



The protective effects of β -sitosterol and vermicularin from *Thamnia vermicularis* (Sw.) Ach. against skin aging *in vitro*

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Abstract: Aged skin, featured with dryness and wrinkles, has received mounting attention due to its adverse influences on beauty. β -Sitosterol and vermicularin are two common active ingredients of *Thamnia vermicularis* (Sw.) Ach., a traditional Chinese medicine, of which the anti-aging effect has been discovered. Their protective performance against skin aging was assayed by co-culturing with skin cells in this work. Results showed that β -sitosterol promoted the biosynthesis of hyaluronic acid by increasing the expression of hyaluronic acid synthases in fibroblasts and enhanced the expression of skin barrier functional proteins including aquaporin 3, loricrin, filaggrin and involucrin in keratinocytes, which conduced to the moisture retention within skin. Moreover, vermicularin might function as an anti-wrinkle agent by preventing the loss of collagen type I. Specifically, vermicularin reduced the amount of reactive oxygen species within hydrogen-peroxide-induced fibroblasts; together with suppressing the activation of mitogen-activated protein kinases, it could inhibit the production of matrix metalloproteinases-1. The present research will contribute to the development of the compounds as anti-aging ingredients for future applications in cosmetic formulations and functional food as well as promote further studies of raw materials containing alike compounds.

Key words: anti-wrinkle, moisturize, β -sitosterol, skin aging, *Thamnia vermicularis* (Sw.) Ach., vermicularin.

INTRODUCTION

Aging is a spontaneous and inescapable process during biological development. The outermost organ of the body, skin can be subjected to various

internal and external causes of aging (Tobin 2017, Farage et al. 2008). Moreover, skin changes such as dryness, sagging and wrinkles are commonly observed as the most apparent characteristics of body aging (Zouboulis and Makrantonaki 2011).

Skin dryness results primarily from the loss of moisture-retaining substances and epidermal barrier dysfunction (Hashizume 2004, Tončić et al. 2018). Hyaluronic acid (HA) is remarkable for its unique ability to hold water within dermis,

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while aquaporin3 (AQP3) and cornified envelope proteins including loricrin (LOR), filaggrin (FLG) and involucrin (IVL) are important due to their contributions to skin barrier function and moisture loss inhibition in epidermis (Papakonstantinou et al. 2012, Kim et al. 2018, Averbek et al. 2007). Therefore, HA and skin barrier functional proteins are main focuses of the research in skin dryness treatment.

A crucial factor upon skin sagging and wrinkling is the loss of collagen type I (COL-I)-the most abundant collagen in dermal connective tissues, and the overexpression of matrix metalloproteinase 1 (MMP-1) (also known as collagenase) is recognized as a vital inducement (Tobin 2017, Contet-Audonneau et al. 1999, Freitas-Rodríguez et al. 2017). Therefore, regulating the contents of COL-I and MMP-1 is an effective strategy for wrinkling prevention. It is known that oxidative stress serves as the molecular mechanism of skin aging (Lephart 2016). Excess of reactive oxygen species (ROS) can damage oxidatively the biomacromolecules and meanwhile cause MMP-1 overexpression via mitogen-activated protein kinases (MAPK) signaling pathway (Kammeyer and Luiten 2015). Given that hydrogen peroxide (H₂O₂) can irritate the overproduction of ROS, it functions as an active pressure source of aging inducement in dermal fibroblasts (Wen et al. 2017).

Growing concerns about skin health lead consumers to prefer cosmetic products that contain functional ingredients. Natural plants account for an important source of functional cosmetic additives (Juhász et al. 2018). Lichen *Thamnolia vermicularis* (Sw.) Ach., a traditional Chinese herbal medicine, has been proved by modern scientific researches to perform multiple pharmacological activities, such as antioxidant, anti-inflammatory, radio-resistance, etc. (Luo et al. 2006, Crawford 2015). Furthermore, the anti-aging function of *T. vermicularis* extract on human dermal fibroblasts has been reported by Chen et al. (2017). Nevertheless, the specific

effective components in *T. vermicularis* extract (TV) along with the underlying mechanisms of skin aging await more in-depth investigations.

A phenolic acid and also the characteristic component in TV, vermicularin (Verm) exists in *T. vermicularis* at a high content (Guo et al. 2011). Equally representative is β -sitosterol (Sito), the most abundant plant sterol in the human diet (Lomenick et al. 2015). In this study, the anti-aging effects of Sito and Verm were investigated by detecting their biological activities against skin drying and wrinkling in cultured human skin fibroblasts and keratinocytes.

