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Research Article

Theme: Advances in Topical Delivery of Drugs
Guest Editor: S. Narasimha Murthy

Enhancement in the Transdermal and Localized Delivery of Honokiol Through Breast Tissue

Xinyi Gao,¹ Meera Gujjar Patel,¹ Pooja Bakshi,¹ Dipali Sharma,² and Ajay K. Banga^{1,3}

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Abstract. Honokiol is a natural phenolic anti-cancer compound isolated from an extract of seed cones from *Magnolia grandiflora*. This study investigated the transdermal delivery of honokiol using various enhancement methods and to explore the potential of honokiol to treat breast cancer directly *via* delivery through mammary papilla. Poration of dermatomed human skin with microneedles significantly increased the delivery of honokiol by nearly 3-fold ($97.81 \pm 18.96 \mu\text{g}/\text{cm}^2$) compared with passive delivery ($32.56 \pm 5.67 \mu\text{g}/\text{cm}^2$). Oleic acid was found to be the best chemical penetration enhancer, increasing the delivery almost 27-fold ($868.06 \pm 100.91 \mu\text{g}/\text{cm}^2$). Addition of oleic acid also resulted in better retention of drug in the porcine mammary papilla ($965.41 \pm 80.26 \mu\text{g}/\text{cm}^2$) compared with breast skin ($294.16 \pm 8.49 \mu\text{g}/\text{cm}^2$). Anti-cancer effect of honokiol was demonstrated with the decrease in the release of cytokine IL-6 and further suppression of Ki-67 proliferative protein. In addition, the topical honokiol formulation investigated was found to be safe and non-irritant. In summary, both microneedles and chemical enhancers can improve the absorption of honokiol through skin. Directly applying honokiol on mammary papilla is a potential administration route which can increase localized delivery into breast tissue.

KEY WORDS: honokiol; breast cancer; transdermal drug delivery; microneedle; chemical enhancer.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women, representing nearly a quarter (23%) of all cancers in women (1). Breast cancer is also the second leading cause of cancer death in women, leading to about 1 in 37 death rate (2). From the latest estimates by the American Cancer Society for 2017, over 252,710 new cases of invasive breast cancer and over 63,410 new cases of carcinoma *in situ* will be diagnosed in women in the USA. In 2017, an estimated number of deaths by breast cancer in united states has been found to be more than 40,000 (3). Conventional chemotherapy to breast cancer is often limited by high toxicity and therapeutic resistance, both of which can lower efficacy. Therefore, other therapeutic strategies for breast cancer are needed to improve efficacy and reduce morbidity and mortality. Most breast cancer (carcinomas) originates in the cells of lobules, the milk-producing glands, or ducts which can carry the

milk from the lobules to the nipple (4). Lee *et al.* investigated the delivery of anti-breast cancer agents to the breast *via* the mammary papilla and found that the delivered drug amount was enough to inhibit the growth of breast cancer cells markedly (5). Therefore, localized delivery of chemotherapeutic agents through the mammary papillae can be explored as a promising approach for treatment of breast cancer, especially non-invasive carcinomas and early stage of invasive carcinomas, that can increase local concentration and minimize systemic exposure.

Honokiol is a natural small-molecule biphenyl compound (266.33 g/mol) isolated from the root, stem bark, and seed cones of *Magnolia grandiflora* which possesses anti-inflammatory, anti-microbial, anti-anxiety, anti-diabetic, and anti-oxidant effects (6,7). It has been reported to also show a significant anti-tumor effect and was demonstrated to inhibit growth and induce apoptosis of various cancer cell lines, such as breast cancer, prostate cancer, and hepatoma cells (8–10). Although honokiol has multiple pharmacological activities, its use is restricted by poor solubility and poor oral bioavailability which is only around 5% due to extensive first-pass metabolism and low absorption (11). Thus, transdermal delivery system can be explored as an attractive alternative to oral delivery of honokiol because it can avoid first-pass elimination and gastrointestinal irritation (12). In addition, transdermal system maintains plasma drug concentrations and increases drug targeting (13). However, not all the drugs are good candidates for this route due to the presence of the rate-

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limiting barrier, stratum corneum, the outermost layer of skin. This skin layer, which is composed of dead, flattened, keratin-filled cells and around 10 to 15 μm thick, has low permeability for many pharmaceutical molecules (14). Only moderately lipophilic drugs with molecular weight less than 500 Da are able to be delivered through skin passively. Due to the high lipophilicity of honokiol ($\log P = 5.2$), physical enhancement techniques like microneedles, and chemical permeation enhancers may improve the penetration of it through skin.

Microneedle technology creates micron-sized channels in the skin by disrupting the stratum corneum, and therefore allowing the delivery of drugs into the epidermis or dermis layers without reaching dermal nerves to stimulate pain, thereby making this technique minimally invasive and painless (15). Both solid and hollow microneedles are available in the market and are made of various materials such as silicon, metal, sugars, or polymers. In the present study, arrays of maltose microneedles, 500 μm long (arranged in three rows of 27 needles each) were used to pierce skin. Chemical penetration enhancer is another enhancement technique which has been widely used in skin delivery system to achieve desirable percutaneous absorption, by reversibly reducing resistance of the stratum corneum (16). Propylene glycol (PG), a commonly used non-irritant vehicle, was used for transdermal delivery of honokiol. It is also a non-toxic penetration enhancer showing synergistic action when used with other chemical penetration enhancers such as oleic acid (17). Three different chemical penetration enhancers, isopropyl myristate (IPM), oleyl alcohol or oleic acid (5%) were added in the PG solution separately to compare their enhancement effects on the permeation of honokiol through skin.

