Pomegranate Seed Oil Enhances the Percutaneous Absorption of trans-Resveratrol

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Abstract: Pomegranate seed oil (PSO) is primarily composed of unsaturated fatty acids, implying its potential application as a transdermal enhancer. In this study, the function of PSO in prompting the percutaneous absorption of resveratrol was examined and compared with isopropyl palmitate (IP). IP of 10% enhanced the cumulative permeation amount of resveratrol by 50% but did not influence the permeation velocity. Though 2.5% and 5.0% IP accelerated the penetration process of resveratrol, they had no discernable impact on total permeation amount. In contrast, the cumulative percutaneous amount of the drug with 2.5%, 5.0% and 10% PSO was 1.25, 2.25, and 3.14-fold that of resveratrol alone, respectively. Moreover, PSO of different concentrations speeded up resveratrol to permeate through skin in the whole process, exhibiting its superior capacity over IP in enhancing the transdermal absorption of resveratrol. IP of 2.5% substantially augmented resveratrol retention in stratum corneum (SC), epidermis, and dermis (p < 0.05) while 2.5% PSO only increased the drug detaining in SC. Involvement of oils also aided in resveratrol diffusion within skin. The study demonstrates that both IP and PSO prompted the percutaneous transport of resveratrol. PSO presents more promising opportunities in serving as a percutaneous enhancer for transdermal preparations.

Key words: trans-resveratrol, isopropyl palmitate, pomegranate seed oil, transdermal enhancer, skin

1 Introduction

Pomegranate seed oil (PSO) is a mixture of fatty acids. Punicic acid, also called conjugated linolenic acids (CLA), is the major constituent of PSO, accounting for 64%-83% of PSO¹. Punicic acid is a polyunsaturated fatty acid, including 18 carbons and 3 double bonds. Apart from punicic acid, PSO contains low proportions of oleic acid and linoleic acid². PSO has diverse biological activities. It possesses anti-inflammation and anti-proliferation properties through regulating multiple signal pathways, implying a promising chemotherapeutic application³, ⁴. PSO as well as its primary component punicic acid, were able to inhibit the growth of several cancer lines, such as breast cancer⁵, colon cancer, prostate cancer, skin cancer, and so on⁶. PSO also exerted protective activity against diabetes complications⁷-⁹. Besides, PSO showed anti-atherogenic effects, able to ameliorate the lipid profiles of hyperlipidaemic subjects¹⁰.

Recently, transdermal drug delivery system has gained increasingly attention due to its convenience, sustained and stable blood level in vivo, and easy removal of drug when some unexpected side effects take place. Stratum corneum (SC) is the first barrier for the drug entry into skin. To overcome the resistance of SC, transdermal enhancers are often employed in transdermal delivery systems¹¹-¹⁰. Among them, natural fatty acids attract particular interests owing to their nontoxicity and inertness to most components. Numerous studies validated the effect of unsaturated fatty acids, such as oleic acid and linoleic acid, in prompting percutaneous transport of various drugs¹⁴-¹⁶. Since PSO is abundant in punicic acid, a polyunsaturated fatty acid, we assume that it may have the feature of enhancing permeation. PSO possesses some advantages over oleic acid and linoleic acid in transdermal application. Firstly, PSO is a natural oil present in pomegranate seed and is easily to obtain, while the production of oleic acid and linoleic acid seems relatively complex, including the saponification of plant oils followed by purifica-
tion process. Secondly, PSO contains versatile fatty acids, including oleic acid and linoleic acid, which may exert synergistic effect with its major ingredient punicic acid. Thirdly, PSO itself has diverse biological functions. Not only may PSO assist in drug transdermal diffusion, it will also act in parallel with the drug to generate stronger remedy effect.