MATERIALS AND METHODS

MATERIALS

TV was provided by Shanghai Inoherb Co., Ltd (Shanghai, China). Sito standard substance was purchased from PureOne Biotechnology (Shanghai, China). Human dermal fibroblast-adult (HDF) and human permanent epidermal cell (HaCaT) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). Human COL-I ELISA kits were purchased from Shanghai MLBIO Biotechnology (Shanghai, China). ELISA kits for Human MMP-1, Human HA, Human AQP3 and Human LOR were purchased from ExCell Biotech (Shanghai, China). Primary antibodies against ERK, phosphorylated ERK (p-ERK), phosphorylated JNK (p-JNK) and phosphorylated p38 MAPK (p-p38), and HRP-conjugated secondary antibodies were from Signalway Antibody (SAB, MD, USA). Primary antibodies against JNK, p38 MAPK, HAS1 and GAPDH were from Proteintech (Chicago, USA). INV, HAS2 and HAS3 antibodies were purchased from ABclonal Technology (Wuhan, China). Antibody against FLG was from Wanleibio (Shenyang, China). Other chemical reagents without special description were from Sinopharm Chemical Reagent (Shanghai, China).

ISOLATION OF SITO AND VERM

TV was dissolved in 50% methanol and separated by silica-column chromatography (200 mesh). Sito (yield 2.5%) was separated from methanol stream by preparative reversed-phase high performance liquid chromatography using a C18 column (20 x 250 mm, 10 mm; Shimadzu, Kyoto, Japan) and identified by HPLC (Agilent 1100) and MS ((+) ESI-TOF: 437.2 M + Na⁺) (showed as Figures S1, S2 in Supplementary Material). Verm (yield 16.7%) was isolated from ethyl acetate stream by recrystallization purification and identified with MS ((-) ESI-TOF: 375.1 M - H⁺) and ¹H NMR (CDCl₃, 400MHz) (δ H: 10.32 (1H, s, CHO), 6.53 (1H, s, H-2'), 6.35 (1H, s, H-5), 4.17 (3H, s, OCH₃), 2.54 (3H, s, Me-8), 2.24 (3H, s, Me-9')) (showed as Figures S3, S4). The chemical structures of Sito and Verm (Fig. 1a) were consistent with literature (Ma and Chen 2001).

CELLS CULTURE

The HDF and HaCaT were both cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (ExCell Bio, Shanghai, China) in an incubator (Sanyo, Osaka, Japan) with humidified atmosphere and 5% CO₂ at 37 °C. HDF cells within 10 generations were used for experiments.

MTT ASSAY

Effects of compounds on cell viability of HDF and HaCaT were detected via MTT assay. Cells (1×10⁵ cells/mL) were inoculated in 96-well plate and allowed to adhere for 12 h. HDF and HaCaT were treated with different concentrations of drugs (TV at 12.5-100 µg/mL, Sito at 12.5-100 µM and Verm at 12.5-100 µM) for 48 and 24 h, respectively. Then, cells were co-cultured with 0.5 mg/mL MTT (Biotech well, Shanghai, China) for 4 h, and the formazan was dissolved with dimethyl sulfoxide. The absorbance at 570 nm was detected

by a microplate reader (SpectraMax i3; Molecular Devices, Sunnyvale, CA, USA).

H₂O₂ IRRITATION AND SAMPLES TREATMENT IN HDF

H₂O₂-induced HDFs were performed as described previously with minor modifications (Zhou et al. 2014). Briefly, HDF cells (2×10⁵ cells/mL) were inoculated and allowed to adhere for 12 h. Then, cells were treated with compounds for 24 h and co-incubated with H₂O₂ (at the final concentration of 200 µM) for another 24 h to induce oxidative stress. The HDF cells incubated in fresh DMEM served as control group (Ctrl) and the HDF cells treated only with 200 µM H₂O₂ served as the oxidative stress control (H₂O₂).

ELISA ANALYSIS

HDF cells (2×10⁵ cells/mL) were inoculated in 96-well plates. After treatment with compounds for 24 h, cells were stimulated or non-stimulated with H₂O₂. Cells culture supernatants were harvested and evaluated the levels of HA, COL-I and MMP-1 by commercially available ELISA kits.

