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Effect of pretreatment with paste and sauce extract made using Tenebrio molitor larvae on ethanol-damaged HepG2 cells

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Abstract
In this study, we made koji using protein-rich Tenebrio molitor larvae (TML) inoculated with Aspergillus oryzae, and then used the koji to prepare a paste and a sauce. The TML koji showed the highest amino nitrogen, protease activity, and free amino acids content when it was fermented for 72 h after inoculation with 0.8% A. oryzae. The koji was aged in 20% saltwater for 50 days, and then the paste and sauce were separated. To evaluate the effect of TML paste and sauce on ethanol (EtOH)-damaged hepatocytes, aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were measured in EtOH-treated HepG2 cells after pretreatment with TML paste and sauce extract. As a result, we confirmed that TML paste and sauce extracts lowered the AST and ALT content in the medium, compared to soybean sauce and paste extracts. TML paste and sauce extract significantly reduced the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6, a biomarker of inflammation, and significantly increased the activity of the antioxidants superoxide dismutase (SOD) and reduced glutathione (GSH) in proportion to the amount of TML added to the paste and sauce. These results suggest that the intake of TML paste and sauce, a new type of fermented food made from insects, may provide effective protection to the liver against hepatocyte injury by EtOH via anti-inflammatory and antioxidative effects.

Key words: hepatoprotective, paste, sauce, Tenebrio molitor

Introduction
Soybean paste (Doenjang) and sauce (Ganjang) are made using the soybean koji fermented by inoculating microorganisms, or by inducing adhesion of microorganisms under natural conditions (Kang et al. 2011; Kim et al. 2011). The fermented soybean koji are matured for 2–3 months in saltwater, before separating the solid from the liquid parts. The solid part (or paste) is Doenjang and the liquid (sauce) is Ganjang (Kang et al. 2011). During aging, the protein, starch, and fat in these soybean products are decomposed into free amino acids, free sugars, and organic acids by microorganisms to facilitate absorption into the body (Park et al. 2007). Because Doenjang and Ganjang have a unique taste and aroma, in addition to a salty taste (Kim et al. 2013; Park et al. 2007), they are used as seasoning in Korea. In addition, as with other fermented foods, such as Kimchi (Park et al. 2014) and cheese (Guinee 2002), they have been reported to have anticancer, antioxidant, and hypoglycemic effects, as well as thrombolytic activity, and have been demonstrate to suppress other adult diseases (Kang 1999; Kim 1998; Kwon et al. 2014; Lee et al. 2012; Lim et al. 1999; Park et al. 1990; Shin et al. 2014).

Many researchers have developed fermented foods using not only soybean as a vegetable protein source but also...
other ingredients, which they have then used to conduct functional studies. An oxygen radical absorbance capacity experiment using fish sauce, another fermented food using animal protein foods, showed that it has more antioxidant activity than apple, pear, orange, white grape, or lime, foods that have been previously reported to have good antioxidant properties (Harada et al. 2010). In addition, fermented shrimp sauce was found to exert increased antioxidant activity through fermentation in a time-dependent manner (Faithong & Benjakul 2014; Park et al. 2015). Although there has been a report on the physicochemical and sensory properties of Tenebrio molitor larvae (TML, or mealworms) sauce made by the inoculation of Aspergillus oryzae and Bacillus licheniformis (Cho et al. 2018), there are currently no reports on the functional analysis of fermented food made from insects.

Tenebrio molitor belong to the Coleoptera Tenebrionidae family. TML have been traditionally used in Asian countries, including Korea, for the purpose of treating liver diseases, including liver cancer (Pemberton 1999). They have been consumed worldwide for their nutritional value, which includes high protein, mineral, and unsaturated fatty acids, and were officially registered as a new food ingredient in Korea in 2015 (Baek et al. 2017; Youn et al. 2014). In previous studies, analyses of their functional properties revealed that TML ethanol (EtOH) extract inhibited the expression of TNF-α and IL-6 in the inflammation-induced RAW 264.7 cell line and had a DPPH radical scavenging ability similar to that of blueberry extract (Baek et al. 2017; Kang et al. 2017). In addition, it has been reported that TML may inhibit beta-secretase 1 enzyme activity, which is related to the accumulation of beta-amyloid (Youn et al. 2014), as well as protect against hepatocellular carcinoma (Park et al. 2015) and alleviate obesity (Seo et al. 2017). TML have also shown to be effective for hair growth and the prevention of hair loss by supporting the proliferation of fibroblasts (Baek et al. 2017).