Trans-resveratrol is a natural compound present in a variety of edible plants\(^\text{17}\). Like PSO, resveratrol has multiple pharmaceutical activities as well, for example, anticancer\(^\text{18, 19}\), anti-inflammation\(^\text{20, 21}\), alleviating neurodegenerative disorders\(^\text{22}\), lowering blood pressure and lipid level\(^\text{23, 24}\), and so on\(^\text{25}\). However, the utility of resveratrol in clinic is limited by its poor solubility, short half time \textit{in vivo}\(^\text{26}\), and the resultant undesirable oral bioavailability\(^\text{27}\). Transdermal route may be an alternative choice for resveratrol administration to reduce its decomposition in digestive tract and improve its bioavailability\(^\text{28}\).

In this study, resveratrol transdermal process was examined and the ability of PSO to enhance resveratrol skin permeation was assessed. Since PSO has synergistic effect with resveratrol\(^\text{29}\), it is hypothesized that not only may PSO be beneficial in resveratrol transdermal diffusion, they would also act together to yield more potent therapeutic outcome. Isopropyl palmitate (IP), a commonly used oil phase of microemulsions and cream\(^\text{30, 31}\), was set as a control to compare the capacity of augmenting resveratrol percutaneous diffusion. IP was proved to be efficient in prompting transdermal absorption of a few drugs\(^\text{32, 33}\). Both IP and PSO are good candidates of oil phases to fabricate microemulsions, which is one of the most popular drug delivery systems utilized to increase percutaneous transport of active ingredients\(^\text{34, 35}\). Thus, our study would provide an insight into the latent contribution of oil phase in microemulsions to the enhancement of transdermal absorption.

2 Materials and methods

2.1 Materials and reagents

Trans-resveratrol, with the purity over 99.0\%, and PSO, were purchased from Xian Plant Biological Engineering Co., Ltd. (Xian, China). IP was from Labcaster Company. Other reagents were obtained from Chengdu Kelun Chemical Reagent Company (Chengdu, China).

2.2 Fatty acid analysis of PSO by GC-MS

The methylation of PSO was conducted following the method proposed by Yu \textit{et al} with some modification\(^\text{36}\). Briefly, 10 mg PSO was dissolved in 4 mL of 0.5 mol/L NaOH-MeOH solution and was subject to steam bath heating for 5 min. When the solution was cooled to room temperature, diluted hydrochloric acid was added to neutralize the excess alkaline, followed by the extraction with 1 mL of hexane. After filtration, the extract of 1 \(\mu L\) was injected into a Clarus 680 GC-MS instrument (PerkinElmer, Inc., USA) with a quadrupole MS for fatty acid analysis. The sample was isolated on a HP-5 capillary column (30 m \(\times\) 0.25 mm). The injector temperature was 270°C with the split ratio of 1:50. The carrier gas was helium. The oven temperature started at 90°C and held for 3 min, then was raised to 180°C at 15°C/min. After holding for 10 min, the temperature was elevated to 250°C at 2.5°C/min. For MS detection, the solvent delay time was 4 min. Both the transfer temperature and source temperature were set 180°C. The scan ranged from 40 to 600 Da. The peaks of fatty acids were identified through the MS data bank of the software.

2.3 \textit{In vitro} cutaneous permeability studies

2.3.1 Skin membranes

The fresh abdominal skin samples were obtained from the pigs of 3 to 4 months old, which were slaughtered in Bingxi slaughter house, Chengdu, China. The experiment protocol was approved by the Ethical Committee of Experimental Animal Care of Chengdu University. The skin was cleansed with saline, followed by careful removal of hair and subcutaneous fat and cutting into round pieces with a diameter of approximate 1.7 cm. After being rinsed with saline again, the skin was stored at \(-80°C\) until use (for a maximum of 3 months). Prior to use, the frozen skin was put into a conical bottle and immersed with saline. The bottle was placed in a 37°C water-bath shaker and shaken at 100 r/min for 1 h.