HaCaT cells (2×10⁵ cells/mL) were inoculated in 6-well plates and treated with compounds. Then, cells were rinsed with precooling phosphate buffer solution (PBS) and lysed using cell lysis buffer (Biotech well, Shanghai, China). The lysates were performed human AQP3 and LOR assay by ELISA. The levels of target proteins were normalized to total proteins that quantified with a BCA kit (Biotech well, Shanghai, China).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

HDF or HaCaT cells (2×10⁵ cells/mL) were inoculated in 6-well plates. After 24 h of treatment, cells were rinsed twice with precooling PBS. Total RNA of the cells was extracted with a total RNA isolation kit (KeyGEN, Nanjing, China). And then a reverse transcription kit (Takara, Shiga,

Japan) was used to obtain cDNA. SYBR real-time fluorescent quantitative polymerase chain reaction was performed using 2×RealStar Green Fast Mixture (Genstar, Beijing, China). The qRT-PCR reaction was conducted with a program as follows: pre-denaturation at 95 °C for 30 s, then forty cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 1 min. The expression levels of target genes were conducted by $\Delta\Delta C_T$ method and normalized to GAPDH. The sequences of primers were as follow: HAS1: 5'-GCG ATA CTG GGT AGC CTT CA-3' (forward), 5'-GGT TGT ACC AGG CCT CAA GA-3' (reverse); HAS2: 5'- CCT CAT CAT CCA AAG CCT GT -3' (forward), 5'- AAA CAG TTG CCC TTT GCA TC -3' (reverse); HAS3: 5'- GTC ATG TAC ACG GCC TTC AA -3' (forward), 5'- CCT ACT TGG GGA TCC TCC TC -3' (reverse); HYAL1: 5'- GTG CTG CCC TAT GTC CAG AT -3' (forward), 5'- ATT TTC CCA GCT CAC CCA GA -3' (reverse); HYAL2: 5'- TCT ACC ATT GGC GAG AGT G -3' (forward), 5'- AGC AGC CGT GTC AGG TAA T -3' (reverse); HYAL3: 5'- CCT CCA GTG CCC TCT TCC -3' (forward), 5'- CTG TCC CAG GAT GAC CTT GT -3' (reverse); AQP3: 5'- CAT CTA CAC CCT GGC ACA GA -3' (forward), 5'- GGC TGT GCC TAT GAA CTG GT -3' (reverse); LOR: 5'- CAT GAT GCT ACC CGA GGT TT -3' (forward), 5'- ACT GGG GTT GGG AGG TAG TT -3' (reverse); IVL: 5'- GTG GGG GAG AGA GGG AAT TA -3' (forward), 5'- CTC ACC TGA GGT TGG GAT TG -3' (reverse); FLG: 5'- GTT ACA ATT CCA ATC CTG TTG TTT TC -3' (forward), 5'- CGT TGC ATA ATA CCT TGG ATG ATC -3' (reverse); GAPDH: 5'- TCC ACT GGC GTC TTC ACC -3' (forward), 5'- GGC AGA GAT GAT GAC CCT TTT -3' (reverse).

MEASUREMENT OF INTRACELLULAR ROS LEVEL

Intracellular ROS level was monitored by a ROS assay kit (KeyGEN, Nanjing, China). Briefly, HDF cells were inoculated in 6-well plates and treated with compounds and H₂O₂. Then, cells were stained with 10 μM DCFH-DA at 37 °C for 20 min. The cells were harvested by trypsinization

and the fluorescence intensity (λ excitation=485 nm, λ emission =525 nm) was determined using a microplate reader (SpectraMax i3; Molecular Devices, Sunnyvale, CA, USA).

WESTERN BLOT ANALYSIS

HDF cells were inoculated and incubated with compounds and H₂O₂. HaCaT cells were treated with compounds only. The cells were rinsed by PBS and lysed using cell lysis buffer with PMSF and Phosphatase Inhibitor cocktail (Biotech well, Shanghai, China) in ice-bath. The total proteins extracted were quantified using a BCA kit. The mixtures of protein samples (20 μg) and loading buffer were boiled and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then protein bands in the gel were transferred to PVDF membrane (Millipore, MA, USA), followed by blocking with 5% BSA and incubation with primary antibodies and HRP-conjugated secondary antibodies. The protein bands were visualized by ECL luminescence reagent (Biotech well, Shanghai, China) via biological image analysis system (FR-1800, Furi Co., Ltd, Shanghai, China).

STATISTICAL ANALYSIS

Experiments were performed independently at least three times. The data were expressed as the mean ± SD and analyzed using SPSS program (V22.0, IBM Co., NY, USA). The statistical differences between groups were analyzed via one-way analysis of variance (ANOVA) and $P < 0.05$ was considered significant.