Immune system plays a key role in cancer and psoriasis and can cause release of various interleukins and chemokines as a response. Psoriasis is a common skin disorder, with red, flaky patches on elbows, knees, and back. Skin infected with psoriasis has similar morphological characteristics as cancerous tissue, inflammation, and excessive proliferation of cells. Thus, psoriatic skin tissue can serve as a skin model for cancer drugs to observe any decrease in expression of proliferative proteins (Ki-67) or cytokines (IL-6) (18). In the present study, anti-cancer activity of the honokiol was investigated, measuring the amount of cytokine, IL-6 expressed after treating the skin with the drug. Anti-cancer activity was further confirmed by performing immunochemistry for the specific hyperproliferative protein (Ki-67) responsible for inflammation and proliferation in cancerous tissue. In addition, the skin irritation potential of the honokiol formulation was further investigated by using *in vitro*-reconstructed human epidermis tissue model.

There is not much evidence for significant delivery of honokiol across skin. Thus, our aim was to enhance the transdermal delivery of honokiol through dermatomed human skin by using techniques such as microneedles and chemical penetration enhancers. For the first time, localized delivery of honokiol through porcine mammary papillae was investigated to improve the drug targeting and decrease systemic side-effects.

MATERIALS AND METHODS

Materials

Honokiol was purchased from MedChemExpress LLC (Princeton, NJ). Propylene glycol (PG) and phosphate-

buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl myristate, oleyl alcohol, and oleic acid were given by Croda Inc. (NJ, USA) as free samples. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human dermatomed skin were purchased from fire fighters skin bank (NY, USA). Porcine mammary papillae and surrounding breast skin were procured from the local slaughterhouse (Atlanta, USA). Materials for *in vitro* skin irritation test were provided by MatTek Corporation (Ashland, MA, USA). Fluoresoft (0.35%) was purchased from Holles Laboratories, Inc. (Cohasset, MA, USA) and methylene blue dye from Eastman Kodak Co. (Rochester, NY, USA). Maltose microneedles were purchased from Elegaphy, Inc. (Tokyo, Japan).

Methods

Preparation of Skin Samples

Human dermatomed skin was obtained from fire fighters skin bank (NY, USA) and was stored in -80°C freezer. The stored skin was thawed in 10 mM PBS solution at 37°C for 1 min. After being completely thawed, the skin was cut into $2 \times 2 \text{ cm}^2$ pieces for permeation study. Freshly excised porcine mammary papillae and surrounding breast skin were washed with water and 10 mM PBS and stored at -80°C . Before the experiment, the stored skin was thawed at room temperature until completely thawed, and then mammary papillae were excised by blunt dissection. Surrounding breast skin was also cut into $2 \times 2 \text{ cm}^2$ pieces for permeation study. Subcutaneous fat was removed by blunt dissection.

Visualization of Microneedles and Microchannels

Maltose microneedles with 500 μm length were used for the creation of microchannels on dermatomed human skin (approximately 0.6 mm thick). The skin was placed on the parafilm with stratum corneum toward the top. The treatment site of the skin was stretched with fingers and microneedles were inserted vertically by using thumb with a relatively uniform force for 1 min. After the microarray was removed, histological studies, pore uniformity, and confocal studies were performed to confirm successful microporation of skin.

Scanning Electron Microscopy. The length of maltose microneedles before and after insertion into the skin was investigated using field emission scanning electron microscopy (SEM) system (Nanoscience Instruments, Inc., Phoenix, AZ, USA). Microneedles were mounted on a metal stub using double-sided carbon sticky tape and sputter coated using Denton vacuum desk sputtering system with gold target (Denton Vacuum LLC, Moorestown, NJ, USA) to increase the signal to noise ratio by forming a thin layer of conductive coating. Five kilovolts was used as primary beam accelerating voltage to collect secondary ion images of different magnifications.

Pore Uniformity Studies. After insertion of microneedle, Fluoresoft (0.35%) solution was applied on the microporation site of the dermatomed human skin for 1 min and wiped off using Kimwipes and alcohol swabs. A two-dimensional

fluorescent image was taken by digital camera (Canon, USA) with macrolens and hollow aluminum cone attached to measure the fluorescent intensity in and around the microchannels created by the microneedle, which can be converted into pore permeability index (PPI) by Fluorophore software, to estimate the relative calcein flux value of each pore and further the relative permeability of skin after being treated with maltose microneedle.