Therefore, in this study, we evaluated the degree of fermentation after preparing a fermented food by the inoculation of A. oryzae using protein-rich (50.32% by dry weight basis) mealworms, which are officially registered as an edible insect in Korea (Yoo et al. 2013). As part of this functional study of TML fermented food, we analyzed its effect on alcoholic liver disease, which can develop into acute liver damage, such as alcoholic hepatitis, and chronic liver disease, such as steatosis, steatohepatitis, fibrosis, and cirrhosis (Bruha et al. 2012). Previous studies have reported its effect on hepatocellular carcinoma (Park et al. 2015).

Materials and Methods

Preparation of TML koji, paste, and sauce

The soybean Baektae (crop year 2017) was grown in Sunchang, Korea. A. oryzae was purchased from Chungmu fermentation (Korea). The soybeans were soaked in distilled water for 12 h and steamed for 1.5 h. For the preparation of koji, the steamed TMLs and soybeans were used alone or mixed at a ratio of 1:1 or 3:7, then crushed and inoculated with 0.2, 0.4, and 0.8% A. oryzae. Then, the TMLs and/or soybeans were molded using a square mold and fermented for 72 h at 30°C with a relative humidity 70%. After the fermentation, the samples were oven-dried for 24 h at 60°C to finish the koji preparation. The koji was then soaked in 20% saltwater for 50 days. After 50 days, the paste (solid) and the sauce (liquid) were separated. Depending on the proportion of TML added to the koji, the following extracts were obtained: GMP100 (100% Glycine max, GM paste), TMP100 (100% TML paste), TMP50 (50% TML/50% GM), and TMP30 (30% TML/70% GM) pastes and GMS100 (100% GM sauce), TMS100 (100% TML sauce), TMS50 (50% TML/50% GM), and TMS30 (30% TML/70% GM) sauces (Fig. 1).

Figure 1 Paste (A) and sauce (B) from koji made with Tenebrio molitor larvae (TML).
Preparation of koji, paste, and sauce extract

The prepared koji was grinded and diluted with distilled water at a ratio 1:10 (w/v). The diluted samples were mixed for 1 h using a rocker, before centrifuging at 4,000 rpm for 10 min. The samples were then filtered using Whatman No.1 filter paper for a primary filtration and a 0.45-μm syringe filter for a secondary filtration. The koji paste was then freeze-dried, grinded, diluted with 95% EtOH at a ratio 1:5 (w/v), and extracted over 24 h. After centrifugation at 3,000 rpm for 10 min, the supernatant was filtered using Whatman No.1 filter paper for a primary filtration and a 0.45-μm syringe filter for a secondary filtration. Then, the supernatant was concentrated using a speed vacuum (Eyela, Tokyo, Japan) for 48 h before dissolving in 20% DMSO. The sauce was centrifuged in 3000 rpm for 10 min and then filtered using Whatman No.1 filter paper for a primary filtration and a 0.45-μm syringe filter for a secondary filtration. The koji, paste, and sauce extracts obtained were used in the subsequent experiments.

Amino nitrogen, protease activity, and free amino acid analysis

The 95% EtOH extract of the koji, paste, and sauce samples were used in amino nitrogen and protease activity assays. Amino nitrogen was measured using a Primary Amino Nitrogen Kit (Megazyme, Chicago, IL, USA), and protease activity was measured using a Pierce Protease Assay Kit (Thermofisher, Waltham, MA, USA), according to the manufacturer’s instructions. The extracted samples were analyzed for free amino acids according to the method provided by the Korean Food Standard Codex (Ministry of Food and Drug Safety 2019). The prepared samples were mixed with 16% trichloroacetate and shaken for 15 min. After centrifugation at 3,000 rpm for 15 min, the supernatant was used for the analysis of free amino acids by liquid chromatography (Agilent, Santa Clara, CA, USA).