2.3.2 Transdermal experiment

The Franz diffusion cell system consisted of 6 donor chambers, 6 receptor compartments, pig skin, and an advanced transdermal diffusion apparatus (Tianjin Fulan Electronic Technology Co. Ltd., China). After the volume of a receptor cell was measured, the cell was filled with saline and maintained at 37°C. Then the thickness of a piece of pig abdominal skin was determined by a Vernier caliper and the skin was mounted on the receptor compartment with the SC layer facing the donor cell. The skin was closely contacted with both donor compartment and receptor chamber by a special clip with the diffusion area of around 1.5 cm\(^2\). Subsequently, 4.0 mL of the test samples at 37°C were added to donor compartments, respectively. In the experiment, the test samples included 3 series of solutions. Group 1 was saturated resveratrol solution. Group 2 included saturated resveratrol solution along with 2.5%, 5.0%, and 10% PSO (v/v), respectively. Group 3 was composed of saturated resveratrol solution plus 2.5%, 5.0%, and 10% IP (v/v), respectively. The donor compartments and the sample outlets of receptor cells were sealed with Parafilm\(^\text{8}\) to avoid solvent evaporation. When the instrument was ready, magnetic stirring at 500 r/min in the receptor cells was initiated, which worked constantly till the
end of experiment. At specified time intervals, 0.5 mL of samples were withdrawn from the outlet of receptor cells, and 0.5 mL of fresh saline incubated at 37°C was replenished into the receptor compartments, respectively. The withdrawn aliquots were centrifuged at 1560 g for 10 min and the supernatant was applied for HPLC analysis. The permeation test of each sample was replicated in 6 diffusion cells.

As soon as the experiment was over, the skin was taken off from the diffusion cells, washed with saline, and dried on filter paper. The diffusion areas were accurately measured by a Vernier caliper. Then the SC layer was carefully detached from the epidermis and dermis using a knife, weighed, and collected in a volumetric flask of 25 mL. The remnant skin was cut into small pieces, weighed, and placed into a volumetric flask of 25 mL as well. Ethanol of 15 mL was added into each flask to extract resveratrol from either SC layer or epidermis and dermis, respectively, under ultrasound treatment for 30 min. Afterward, the solutions were diluted to the designated volume with distilled water.

2.3.3 HPLC analysis

Samples of 100 μL were injected into an Agilent 1100 HPLC instrument for the determination of trans-resveratrol content. Sample separation was performed on a Diamonsil C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was composed of methanol and 0.1 mol/L acetic acid (4:6, v/v) with the flow rate of 1 mL/min. The column temperature was maintained at 35°C and the detection wavelength was 307 nm.

2.3.4 Calculation of permeation parameters

According to the first diffusion law of Fick’s, the relationship between the diffusion velocity and drug concentration is expressed as the following:

\[
\frac{dQ}{dt} = K_p(C_D - C_R)
\]

(1)

where \(Q (\mu g/cm^2)\) is the cumulative amount of a drug permeating through the skin of the area of 1 cm² and entering the receptor cell. \(K_p (cm/h)\) represented the permeating coefficient. \(C_D (\mu g/mL)\) and \(C_R (\mu g/mL)\) are the drug concentration in donor chamber and receptor cell, respectively.

The system is usually under the condition of \(C_D \gg C_R\) and \(C_D\) could be considered as the initial concentration of the donor compartment \(C_0\). Thus, equation (2) is obtained via the integration of equation (1).

\[
Q = K_pC_D(t - t_0)
\]

(2)

where \(t_0\) is the lag time. When permeation reaches steady state, the diffusion velocity tends to be constant. If a permeating curve is plotted with the cumulative permeating amount against time, the diffusion velocity of steady state \(J_w\) is equal to the slope of the curve. Then \(K_p\) can be reckoned through equation (3):

\[
K_p = \frac{J_w}{C_0}
\]

(3)

The permeation rate across skin is mainly dependent on the thickness of skin. In the Franz cells, drug diffusion is driven by concentration gradient and occurs from SC layer to dermis. The diffusion coefficient within skin \(D_s, cm^2/h\) can be depicted as equation (4):

\[
D_s = \frac{h^2}{6 t_s}
\]

(4)

where \(h\) represents the thickness of skin.