Confocal Microscopy Studies. A computerized Zeiss confocal laser scanning microscope LSM410 (Goettinger, Germany) was used to visualize the distribution pathways of calcein in the microchannels on the dermatomed human skin after being treated with maltose microneedle. Fluorescent (0.35%) solution was applied on the treated skin for 1 min and then removed using Kimwipes and alcohol swabs. The treated skin was placed on microscope slide and scanned under confocal microscope with $\times 10$ objective to obtain the fluorescent images. The excitation wavelength was 496 nm and X-Z sectioning was used to estimate the depth of the microchannels.

In Vitro Permeation Studies

In Vitro Permeation Study Across Dermatomed Human Skin

In the present study, permeation of honokiol was performed on dermatomed human skin using static Franz-type diffusion cells (PermeGearV6 station vertical cell stirrer and Logan FDC-6 console system). The objective of the study was to compare the enhancement efficiency of the delivery of honokiol across dermatomed human skin by using maltose microneedles and different permeation enhancers. The water bath was set at 37 °C to maintain the temperature of the skin surface at 32 °C. Receptor compartments were filled with 10 mM phosphate-buffered saline: polyethylene glycol 400 (60:40), continuously stirred with a magnetic stirrer. For the control group, human dermatomed skin was mounted on the Franz diffusion cell with stratum corneum facing up and 100 μ l of 10 mg/ml honokiol solution in PG was added in the donor compartment. For the microneedle group, maltose microneedles were manually inserted vertically and held in place for 1 min. The microporated skin were mounted on the Franz diffusion cell with stratum corneum facing up, and the same drug solution was added in the donor compartment. In the chemical enhancer group, honokiol solution in PG with 5% IPM, oleyl alcohol, or oleic acid was added on the surface of the untreated skin. The permeation area between compartments was 0.64 cm². Samples (300 μ l) were taken from the receptor chamber at predetermined time points over 72 h. Samples were replaced by receptor solution, and all the samples were analyzed using a reversed-phase high-performance liquid chromatography (HPLC) method.

In Vitro Permeation Study Across Porcine Mammary Papillae

In vitro permeation study of honokiol was also performed across porcine mammary papillae and surrounding breast skin to investigate the delivery of honokiol across such

tissues using optimal permeation enhancers selected from the previous study. In the donor compartment, 200 μ l of 10 mg/ml honokiol in PG solution with 5% chemical enhancer was added in the donor compartment to ensure the coverage of the tissue. Receptor (300 μ l) was taken from each cell at predetermined time points over 72 h followed by replacement with fresh receptor solution. All the samples were analyzed by HPLC.

Skin Extraction Studies

Extraction of Drug from Dermatomed Human Skin and Porcine Breast Skin

After 72 h, the drug solution remaining on the skin was removed with Q-tips soaked in fresh receptor solution. The skin pieces were then taken from Franz diffusion cells, minced, and placed in six-well plates. Methanol (2 ml) was added to each well, and the plates were shaken at 150 rpm for 4 h. The samples were then filtered through a 0.22- μ m nylon filters and analyzed by HPLC.

Extraction of Drug from Porcine Mammary Papillae

The method of extracting honokiol from porcine mammary papillae was modified from previous literature (5). After 72 h, excess dose in the donor compartment was wiped away with Q-tips, and the diffused areas were cut into approximately 1 \times 1 \times 1 mm cubes and placed into a 15-ml centrifuge tube. Methanol (2 ml) was added into the tube and vortexed for 30 min. The tubes were then centrifuged at 5700 \times g, and the supernatants were decanted into a clean centrifuge tube. This process was repeated twice before the pooled supernatants were dried down in a rotary evaporator. The residues were then reconstituted with 2 ml of methanol and analyzed by HPLC.

Quantitative Analysis

A reversed-phase high-performance liquid chromatography (RP-HPLC) method based on literature (19) was utilized for all the samples to determine the levels of honokiol. All chromatographic analysis was performed in a Waters Alliance 2685 system (Milford, MA, USA) coupled with a 2996 photodiode array detector. The column used was Phenomenex Prodigy (150 \times 4.6 mm, 5 μ m) at room temperature with methanol and water (78:22%, v/v) as mobile phase. A sample volume of 10 μ l was injected at a flow rate of 1 ml/min and analyzed at the absorption wavelength of 290 nm.

The standards were prepared in 10 mM PBS solution, and linearity was observed in the concentration range of 0.1–50 μ g/ml.

Skin Irritation Testing and Determination of Cytotoxicity by MTT Assay

The Honokiol formulation was tested for skin irritation using 3D tissue culture Epiderm™ model (MatTek Corporation, Ashland, MA, USA). The protocol was followed according to the manufacturer's instructions (20). SDS solution (5%) was used as positive control, while the negative

control (NC) was sterile Dulbecco's phosphate buffer saline (DPBS). Tissues were exposed to 100 μ l of PC, NC, and 50 μ l of 25 μ M honokiol in PG solution with 5% oleic acid for 1 h. Tissues were then rinsed with DPBS, replaced with fresh media, and tissues were further incubated for 48 h. On the completion of 72 h, cytotoxicity or irritation of the dose delivered was analyzed by using methyl thiazolyl tetrazolium (MTT) assay. MTT assay was then carried out to determine the cell viability which would specify if the formulation was irritant or non-irritant. MTT reagent (1 mg/ml) was added to the tissue inserts after 72 h treatment and incubated for 3 h at 37 °C with 5% CO₂. After 3 h, MTT was extracted from the tissues by extractant solution, placing the tissues on a shaker for 2 h. Absorbance of extracted solution was measured at 570 nm. Tissues, if viable, convert MTT to a purple dye and conversion is proportional to the viability of the tissues. Cell viability was calculated using a spreadsheet provided by MatTek; viability of less than 50% was determined to be irritant and cytotoxic.