Cell culture, EtOH-damaged HepG2 cells, and cell viability assay

HepG2 cells were grown in DMEM (LM001–11, WELGENE, Gyeongsan, Korea) containing 1000 mg/L glucose, 10% FBS and 1% antibiotic-antimycotic (AA) (Life Technology, Carlsbad, CA, USA) at 37°C in a 5% carbon dioxide atmosphere. Once the cells reached 80% confluence, they were suspended in trypsin, plated at a density of 1 × 10^5 cells per well in a 6-well plate, and incubated for 24 h. The media was then replaced with 2 mL of DMEM containing 10% FBS, 1% AA, and the paste or sauce extracts. After incubation for 4 h, the media was replaced with 2 mL DMEM containing 10% FBS, 1% AA, and 300 mM EtOH. The wells were covered with parafilm to prevent EtOH evaporation and incubated for 24 h. After incubation, the cells and media were separated by centrifugation (3,000 rpm, 5 min). The HepG2 cells and media were used in the subsequent experiments.

HepG2 cell viability was measured by CellTiter 96 Aqueous One Solution Cytotoxicity Assay (Promega, Madison, WI, USA). HepG2 cells were plated at a density of 1 × 10^5 cells per well in a 6-well plate and incubated for 24 h. The media was then replaced with 2 mL of DMEM containing 10% FBS, 1% AA, and the paste or sauce extracts. After incubation for 4 h, the media was replaced with 2 mL DMEM containing 10% FBS, 1% AA, and 300 mM EtOH. After incubation for 24 h, 10 μL CellTiter 96 Aqueous One Solution reagent was added to each well. After incubation for 4 h, we measured the absorbance for the solution in each well at 450 nm.

Aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) analysis

The media isolated in the previous section (Cell culture and EtOH-damaged HepG2 cells) was used for AST and ALT analysis. The AST and ASL levels were measured by Asan GOT and GPT (ASAN Pharmaceutical, Seoul, Korea), respectively, according to manufacturer’s instructions. Silymarin was used as a positive control. Silymarin has been previously reported to exert an antioxidant effect by removing oxygen free radicals and reducing lipid peroxidation, thereby promoting hepatocyte regeneration (Lucena et al. 2002; Montvale 2000; Pradeep et al. 2007; Rainone 2005). The AST and ALT levels were expressed in IU/L.

Total RNA extraction and quantitative PCR analysis

The total RNA of HepG2 cells was isolated using TRIzol Reagent (Ambion, Austin, TX, USA). The cDNA was synthesized using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The TNF-α and IL-6 levels were analyzed using BrightGreen 2X qPCR MasterMix-No Dye (Applied Biological Materials, Richmond, BC, Canada), according to the manufacturer’s instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and the relative gene expression levels were analyzed using the 2^−ΔΔCt method. Primers for GAPDH (forward 5′- ACCATCCCTCAACCTTTGA-3′, reverse 5′-CTGTTGCGTGA GCCAAATTCTG-3′), TNF-α (forward 5′-GGAGAAGGG TGACCGACTCA-3′, reverse 5′-CTGCCAGACTCGG CAA-3′), and IL-6 (forward 5′-GGAGACTTGCCCTGGTG AAAA, reverse 5′- GTCAGGCGTGGTTATTGCAT-3′) were used for cDNA amplification (Malapel et al. 2008).
Glutathione (GSH) activity and superoxide dismutase (SOD) inhibition rate analysis.

The cells isolated in a previous section (Cell culture, EtOH-induced HepG2 cells, and cell viability assay) were lysed with RIPA buffer and used to determine the GSH activity and SOD inhibition rate. GSH activity was measured using a Glutathione Assay Kit (Sigma Aldrich, St Louis, MO, USA) and SOD inhibition rate was measured using a SOD Assay Kit (Sigma Aldrich), according to the manufacturer’s instructions. Silimarin was used as a positive control. GSH activity and SOD inhibition rate were expressed in relative values of samples for the non-treatment control.