During the steady state, the diffusion velocity can also be expressed as equation (5):

\[
J_w = \frac{(D_s \ast P_{sv} \ast C_0)}{h}
\]

(5)

where \(P_{sv}\) was the partition coefficient of drug in skin and vehicle. By integrating equation (3) and (5), another equation is attained:

\[
P_{sv} = \frac{K_p \ast h}{D_s}
\]

(6)

2.4 Statistical analysis

All data were presented as mean ± standard deviation (SD), calculated from 6 skin permeation experiments. ANOVA analysis and Turkey test were conducted to compare the difference among results. Statistical significant variation was set as \(p<0.05\).

3 Results and discussion

3.1 Fatty acid composition of PSO

The GC-MS total ion chromatogram of PSO after methylolation was displayed in Fig. 1. The analysis result of fatty acid composition was summarized in Table 1. The numbers displayed in the first column of Table 1 corresponded to

![Fig. 1](image-url)
Table 1 The identification of fatty acids in PSO.

| No. | Fatty acid                     | Chemical formula | Acronym | Peak area% |
|-----|--------------------------------|------------------|---------|------------|
| 1   | Hexadecanoic acid              | C_{16}H_{36}O_{2} | C16:0   | 3.54       |
| 2   | (9Z, 12Z)-Octadecadienoic acid | C_{18}H_{36}O_{2} | C18:2(9,12)| 6.49     |
| 3   | 11-Octadecenoic acid           | C_{18}H_{36}O_{2} | C18:1(11)| 6.38      |
| 4   | Stearic acid                   | C_{18}H_{36}O_{2} | C18:0   | 3.28       |
| 5-8 | Octadecatrienoic acid          | C_{18}H_{38}O_{2} | C18:3   | 73.93      |
| 9   | 11-Eicosenoic acid             | C_{20}H_{40}O_{2} | C20:1(11)| 0.59      |
| 10  | Eicosanoic acid                | C_{20}H_{42}O_{2} | C20:0   | 0.64       |

Note: C18:2 indicates a C18 chain with two ethylenic bonds.

3.2 HPLC analysis of trans-resveratrol

HPLC chromatograms (Fig. 2) implied that the blank skin permeation solution with either 10% PSO or 10% IP did not interfere with the detection of trans-resveratrol. The peak areas were well linear with resveratrol concentrations in the range of 0.032 – 100 ng/mL, following the relationship of $Y = 271861 X + 202687$ ($r^2 = 0.9987$). The limit of detection (LOD) and quantitation (LOQ) were 15 and 25 ng/mL, respectively.

3.3 Permeating curves

Cutaneous permeating processes of saturated trans-resveratrol solutions containing various proportions of oil were displayed in Fig. 3. The transdermal curves showed a relatively long lag time of the drug. Resveratrol kept undetectable in donor compartments until after 15 h. Oil incorporation reduced the lag time of resveratrol with different extents. IP of 2.5% and 5.0% accelerated the transdermal absorption of resveratrol and shortened the lag time remarkably, but contributed little to the increase of cumulative permeation amount. On the contrary, though the effect of 10% IP on reducing the lag time of resveratrol was not so evident as 2.5% and 5.0% IP, the total penetration amount with 10% IP reached 1.5-fold that of resveratrol alone. Compared to IP, PSO exhibited more potent capacity in enhancing penetration. PSO of different ratios speeded up the transdermal absorption of resveratrol in the whole process and reduced the lag time dramatically. As the increment of PSO content, the penetration amount of drug at 60 h ascended gradually. The cumulative percutaneous amount of resveratrol with the aid of 10% PSO was 3.4-fold that of resveratrol itself.