RESULTS

EFFECTS OF TV, SITO AND VERM ON CELL VIABILITY OF HDF AND HACAT

The cytotoxicity of TV, Sito and Verm towards HDF and HaCaT was determined with MTT assay. TV at 0-50 μg/mL, Sito at 0-25 μM and Verm at

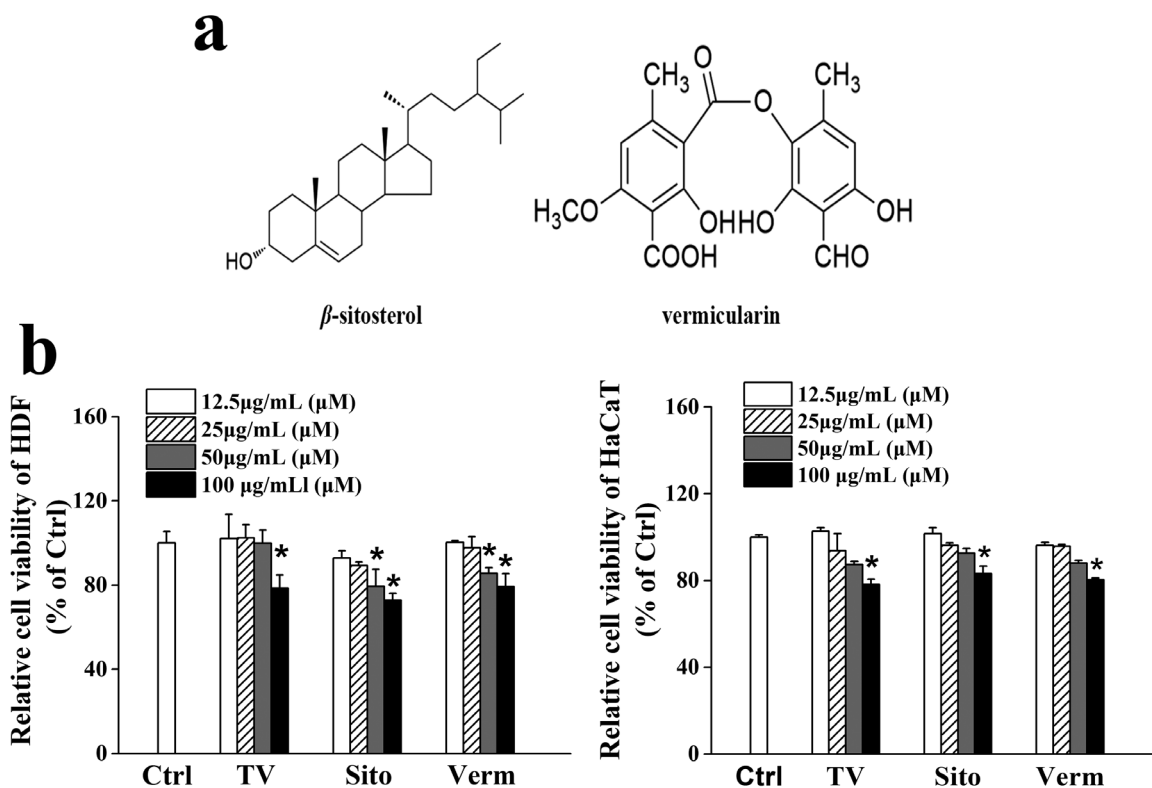


Figure 1 - Structures of β -sitosterol and vermicularin (a) and their effects on cell viability of HDF and HaCaT (b). * $P < 0.05$ vs. Ctrl group.

0-25 μM barely influenced HDF viability, and TV at 0-50 $\mu\text{g/mL}$, Sito at 0-50 μM and Verm at 0-50 μM exerted negligible effects on HaCaT viability (Fig. 1b). Compounds at as-designated concentrations were not cytotoxic and thus applied in subsequent experiments.

SITO-PROMOTED HA PRODUCTION VIA UP-REGULATING HAS AND DOWN-REGULATING HYAL IN HDF

As a measure of moisturizing capacity of compounds, HA production in HDF cells was assessed by ELISA, with nacetylglucosamine (GlcNAc) the HA precursor taken as a positive control. Results showed that Sito improved effectively the HA secretion in a dose-dependent manner and by 18% at high concentration, while Verm hardly altered the level of HA (Fig. 2a, b).

In order to clarify the specific mechanism of Sito promoting HA secretion, the expression

of hyaluronic acid synthases (HAS1, -2, -3) and hyaluronidases (HYAL1, -2, -3) in HDF was further investigated. According to the measurements, Sito dose-dependently increased the mRNA and protein levels of HAS1, -2, -3 (Fig. 2c, e), whereas a decrement in HYAL3 mRNA level was observed as the concentration of Sito reached 25 μM (Fig. 2d).

SITO PROMOTED THE EXPRESSION OF AQP3, LOR, FLG AND IVL IN HACAT

For exploring effects of compounds on epidermal barrier function, the protein levels of AQP3 and LOR were measured by ELISA and the levels of FLG and IVL were measured by western blot. All-trans retinoic acid (ATRA) or calcium chloride (CaCl_2) was taken as the positive control promoting the expression of target protein (Bellemère et al. 2008, Pleguezuelos and Kapas 2006). Sito outperformed remarkably Verm in increasing the protein levels of AQP3 and LOR (Fig. 3a, b).