Statistical analysis

All measurements were repeated in triplicate. The results represent the mean ± standard deviation (SD). Comparisons between two groups were performed using the Student’s t-test. SPSS version 18 (SPSS Inc., Chicago, IL, USA) was used for analysis, where a P-value <0.05 was considered statistically significant.

Results and Discussion

Evaluation of fermentation on koji made with TML

The amino nitrogen content, protease activity, and free amino acid content were determined in order to evaluate the degree of TML fermentation according to the amount of A. oryzae inoculated and the fermentation time. The amino nitrogen content and the protease activity were found to increase in proportion to the quantity of A. oryzae inoculum and fermentation time. The maximum value of 0.62 mg/mL was measured when the TML koji had been fermented for 72 h after inoculation with 0.8% A. oryzae (Fig. 2A). In terms of the protease activity, the maximum value of 174% compared with the control (non-fermented TML) was also found in the TML koji that had been fermented for 72 h after inoculation with 0.8% A. oryzae. The analysis of the free amino acid content before and after fermentation demonstrated that the levels of all free amino acids, except arginine, increased after fermentation (Fig. 2C). Therefore, the TML paste and sauce made with TML koji fermented with 0.8% A. oryzae for 72 h was investigated in subsequent experiments.

Figure 2  Evaluation of fermentation on koji made with Tenebrio molitor larvae (TML). Quantitative comparison of amino nitrogen content (A), protease activity (B), and free amino acid (C) of TML koji. Values represent the mean ± standard deviation (SD) of experiments carried out in triplicate. **P < 0.01 and ***P < 0.001 indicate significant differences between the non-aging and aging groups.
Evaluation of aging on paste and sauce made with TML koji

To evaluate the degree of aging in the TML paste and sauce made with TML koji, the amino nitrogen and protease activity in the TML paste and sauce was measured. The amino nitrogen content and protease activity of paste were found to increase in proportion to the TML concentration in the koji. In the case of the paste, the maximum value of amino nitrogen (0.44 mg/mL) and protease activity (142.60%) were found in the TMP100 extract (Fig. 3A, C). In addition, in the case of the sauce, the amino nitrogen content and protease activity were also found to increase in a TML concentration-dependent manner. The maximum value of the amino nitrogen (3.78 mg/mL) and protease activity (135.27%) was found in the TMS100 extract (Fig. 3B, D). Accordingly, the TML paste and sauce were found to be effectively aged in proportion to the content of TML in the koji.

Hepatoprotective effect of pretreatment of TML paste and sauce on EtOH-damaged hepatocytes

Previous studies have reported no toxicity in mice fed orally administered lyophilized TML powder 3000 mg/kg/day (Han et al. 2014). Accordingly, TML has been legally recognized as a new food source in Korea. In this study, to determine whether TML paste and sauce protect against hepatic cell damage by alcohol, we examined the viability of hepatocytes treated with alcohol after pretreatment with TML paste and sauce extract. First we confirmed that the cell viability of HepG2 cells after only EtOH treatment were 65%. The cell viability of EtOH-damaged HepG2 cell pretreated with GMP100, TMP30, GMS100 and TMS30 were 90%, 95%, 80%, and 87%, respectively, compared with EtOH(−)/treatment(−) (Fig. 4). The result shows that the EtOH induced liver damage was protected when the cells were pretreated with GMP100, TMP30, GMS100, or TMS30 extract. In addition, the protective effect was better in TMP30 or TMS30 than in GMP100 or GMS100. Even the pretreatment of TMP30 and TMS30 with the lowest TML content among kojis made in this study was effective in protecting liver cells from alcohol damage. The hepatic enzymes AST and ALT have been commonly used as an index to determine the severity of hepatocyte injury, as their levels in the blood tend to increase with necrosis and tissue destruction of hepatocytes during hepatocellular injury (Huang et al. 2006; Jung 2001; Kim 2009). Therefore, the AST and ALT levels were measured to evaluate the hepatoprotective potential of TML paste and sauce. First, we confirmed that the AST and ALT levels in HepG2 cells after EtOH treatment were 56.45 IU/L and 38.15 IU/L, respectively, and significantly increased compared to those without EtOH (25.23 IU/L and 25.67 IU/L). Then, we identified whether pretreatment with TML paste (400 μg/mL) and sauce (40 μL/mL) extract before EtOH treatment significantly decreased the levels of AST and ALT in the HepG2 cells, as in cells pretreated with silymarin (100 μg/mL) (23.35 IU/L and 16.99 IU/L), which was used as a positive control (Fig. 5).