H&E Staining

All the tissues from the 3D tissue culture inserts, EpiDerm™ model (MatTek Corporation, Ashland, MA, USA) were taken and embedded in OCT media and stored at -80 °C. Seven-micrometer-thick tissue sections were obtained by cryotome, and polysine slides were used for the histology. Tissue samples were fixed using 10% formaldehyde. All tissues were stained using hematoxylin and eosin; subsequent washing was done using water, 95% alcohol, acid alcohol, and Scott's reagent. Slides were stored in xylene until sealed with cyostat-60. Acid alcohol solution was prepared by adding 2 ml of HCL in 198 ml of 70% EtOH. Images were viewed using Leica-polarizing microscope, and image analysis was done using ImageJ software.

IL-6 Determination

3D Psoriatic cell culture model was purchased from MatTek Corporation (Ashland, MA, USA), and tissue inserts were treated with test samples as per manufacturer's instructions. Upon receipt of the kit, fresh media were replaced and tissue inserts were incubated overnight at 37 °C with 5% CO₂. After 18 h, 50 μ l of 25 μ M honokiol in PG solution with 5% oleic acid was applied on the apical surface of the psoriatic skin tissue. Psoriasis ointment treatment and untreated tissues were taken as positive and negative control, respectively ($n=4$ for each group). Tissues were placed in the incubator; every other day, the tissue surface was rinsed with sterile saline, re-dosed, and replaced with 5 ml of fresh media. The tissues were dosed for a total of 96 h, cell culture media was collected, and levels of IL-6 were analyzed after 96 h using ELISA (R&D systems).

Immunohistochemistry

Tissue sections (5 μ m) were cut from the paraffin-embedded samples and placed on polylysine-coated glass slides for immunohistochemistry. The method was followed according to the protocol provided from MatTek. Each slide had an immunohistochemical sample and a negative control.

The samples were prepped for antigen retrieval process by first rehydrating in tris buffer saline (TBS) for 5 min and were incubated in 0.05% citraconic anhydride at 98 °C for 45 min. The slides were allowed to cool down and followed with washing for 5 min with 1 \times TBS. The samples were then blocked with 10% donkey serum and 1% BSA for 1 h at room temperature in a light-proof box. The slides were then incubated with 5 μ g/ml primary antibody (Ki-67) for 1 h at room temperature. After incubation with primary antibody, the slides were then washed twice with 5 \times TBS for 5 min. This was followed by adding 1 μ g/ml secondary antibody (Anti-sheep 488 AlexaFluor) for 1 h at room temperature. The slides were then washed twice with 1 \times TBS and incubated in DAPI for 5 min, followed by repeated washing with 1 \times TBS and distilled water. The samples were then covered with coverslip and allowed to dry. The treated samples were observed under Leica SP8 confocal laser microscope (Switzerland) with $\times 10$ objective at an excitation/emission wavelength of 493/519 nm for AlexaFluor 488 (green channel) and 405 nm for DAPI (blue channel). Fluorescent images were processed by Leica Application Suite-Advanced Fluorescence (LAS-AF) software. The surface area of sectioned samples was calculated from the images using ImageJ 1.41o (National Institute of Health, USA).

Data Analysis

All statistical evaluations were performed using Student's *t* test and analysis of variance (ANOVA). Further, in order to determine the significant difference between the groups Tukey's test was performed and $p \leq 0.05$ was considered for significant difference.

RESULTS

Visualization of Microneedles and Microchannels

Scanning Electron Microscopy

Microneedle arrays were observed under SEM and characterized to have a pyramid-shaped structure with the needle length around 500 μ m (Fig. 1a) and needle base dimension around 200 μ m. After 1 min of insertion in human dermatomed skin, maltose microneedle was dissolved and the length of the needle was found to significantly decrease to less than 200 μ m (Fig. 1b) while the needle base dimension was no difference, which indicated the likeliness of maltose microneedle to penetrate through the stratum corneum and epidermis of human dermatomed skin.

Pore Uniformity Studies

The florescent image was taken, and three rows of micropores were generated on the skin (Fig. 2a). The uniformity of the microchannels were analyzed and confirmed by Fluorophore software by using pore permeability index (PPI) to show the relative calcein flux value of each pore. The average PPI value of pores created by maltose microneedle was 3.8 ± 1.94 for 80 micropores, and a histogram was generated depicting a relatively uniform distribution of pores (Fig. 2b). A bell-shaped distribution pattern was observed for