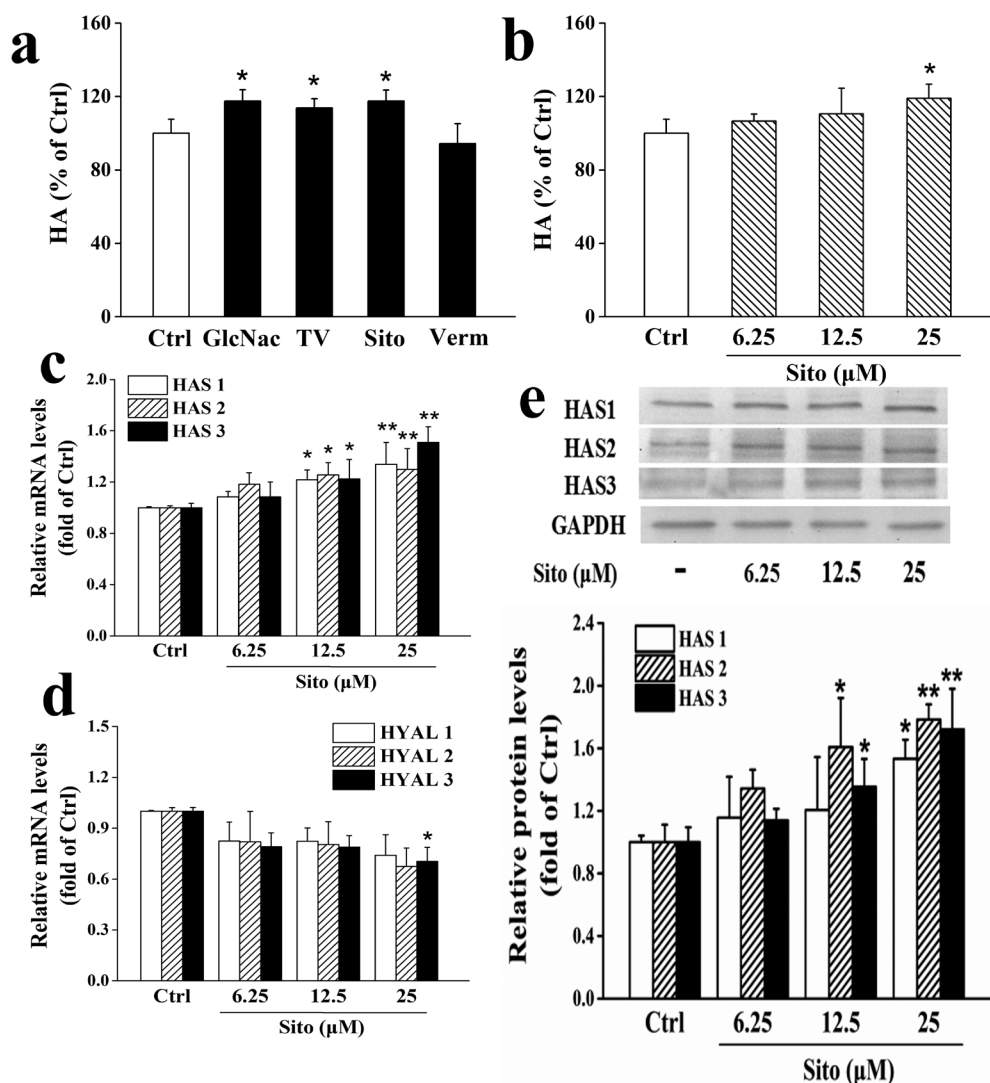


Figure 2 - Effects of compounds on HA content and on the expression of HASs and HYALs in HDF. Amount of HA (a, b) was measured by ELISA kit. The mRNA expression of HAS1-3 (c) and HYAL1-3 (d) was measured by qRT-PCR. The protein expression of HAS1-3 (e) was measured by western blot. * $P < 0.05$ vs. Ctrl group.

Furthermore, Sito significantly enhanced the mRNA and protein levels of AQP3, LOR, FLG and IVL in a dose dependent manner (Fig. 3c-f).