The AST levels in the EtOH-damaged HepG2 cells after pretreatment with the GMP100, TMP30, TMP50, and

Figure 3 Evaluation of fermentation on paste and sauce made with Tenebrio molitor larvae (TML) koji. Amino nitrogen of each paste (A) and sauce (B) was analyzed. In addition, the protease activity of each paste (C) and sauce (D) was evaluated. GMP100, paste made with Glycine max (GM) 100%; TMP30, paste made with TML 30%/GM 70%; TMP50, paste made with TML 50%/GM 50%; TMP100, paste made with TML 100%; GMS100, sauce made with GM 100%; TMS30, sauce made with TML 30%/GM 70%; TMS50, sauce from with TML 50%/GM 50%; TMS100, sauce made with TML 100%. The concentration of the paste and sauce was 400 μg/mL and 40 μL/mL, respectively. Values represent the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate significant differences between the control and each sample made with paste and sauce.
Figure 4  The viability of HepG2 cells treated with alcohol after pretreatment with Tenebrio molitor larvae (TML) paste and sauce extract. Cell viability after 300 mM EtOH treatment of HepG2 cells pretreated with 95% EtOH extract of GMP100, TMP30, GMS100 and TMS30. GMP100, paste made with Glycine max (GM) 100%; TMP30, paste made with TML 30%/GM 70%; GMS100, sauce made with GM 100%; TMS30, sauce made with TML 30%/GM 70%. The concentration of the paste and sauce was 400 μg/mL and 40 μL/mL, respectively. Values represent the mean ± SD of triplicate experiments. **P < 0.01 indicate significant differences between the EtOH (−)/treatment (−) and EtOH (+)/treatment (−). *P < 0.05 indicate significant differences between the EtOH (+)/treatment (−) and EtOH (+)/treatment (+).

Figure 5  The hepatoprotective potential of paste and sauce made with Tenebrio molitor larvae (TML) koji. The AST levels in the media were measured after 300 mM EtOH treatment of HepG2 cells pretreated with 95% EtOH extract of each paste (A) and sauce (B). The ALT levels in the media were measured after 300 mM EtOH treatment of HepG2 cells pretreated with 95% EtOH extract of each paste (C) and sauce (D). S, silymarin (100 μg/mL); GMP100, paste made with Glycine max (GM) 100%; TMP30, paste made with TML 30%/GM 70%; TMP50, paste made with TML 50%/GM 50%; TMP100, paste made with TML 100%; GMS100, sauce made with GM 100%; TMS30, sauce made with TML 30%/GM 50%; TMS50, sauce made with TML 50%/GM 50%; TMS100, sauce made with TML 100%. The concentration of the paste and sauce was 400 μg/mL and 40 μL/mL, respectively. Values represent the mean ± SD of triplicate experiments. **P < 0.01 indicate significant differences between the EtOH (−)/treatment (−) and EtOH (+)/treatment (−). *P < 0.05, **P < 0.01 and ***P < 0.001 indicate significant differences between the EtOH (+)/treatment (−) and EtOH (+)/treatment (+).
TMP100 extracts were found to be significantly decreased compared to the HepG2 cells treated only with EtOH (56.45 IU/L). The AST levels in the cells pretreated with GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts was 36.11, 36.25, 28.70, and 26.46 IU/L, respectively (Fig. 4A). On the other hand, in case of the cells pretreated with GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL) extracts, the AST levels were 36.78, 34.73, 29.68, and 19.53 IU/L, respectively (Fig. 5B). Accordingly, pretreatment with GMS100, TMS30, TMS50, and TMS100 significantly decreased the AST levels in the EtOH-damaged HepG2 cells compared to the cells only treated with EtOH.