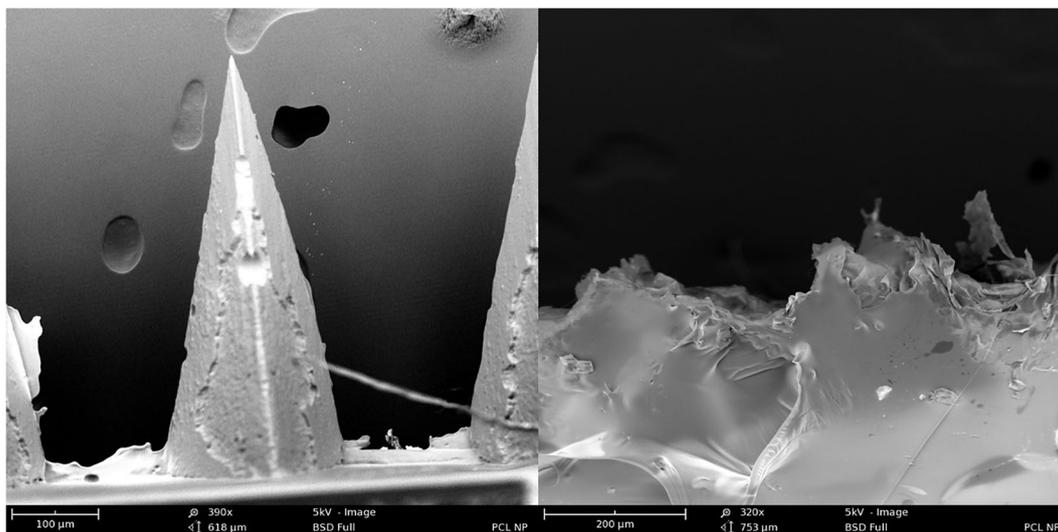


Fig. 1. SEM images of maltose microneedle before (a) and after (b) insertion

the PPI histogram, confirming the uniform channels created by the maltose microneedle.

Confocal Microscopy Studies

The depth of the microchannels in microporated human dermatomed skin was observed under Leica SP8 confocal laser microscopy. A z-stack was conducted starting from the skin surface to the end of microchannels, the point where signal of cacein visually disappeared. The z-stack step size was 5 µm, and depth of the microchannels was found to be around 100 µm (Fig. 3).

In vitro Permeation Studies

Comparing the Enhancement Effect of Maltose Microneedle and Different Chemical Enhancers

The effects of maltose microneedle and different chemical enhancers on permeation of honokiol were evaluated on human dermatomed skin. PG was chosen as delivery vehicle because it is not irritant to skin and honokiol has good

solubility in it. Test groups have been described in Table I. Both the cumulative amount of the drug penetrating through the skin and the total amount retained in the skin were examined in this study. Due to the barrier resistance of stratum corneum and hydrophilic property of honokiol, very little drug was delivered across the skin ($32.56 \pm 5.67 \mu\text{g}/\text{cm}^2$) by passive diffusion. Microneedle technique has been found to facilitate delivery of many molecules through the skin previously by creating microsized channels through stratum corneum and epidermis layers. In this study, maltose microneedle treatment resulted in approximate 3-fold increase ($p < 0.05$) in honokiol delivery ($97.81 \pm 18.96 \mu\text{g}/\text{cm}^2$) as compared with passive delivery (Fig. 4a). However, the drug amount retained in skin after 72 h for MN and control groups are similar, 16.77 ± 10.08 and $27.49 \pm 8.77 \mu\text{g}/\text{cm}^2$, separately (Fig. 4b).

Different chemical enhancers (IPM, oleyl alcohol, or oleic acid) were used to investigate the enhancement efficiency on skin penetration of honokiol without treatment of microneedle. As shown in Fig. 4a, b, the IPM group showed a comparable effect as microneedle, which increased the honokiol permeation to $83.48 \pm 1.86 \mu\text{g}/\text{cm}^2$, whereas it

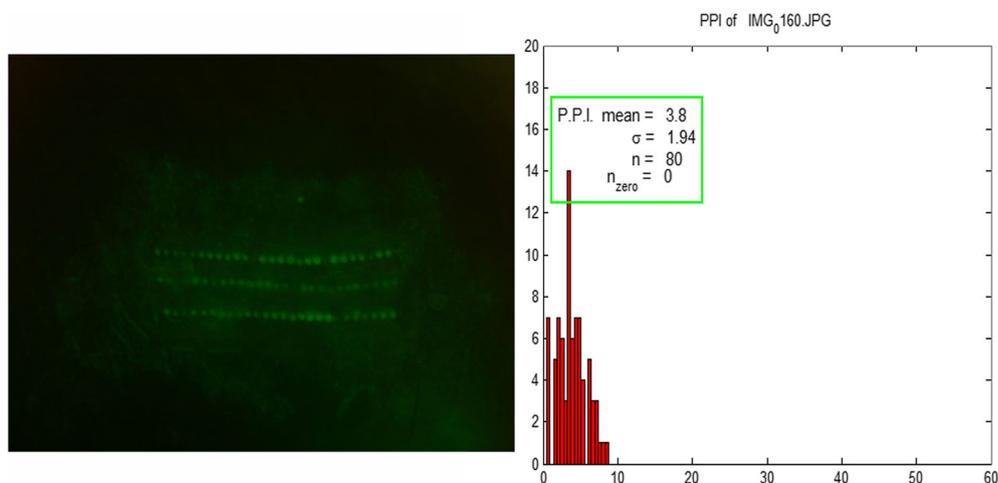


Fig. 2. Fluorescent images of maltose microneedle-treated skin (a) and histogram with PPI values (b)

