforementioned findings, considering the fact that 30% ethanolic SPE exhibited better ROS attenuation properties in *t*-BHP treated fibroblast cells over 70% ethanolic and aqueous extracts. Nevertheless, it is to be noted that the ROS reduction by 30% ethanolic extracts didn't differ significantly across the silkworm varieties, developmental stages and sex which is in contrary to DPPH and ABTS radical scavenging activities as observed in our prior study (Lee *et al*., 2021a). The aforementioned findings indicate that the bioactive components contributing to amelioration of ROS are effectively extracted from silkworm pupae in 30% ethanol over other extraction solvents.

The bioactive components of silkworm pupa are no doubt derived from the silkworm larval stage. Silkworms feed on mulberry leaves which are a potential source of diverse bioactive constituents (quercetin-type flavonoids, kaempferol-type flavonoids, chlorogenic acids, amino acids, unsaturated fatty acids, 1-deoxynojirimycin, γ-aminobutyric acid) (Zou *et al*., 2022; Rahul *et al*., 2022). These constituents undergo various biochemical modifications as a part of the insect metabolism (Nino *et al*., 2021) which varies across the developmental stages, sexes (males and females) and silkworm varieties. Variations in levels of various biomolecules (carbohydrates, proteins, amino acids and lipids) across the developmental stages in silkworm have been previously reported (Gilbert and Schneiderman, 1961). This subsequently results in disparities in qualitative and quantitative accumulation of bioactive components (Shen *et al*., 2018; Dong *et al*., 2017; Yin *et al*., 2010; Rahmathulla *et al*., 2005) which explains the reason for the variations in the enzyme inhibitory properties as well as ROS attenuation activities by SPE belonging to different varieties, sexes and development stages.

Extraction conditions including disintegration methods, solvent of choice, temperature and duration play a very significant role in extraction of target compounds in desired quantities. In this study, the bioactive constituents of silkworm pupae contributing to the anti-aging and antioxidant activities were found to be better extracted in 70% and 30% ethanol, respectively. Earlier reports have also suggested that the most important bioactive components of silkworm pupae i.e. polyphenols as well as fatty acids are reported to be better extracted in ethanol/water solvents (Ebrahimi and Lante, 2022; Saini *et al*., 2021).

Although, from the results of the present study, it is assumed that fatty acids and polyphenols of SPE might have contributed to the skin anti-aging and free radical scavenging properties, it is relatively difficult to comprehend or define a specific

component/s or a class of compound/s of natural products to a particular function, unless examined. So, further analyses like mass spectrometry studies are necessarily required to identify and quantify the bioactive components of silkworm pupae in different extracts which will help to delineate their role in anti-aging as well as ROS scavenging activities.

Conclusion

In the present study, we reported that aqueous and ethanolic (70% and 30%) SPE can inhibit the activities of collagenase, elastase as well as tyrosinase, indicating their anti-skin aging and skin whitening potential. SPE also exhibited cytocompatibility, reduction in ROS and lipid peroxidation levels in fibroblasts, all of which are imperative for maintaining optimal skin health and function. This study is the first comprehensive report on the potential of SPE in possessing the aforementioned properties. These findings provide preliminary data on the potential of SPE as a valuable ingredient in cosmeceuticals.

Acknowledgments

This research activity was supported by the 'Research Program for Agriculture Science and Technology Development' (Grant No. PJ01502202), National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea.

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