The ALT levels in the EtOH-damaged HepG2 cells after pretreatment with TMP30, TMP50, and TMP100 were significantly decreased compared to the cells treated only with EtOH (38.15 IU/L). The ALT levels in the cells pretreated with GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts was 24.59, 27.15, 22.61, and 19.53 IU/L, respectively (Fig. 5C). Likewise, in the case of pretreatment of GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL) extracts, the ALT levels were 26.43, 26.79, 20.97, and 19.67 IU/L, respectively (Fig. 5D). These results showed that the AST and ALT levels in the EtOH-damaged HepG2 cells after pretreatment with TML paste and sauce extract were lower than those in the cells treated with GM paste and sauce extract. The AST and ALT levels are an index of liver damage and were found to be significantly lower in the EtOH-damaged hepatocytes pretreated with TML paste and sauce extract in proportion to the TML content. Accordingly, we suggest that the TML paste and sauce may exert a hepatoprotective effect on EtOH-damaged hepatocytes. Therefore, our results suggest that the ingestion of TML paste and sauce on a daily basis could provide a protective effect against hepatocyte injury by EtOH.

**Evaluation of anti-inflammatory potential of TML paste and sauce in EtOH-damaged hepatocytes**

To determine the anti-inflammatory potential of TML paste and sauce on EtOH-damaged hepatocytes, the expression levels of TNF-α and IL-6 mRNA were measured in HepG2 cells treated with 300 mM EtOH after pretreatment with

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**Figure 6** The anti-inflammatory potential of the paste and sauce made with *Tenebrio molitor* larvae (TML) koji. The TNF-α levels were analyzed after 300 mM EtOH treatment on HepG2 cells pretreated with 95% EtOH extract of each paste (A) and sauce (B). The IL-6 levels were measured after 300 mM EtOH treatment on HepG2 cells pretreated with 95% EtOH extract of each paste (C) and sauce (D). S, silymarin (100 μg/mL); GMP100, paste made with Glycine max (GM) 100%; TMP30, paste made with TML 30%/GM 70%; TMP50, paste made with TML 50%/GM 50%; TMP100, paste made with TML 100%; GMS100, sauce made with GM 100%; TMS30, sauce made with TML 30%/GM 70%; TMS50, sauce made with TML 50%/GM 50%; TMS100, sauce made with TML 100%. The concentration of the paste and sauce was 400 μg/mL and 40 μL/mL, respectively. Values represent the mean ± SD of triplicate experiments. **P < 0.01 and ***P < 0.001 indicate significant differences between the EtOH (+)/treatment (-) and EtOH (+)/treatment (+).
TML paste (400 μg/mL) and sauce (40 μL/mL) extract. First, the expression levels of TNF-α and IL-6 transcript, which are biomarkers for inflammation, were found to be significantly increased in the HepG2 cells only treated with EtOH compared to normal HepG2 cells EtOH (−)/treatment (−). In the EtOH-damaged HepG2 cells pretreated with TML paste and sauce extract, the TNF-α and IL-6 expression levels were found to decrease to levels similar to those of the cells pretreated with silymarin (100 μg/mL), which was used as a positive control (Fig. 6).

The expression levels of TNF-α in the EtOH-damaged HepG2 cells pretreated with silymarin (100 μg/mL), GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts were 0.63, 0.92, 0.75, 0.73, and 0.67-fold, respectively, and lower than those in the cells only treated with EtOH (4.74-fold) (Fig. 6A). On the other hand, in case of cells pretreated with silymarin (100 μg/mL), GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL), the expression levels of TNF-α were 0.64, 1.07, 0.88, 0.67, and 0.75-fold, respectively, and lower than those in the cells only treated with EtOH (2.77-fold) (Fig. 6B).