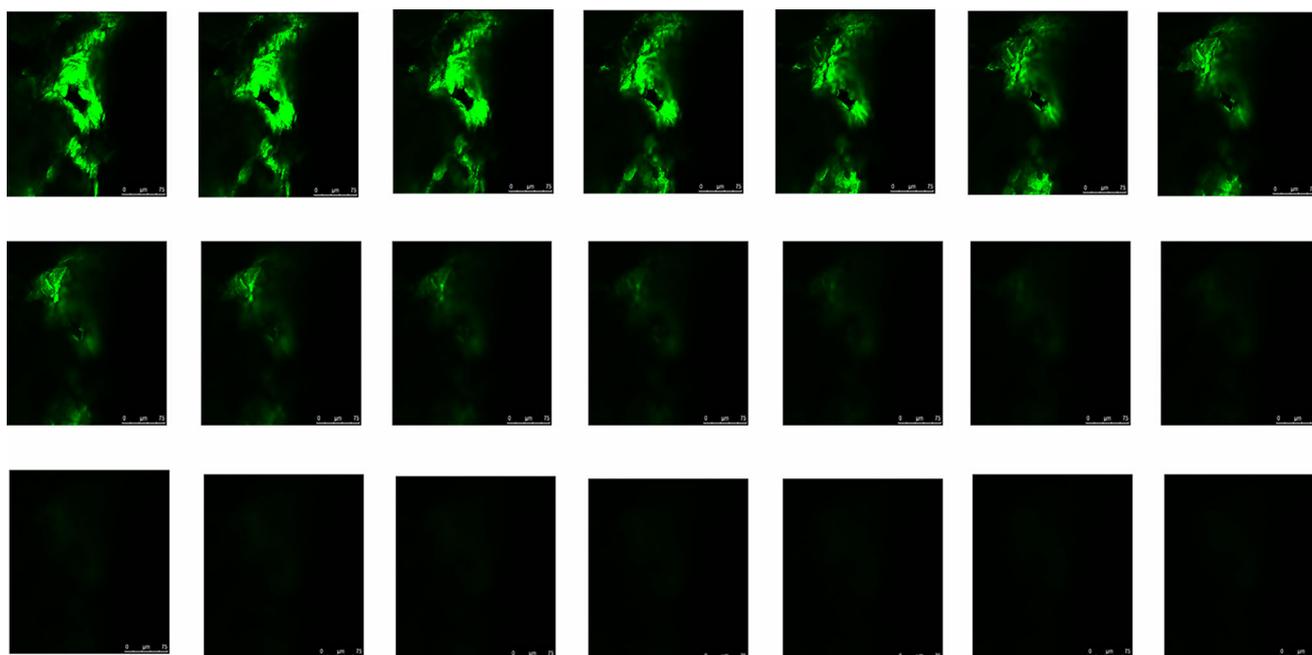


Fig. 3. Confocal microscopy z-stack of microneedle-treated skin

did not change the drug level in the skin significantly ($18.42 \pm 2.82 \mu\text{g}/\text{cm}^2$). However, the permeation of honokiol through the skin was dramatically increased nearly 17-fold ($549.31 \pm 73.20 \mu\text{g}/\text{cm}^2$) and 27-fold ($868.06 \pm 100.91 \mu\text{g}/\text{cm}^2$) as compared with the control group when oleyl alcohol or oleic acid was added in the formulation, respectively, as well as the drug amount remaining in the skin (83.61 ± 26.19 and $84.37 \pm 12.01 \mu\text{g}/\text{cm}^2$ of honokiol) after the permeation study.

Permeation Study Through Porcine Mammary Papillae and Breast Skin

In vitro permeation study was also carried out on porcine mammary papillae and surrounding breast skin to investigate the delivery potential of honokiol into breast tissue. F3 was selected as the optimal formulation in this study because oleic acid showed the greatest enhancement effect on the permeation of the drug through human dermatomed skin previously. After 72 h, the amount of honokiol penetrating across breast skin ($111.54 \pm 19.48 \mu\text{g}/\text{cm}^2$) is higher than across the mammary papillae ($36.92 \pm 3.54 \mu\text{g}/\text{cm}^2$) (Fig. 5a); however, much greater amount of drug was retained in the mammary papillae ($965.41 \pm 80.26 \mu\text{g}/\text{cm}^2$) than breast skin ($294.16 \pm 8.49 \mu\text{g}/\text{cm}^2$) (Fig. 5b).

Table I. Specifications of the Test Groups Evaluated in the *In Vitro* Permeation Study

Group	Formulation	Treatment
Control	PG solution	None
MN	PG solution	Maltose microneedle
F1	PG solution + 5% IPM	None
F2	PG solution + 5% oleyl alcohol	None
F3	PG solution + 5% oleic acid	None

Topical Effect of Honokiol on Cell Viability and Morphology

Skin Irritation Test

The relative percent viability of EpiDerm™ tissues treated with PC, NC, and honokiol formulation is shown in Fig. 6. Formulation being tested is considered to be an irritant if the percentage of cell viability is less than 50% of the NC. Based on this MTT assay, the cell viability for the NC was observed to be close to 100% while the PC and the honokiol formulation showed cell viability of 5.28 ± 1.08 and $87.26 \pm 2.97\%$, respectively.