VERM-INCREASED THE CONTENT OF COL-I BY DECREASING MMP-1 PRODUCTION VIA SUPPRESSING ROS LEVEL AND MAPK SIGNALING IN HDF

As an evaluation of compounds' anti-wrinkle effects, their influences on the contents of COL-I, MMP-1 and ROS were determined with H_2O_2 -

induced HDF. Nicotinamide (VB3) or ascorbic acid (VC) was taken as the positive control (Chen and Damian 2014). The level of COL-I decreased by 23% and the levels of MMP-1 and ROS increased respectively by 36% and 55% in H_2O_2 -induced HDF (Fig. 4a-c). Results indicated that Verm increased surprisingly the content of COL-I and also decreased effectively the levels of MMP-1 and ROS, while Sito hardly regulated these factors (Fig. 4). Moreover, Verm restored the levels

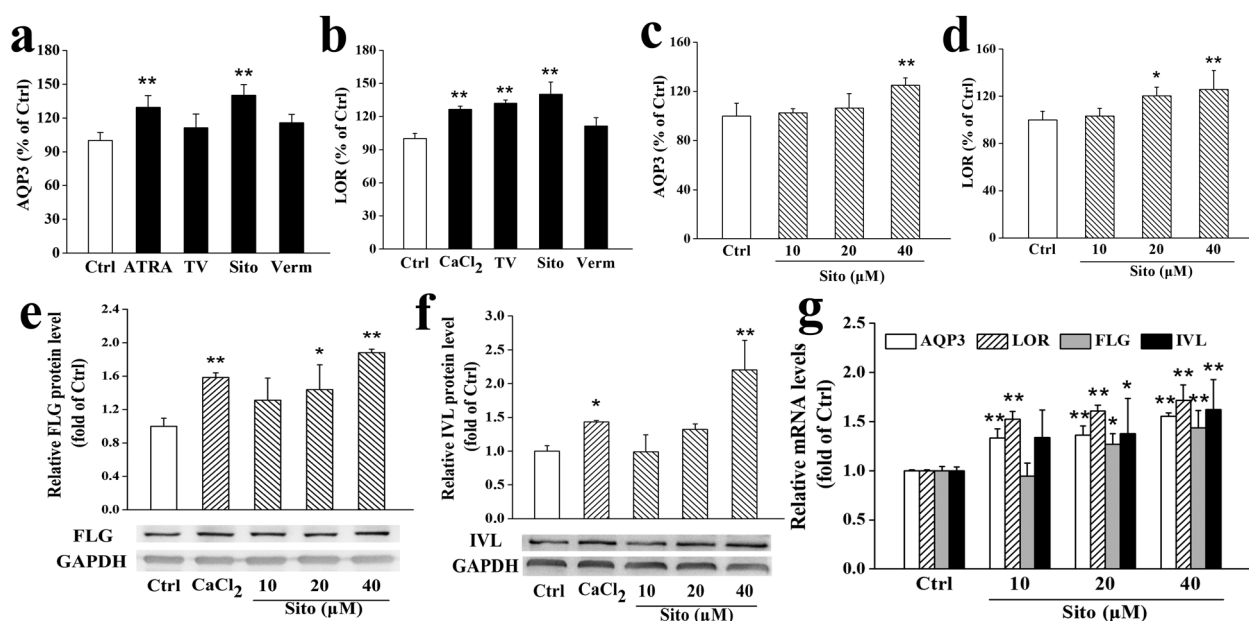


Figure 3 - Effects of compounds on the expression of AQP3, LOR, FLG and IVL in HaCaT. The protein expression of AQP3 (a, c) and LOR (b, d) was measured by ELISA. The protein expression of FLG (e) and IVL (f) was measured by western blot. The mRNA expression of AQP3, LOR, FLG and IVL (g) was measured by qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ vs. Ctrl group.

of COL-I, MMP-1 and ROS in a dose-dependent manner (Fig. 4d-f).

For further explaining the specific role of Verm against skin aging, its effect on the activation of MAPK signaling (ERK, JNK and p38 MAPK) was examined by western blot. The phosphorylation of ERK, JNK and p38 MAPK heightened in H₂O₂-induced HDF (Fig. 5). Verm obviously suppressed the phosphorylation of ERK and JNK in a dose-dependently manner (Fig. 5a, b), and the phosphorylation of p38 (p-p38) was also attenuated as the concentration of Verm reached 20 μ M (Fig. 5c).

DISCUSSION

Dryness and wrinkles are typical features of skin aging. Thereby, materials with moisturizing and anti-wrinkling activities are potential candidates to anti-aging ingredients in skincare formulations. Sito generally exists in human diet as well as many medicinal plants and possesses anti-cancer, anti-inflammatory and analgesic activities (Lomenick et al. 2015, Villaseñor et al. 2002). Verm is a main

characteristic component in *T. vermicularis* with physiological activities of anti-inflammatory and antipyretic (Guo et al. 2011, Crawford 2015). Sito and Verm are typical components of TV which showed anti-aging effect. In this study, their activities on moisturizing and anti-wrinkling were investigated within skin cells and the mechanisms of protection were further discussed.