The expression levels of IL-6 in the EtOH-damaged HepG2 cells pretreated with silymarin (100 μg/mL), GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts were 0.59, 0.82, 0.75, 0.59, and 0.78-fold, respectively, and lower than those in the cells only treated with EtOH (2.85-fold) (Fig. 6C). Likewise, in the case of cells pretreated with silymarin (100 μg/mL), GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL), the expression levels of IL-6 were 0.55, 3.00, 0.87, 0.67, and 0.75-fold, respectively, and lower than those in the cells only treated with EtOH (6.63-fold) (Fig. 6D). According to these findings, the expression levels of TNF-α and IL-6 in EtOH-damaged hepatocytes pretreated with TML paste and sauce extracts were suppressed at levels similar to those in cells pretreated with silymarin, which is known to exert a hepatoprotective, anti-inflammatory, and antioxidant effect and provide protection against apoptosis (Arafa 2009; Avci et al. 2016; Pascual et al. 1993; Schuppan et al. 1999). This suggests that the TML paste and sauce also have an excellent anti-inflammatory effect on EtOH-damaged hepatocytes, as the degree of inhibition of cytokine expression of their crude extracts (400 μg/mL of paste and 40 μL/mL of sauce extracts).
in EtOH-damaged HepG2 cells was similar to that of silymarin (100 μg/mL).

Evaluation of antioxidant potential of TML paste and sauce in EtOH-damaged hepatocytes

To determine the antioxidant potential of TML paste and sauce, we measured the SOD inhibition rate and GSH activity. The SOD inhibition rate and GSH activity were 87.78% and 68.70% in the HepG2 cells only treated with EtOH compared to the non-treatment control.

In the case of cells pretreated with TML paste (400 μg/mL) and sauce (40 μL/mL) extract, the levels of SOD inhibition rate and GSH activity were significantly increased compared to the cells only treated with EtOH. In the HepG2 cells pretreated with TML paste and sauce extract, the levels of SOD and GSH were similar to those in cells pretreated with silymarin (100 μg/mL) (160.19% and 161.77%) (Fig. 7).

The SOD inhibition rate in the EtOH-damaged HepG2 cells after pretreatment with TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) were significantly increased compared with cells only treated with EtOH (68.70%). The relative GSH activity in the case of cells pretreated with GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts was 145.24, 151.07, 150.31, and 152.07%, respectively (Fig. 7A). On the other hand, in the case of cells pretreated with GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL) extracts, the relative GSH activity was 145.03, 148.59, 150.63, and 151.74%, respectively (Fig. 7B).

The SOD inhibition rate in the EtOH-damaged HepG2 cells after pretreatment with TMP30, TMP50, and TMP100 extract were significantly increased compared to the cells treated only with EtOH (87.78%). The relative SOD inhibition rate in the case cells pretreated with GMP100 (400 μg/mL), TMP30 (400 μg/mL), TMP50 (400 μg/mL), and TMP100 (400 μg/mL) extracts was 138.38, 151.75, 156.69, and 158.30%, respectively (Fig. 7C). Likewise, in the case of cells pretreated with GMS100 (40 μL/mL), TMS30 (40 μL/mL), TMS50 (40 μL/mL), and TMS100 (40 μL/mL) extracts was 145.24, 151.37, 151.27, and 151.91%, respectively (Fig. 7D). These results showed that pretreatment with TML paste and sauce extracts has a higher antioxidant effect on EtOH-damaged hepatocytes than pretreatment with GMP or GMS extracts. Since the enzyme GSH plays an important role in liver protection, preventing liver damage by detoxifying various toxic compounds (Kaplowitz et al. 1985), our findings suggest that TML paste and sauce could have a hepatoprotective effect by exerting antioxidant activity.

Our findings demonstrated that TML paste and sauce have an inhibitory effect on AST and ALT enzyme activity, which is an index of hepatocyte injury severity, are able to down-regulate the expression of cytokines, which are biomarkers of inflammation, and increase SOD and GSH activity, which are biomarkers of antioxidant effects, in EtOH-damaged hepatocytes, as their effect on these activities was similar to that of the positive control silymarin. Further studies are needed to isolate a single active substance with hepatoprotective activity from TML paste and sauce in order to analyze the mechanism of this hepatoprotective effect.

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