H&E Staining

The 3D cell culture tissues were treated with honokiol solution and stained by H&E staining procedure. Structural and morphological changes were observed in the stratum corneum thickness for skin treated with honokiol formulation as compared with non-treated skin. Negative control showing the intact stratum corneum (Fig. 7a) while the tissue treated with honokiol formulation shows partial disruption of stratum corneum (Fig. 7b) which can be attributed to the presence of chemical permeation enhancer and oleic acid present in the formulation.

Determination of IL-6 Levels in Psoriasis Tissue Treated with Honokiol

A 3D psoriatic cell culture was dosed with honokiol solution. After 96 h of exposure, cytokine analysis was performed with ELISA. Since psoriatic patients have elevated IL-6 levels, it was chosen as an endpoint to determine efficacy in this study. The untreated group was used as a negative control and had basal levels of $917.81 \pm 87.65 \text{ pg}$ of IL-6. Treatment with honokiol significantly reduced IL-6

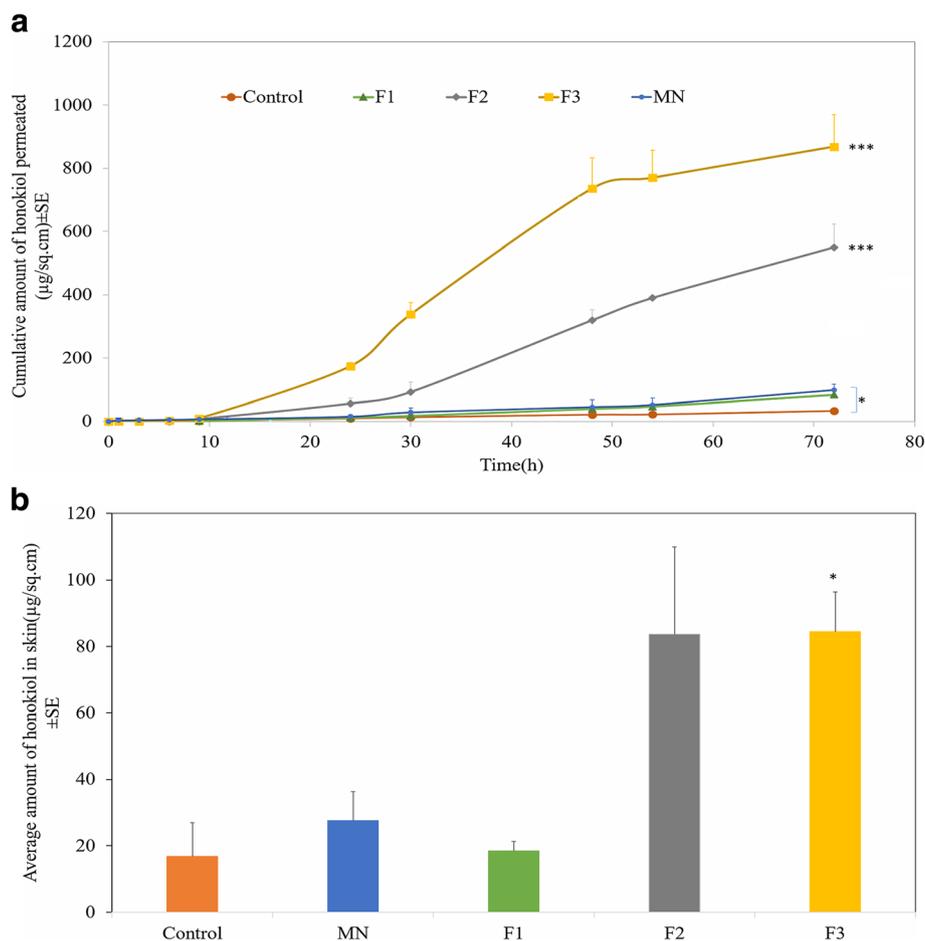


Fig. 4. a Effect of maltose microneedles and different chemical enhancers (IPM, oleyl alcohol, or oleic acid) on permeation of honokiol compared with passive delivery ($n \geq 3$). $*p \leq 0.05$, significant difference between the test groups, ANOVA Turkey's HSD test; $***p \leq 0.001$, significant difference between the test groups, ANOVA Turkey's HSD test. **b** Average amount of honokiol in dermatomed human skin under the use of different treatments ($n \geq 3$). $*p \leq 0.05$, significant difference between the test groups, ANOVA Turkey's HSD test

levels to 374.58 ± 91.33 pg, similar with the positive control which decreased IL-6 levels to 463.91 ± 47.51 pg (Fig. 8).

Physiological Characteristics of Honokiol Topical Application on Psoriasis Tissue Culture Model and Its Effect on Cell Proliferation

Immunohistochemical analysis was performed on 3D psoriatic tissues treated with and without honokiol. The tissues were also treated with an antibody specific for Ki-67, which is a marker for hyperproliferation. The stained tissues showed a morphological decrease in the amount of Ki-67 in honokiol-treated skin as compared with the negative control (Fig. 9).