3.4 Residue in skin

The contents of resveratrol remaining in SC, epidermis and dermis after the completion of the penetration experiment, were illustrated in Fig. 4. It indicated that 2.5% IP dramatically increased the residual amount of resveratrol in SC ($p < 0.02$) while the oil of other ratios diminished resveratrol retention on SC layer. In contrast, PSO at various concentrations notably reduced the remnant of resveratrol on SC ($p < 0.05$). Besides, incorporation of 2.5% IP or 2.5% PSO augmented the retaining of resveratrol in epidermis and dermis. Oils of other proportions reduced resveratrol trap in the deeper skin layer. SC is the first barrier for a drug to permeate through skin. Since SC is hydrophilic, resveratrol with lipophilic IP had more difficulty in transporting across SC layer, resulting in more retaining of resveratrol in SC with IP. As the increase of IP concentration, other properties of IP in prompting penetration, such as dissolving the skin-tissue components, disrupting the highly ordered lamellar structure, interacting with intracellular protein, and so on, began to take effect. As a result, when IP was at a higher concentration, the retention of resveratrol in SC, epidermis and dermis decreased, along with ascending cumulative permeation amounts.

Figures 3 to 4 showed that compared to the resveratrol control, the cumulative permeation amount of the drug with 2.5% IP remained unchangeable, but the residue in SC, epidermis and dermis augmented substantially, indicating that 2.5% IP increased the overall transdermal amount, however, large quantities of drug were trapped in skin. As the increase of IP proportion, the retention of resveratrol in skin decreased, along with gradual ascending of cumulative permeation amount. The study implied that IP at low ratio was capable of accelerating percutaneous transport and making the permeation equilibrium achieved at earlier
time. IP of 10% enhanced the total transdermal absorption of resveratrol, but had little effect on the absorption velocity. Fig. 3 exhibited the strong capacity of PSO in enhancing penetration amount as well as speeding transdermal absorption. The ability of PSO as a percutaneous enhancer originates from its constituents. According to GC-MS analysis, PSO mainly consisted of punicic acid (C18:3, 73.93%), linoleic acid (C18:2, 6.49%), and oleic acid (C18:1, 6.38%). Linoleic acid and oleic acid have been reported to prompt transdermal transport of several drugs on different types of skin. Studies revealed that unsaturated long-chain fatty acids displayed more potent activity in enhancing cutaneous permeation than the analogous saturated fatty acids. Amri et al. reported that fatty acids were present in PSO in the lipid forms of triglycerides, glycolipids, and phospholipids. Saturated fatty acids, such as palmitic acid and stearic acid, were abundant in glycolipids and phospholipids. The ratios of the three lipid forms were similar for monounsaturated fatty acids, while polyunsaturated fatty acids, for example, punicic acid and linoleic acid, enriched in triglyceride form. The study implied that unsaturated fatty acids in lipid forms, like PSO, maintained the capacity of free acids in prompting skin penetration.

3.5 Parameters of percutaneous permeation

The related permeation parameters, such as permeation coefficient ($K_p$), diffusion coefficient within skin ($D_s$), and partition coefficient between skin and vehicle ($P_{sv}$), were listed in Table 2. Both oils showed the same trend that when oil concentration was as low as 2.5%, the oil decreased $K_p$ as well as $P_{sv}$ of resveratrol. As oil ratio ascended to 5.0% and 10%, $K_p$ and $P_{sv}$ elevated correspondingly, accompanied with the enhanced transdermal absorption of resveratrol. The effect of PSO on $D_s$ exhibited a different
PSO of 2.5% accelerated resveratrol diffusion within skin, as evidenced by the increased value of $D_s$. And $D_s$ kept growing till PSO concentration rose to 5.0%. Furthermore, when PSO ratio in the solution reached 10%, $D_s$ began to descend back to the original level of resveratrol in the absence of oil. In contrast, $D_i$ of resveratrol with IP elevated in an oil concentration dependent fashion. The higher the IP ratio was, the greater the $D_i$ would be.