HA functions crucially in moisture holding of the skin, in which the metabolism in mammals is directly regulated by hyaluronic acid synthases (HAS1-3) and hyaluronidases (HYAL1-3) (Lee et al. 2016, Averbeck et al. 2007, Itano and Kimata 2002). That Sito could promote HA secretion and enhance HAS1-3 expression significantly revealed its probable moisturizing activity through promoting the biosynthesis of HA chains and thus raising the HA level. In addition, epidermal barrier function is another important regulation for skin moisture, as it can prevent the moisture from trans-epidermal loss (Tončić et al. 2018, Chaudhuri and Bojanowski 2017, Li et al. 2010). Since Sito was

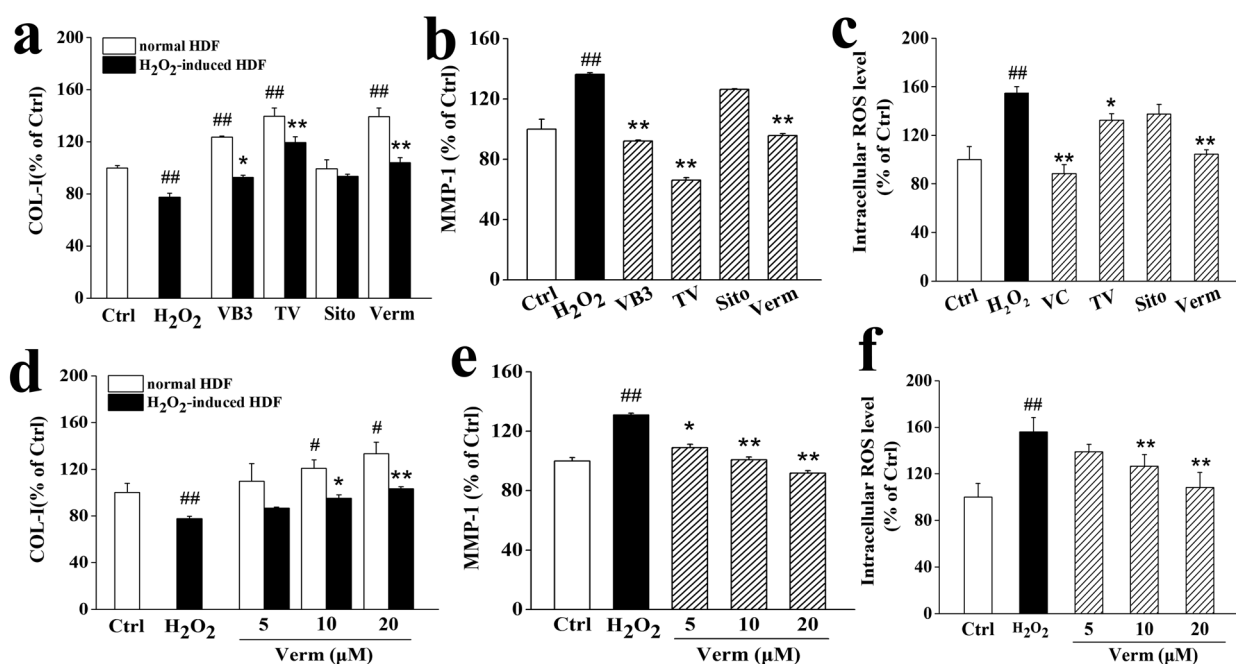


Figure 4 - Effects of compounds on COL-I secretion, MMP-1 production and ROS level in H₂O₂-induced HDF. COL-I secretion (**a**, **d**) and MMP-1 production (**b**, **e**) were measured by ELISA. The level of intracellular ROS (**c**, **f**) was measured using a fluorometric ROS assay kit. # $P < 0.05$ and ## $P < 0.01$ vs. Ctrl group. * $P < 0.05$ and ** $P < 0.01$ vs. H₂O₂ group.

found to enhance the expression levels of water channel protein (AQP3) and the components of cornified envelop (LOR, FLG and IVL) in HaCaT, it could also lock skin moisture through promoting skin barrier function. The dual effect enabled Sito to be an active ingredient for dryness relief, which outperformed Verm. The results were consistent with the previous report of phytosterols being used as cosmetic additives to retain moisture and deter skin aging (Folmer 2003).

COL-I loss can induce fragility of collagen fibers, which further results in the formation of skin wrinkles, one of its major cause is MMP-1 overexpression. Oxidative stress has been recognized as an important molecular mechanism of skin aging, and it was reported that ROS-activated MAPK signaling could stimulate the expression of MMP-1 in HDF (Lephart 2016, Noh et al. 2016, Lu et al. 2017, Sun et al. 2017). In this study, Verm was found to increase COL-I level and inhibit MMP-1 overexpression so as to perform anti-wrinkling effect that Sito could not realize. Moreover, our

results suggested that indeed Verm could attenuate the H₂O₂-induced overproduction of ROS, which further supported the former findings that phenolic compounds with activities in antioxidation, collagen synthesis promotion, as well as collagen degradation inhibition effected skin anti-aging (Kammeyer and Luiten 2015, Svobodová et al. 2003, Fisher et al. 2002).