Usually, percutaneous amount is proportional to the permeation coefficient ($K_p$), as demonstrated by equation (2). The transdermal processes of resveratrol with PSO and IP well conformed to the rule. Cumulative permeating amount of resveratrol at 60 h elevated as the increased concentration of oil, along with the augmenting of $K_p$. SC is the first barrier the drug encounters during percutaneous transport. $K_p$ actually acts as an indicator to assess the ability of a vehicle to permeate through the SC layer. It is notable that regardless of the reduced $K_p$ of resveratrol due to the involvement of 2.5% PSO, the total penetration amount at 60 h with 2.5% PSO was much higher than that without PSO. The same situation occurred on 2.5% IP as well. Inclusion of 2.5% IP decreased the $K_p$ of resveratrol from 2.78 to 1.34 cm/h, nevertheless, the cumulative permeating amount was still comparable with that of resveratrol alone. It implied that though 2.5% PSO and IP attenuated the power of resveratrol in penetrating through SC, they may help to the passage in epidermis and dermis. Transport in epidermis and dermis not only means the diffusion in skin, but also indicates the capacity of moving out of skin and entering blood circulation. PSO and IP at low dose possibly dis-
Pomegranate Seed Oil Enhances Skin Penetration of trans-Resveratrol

Table 2  Percutaneous parameters of trans-resveratrol in the presence of different concentrations of PSO or IP.

| Oil  | Concentration | $K_p \cdot 10^{2}$ (cm/h) | $D_s \cdot 10^{-4}$ (cm²/h) | $P_{sv}$ |
|------|---------------|-------------------------|--------------------------|----------|
|      | 0             | 2.78 ± 0.30             | 6.47 ± 2.40              | 10.44    |
| PSO  | 2.5%          | 1.63 ± 0.77             | 7.47 ± 2.34              | 5.65     |
|      | 5.0%          | 2.87 ± 0.42             | 9.47 ± 2.48              | 8.85     |
|      | 10%           | 4.67 ± 0.15*            | 6.47 ± 4.50              | 17.41*   |
|      | 2.5%          | 1.34 ± 0.92             | 7.42 ± 2.12              | 3.04     |
| IP   | 5.0%          | 2.85 ± 1.17             | 11.24 ± 4.16*            | 8.06     |
|      | 10%           | 3.83 ± 0.68             | 15.12 ± 1.92*            | 9.34     |

* In regard to the control group without oil, $p < 0.05$.

4 Conclusion

The effect of IP and PSO on the transdermal absorption of trans-resveratrol was studied on pig abdominal skin using a Franz diffusion cell system. The permeation curves declared that IP of 2.5% and 5.0% accelerated the permeation of resveratrol, but had no contribution to the cumulative percutaneous amount. IP of 10% improved total transdermal absorption of resveratrol by 50%, however, it had no apparent impact on speeding up the absorption. In contrast, PSO exhibited more potent ability in enhancing the percutaneous absorption as well as accelerating the transdermal process. The cumulative permeation amount of resveratrol with 10% PSO was 3.14-fold that of resveratrol alone. IP of 2.5% dramatically increased the retention of resveratrol in SC, epidermis, and dermis ($p < 0.05$), while 2.5% PSO only boosted the drug trap in SC. Oils at other ratios diminished the detaining of resveratrol in all layers of skin. IP and PSO of 2.5% reduced the permeation coefficient $K_p$ (cm/h) and partition coefficient ($P_{sv}$) of resveratrol. However, as the proportion of oils rose, $K_p$ and $P_{sv}$ increased correspondingly. The diffusion in skin of resveratrol with IP was faster than that with PSO. The study demonstrates that transdermal delivery is an alternative approach for resveratrol administration. Both IP and PSO prompted the percutaneous transport of resveratrol. PSO may be a more promising oil phase to prepare micro-emulsions for transdermal absorption of resveratrol.

Acknowledgements

This study was financially supported by key program of Education Department of Sichuan Province (Grant No. 16ZA0298), Chengdu Industrial Cluster Project (Grant No. 2016XT00-00023-GX), fund of National Education Department for overseas returnees (Grant No. 20131792), and applied fundamental funding from Science and Technology Department of Sichuan Province (Grant No. 2013YJ0042).

Conflicts of interest

The authors declare no conflict of interest.

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