ERK, JNK and p38 MAPK are key members of MAPK signaling pathway. As reported before, phosphorylation of ERK and JNK could mediate the activation of c-fos and/or c-jun; then, activated c-fos and c-jun would constitute AP-1 the transcription factor that further regulated the expression of MMP-1 (Sun et al. 2017). Since experimental results in this work revealed the suppressed phosphorylation of ERK and JNK by Verm in H₂O₂-induced HDF, Verm was proved an anti-aging agent that prevented COL-I loss via inhibiting the expression of MMP-1 in the way of reducing intracellular ROS level and suppressing the MAPK signaling activation. Actually,

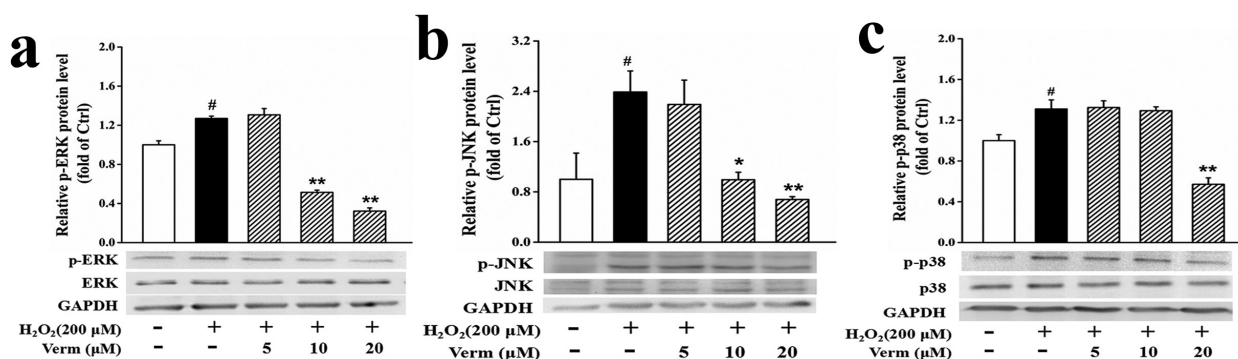


Figure 5 - Effect of Verm on the activation of MAPK signaling in H₂O₂-induced HDF. The phosphorylation levels of ERK (a), JNK (b) and p38 (c) were examined by western blot. # $P < 0.05$ vs. Ctrl group. * $P < 0.05$ and ** $P < 0.01$ vs. H₂O₂ group.

activation of transforming growth factor-beta (TGF- β) signaling pathway was also proposed as another anti-aging strategy, for it could upregulate the collagen expression (Gao et al. 2018, Park et al. 2018). Verm's effects on this pathway, however, are open to further investigation.

In conclusion, β -sitosterol and vermicularin are two active compounds preventing skin aging. Sito could promote the biosynthesis of HA and enhance the skin barrier function. This dual effect enables Sito to be an active compound for skin moisturizing. While, Verm was able to prevent COL-I loss via antioxidation and collagen degradation inhibition so as to perform anti-wrinkling effect. Our findings contributes to the development of β -sitosterol and vermicularin as anti-aging ingredients for application in skincare products, in addition, the research may provide reference for future studies on the bioactivities of raw material containing these compounds.

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AUTHOR CONTRIBUTIONS

Haiyuan Yu and Yanhua Lu originally designed the experiments. Minhua Hong and Dan Liu provided *Thamnia vermicularis* (Sw.) Ach. extract and identified the components. Haiyuan Yu and Xueqing Shen performed the experiments. All authors discussed the results. Haiyuan Yu wrote the manuscript.

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SUPPLEMENTARY MATERIAL

Figure S1 - HPLC chromatographic profile of *T. vermicularis* extract (**a**) and β -sitosterol standard (**b**). HPLC condition: Agilent ZORBAX Eclipse XDB-C18 column (4.6×250

mm, 5 μ m), DAD G1315B Detector, G1311QuatPump, mobile phase methanol/water (90:10, v/v), flow rate 1.0 mL/min, injection volume 20 μ L, detection wavelength 210 nm, column temperature 25 $^{\circ}$ C.

Figure S2 - MS profile of β -sitosterol ((+) ESI-TOF: 437.2 M + Na $^{+}$).

Figure S3 - MS profile of vermicularin ((-) ESI-TOF: 375.1 M - H $^{+}$).

Figure S4 - 1H NMR spectrum of vermicularin in $CDCl_3$ (400 Hz).