DISCUSSION

Most breast carcinomas are expected to originate from milk duct which can deliver the milk to the nipple and lobule which can produce the milk. Rusby *et al.* have demonstrated the structure of the nipple by three-dimensional reconstruction of the nipple (21). They found the ducts are arranged in a

central bundle and most originate from the bundle beneath the skin and narrow in the tip of nipple. These facts can show that the nipple is a leaky tissue and localized delivery of anti-breast cancer agents *via* nipple can be an effective and safe method to treat pre-cancers and carcinomas. Murata *et al.* tried to treat mammary tumors by ductal injection of anti-cancer agents; however, this method still will cause some injury to the patients (22). Dave *et al.*, through confocal microscopy imaging of the nipple after applying different dyes on porcine skin, demonstrate that duct carried the drug to the underlying breast tissue and that the extent of distribution depends on the lipophilicity of the drug, with lipophilic drug being localized to the ducts and hydrophilic drug distributed in the ducts and the surrounding tissue (23). Thus, localized delivery of chemotherapeutic agents through the mammary papillae may be a promising approach in the treatment of breast cancer, especially non-invasive carcinomas and early stage of invasive carcinomas, and our study first investigated the delivery of honokiol through this route.

Honokiol, a natural small molecule biphenyl compound isolated from the stem barks of *Magnolia grandiflora*, is widely known for its anti-inflammatory, anti-oxidant, and

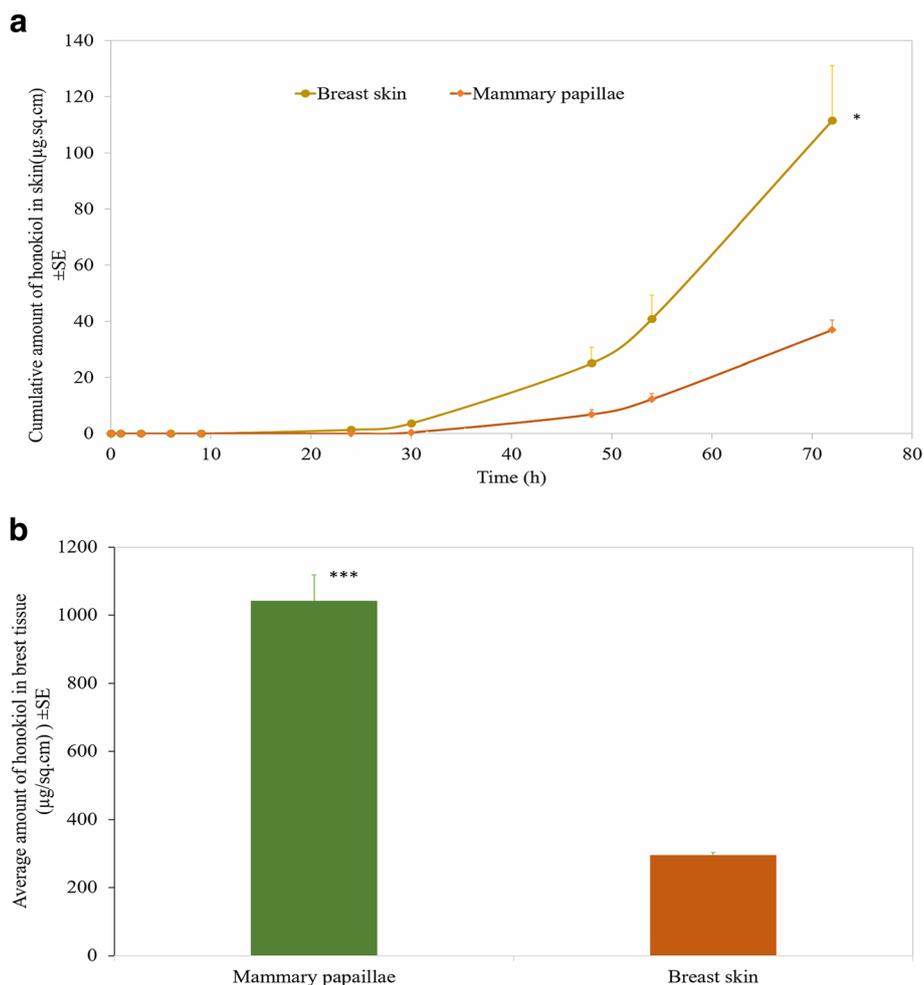


Fig. 5. **a** Profile of honokiol permeation across porcine mammary papillae and breast skin ($n \geq 3$). $*p < 0.05$, significant difference between the test groups, ANOVA Turkey's HSD test. **b** Retention of honokiol in porcine mammary papillae and breast skin ($n \geq 3$). $***p \leq 0.001$, significant difference between the test groups, ANOVA Turkey's HSD test

anti-thrombotic effects. More recently, its anti-tumor effects have been reported in cancer cell lines, such as hepatoma, fibrosarcoma, prostate, ovarian, breast cancer, and lymphocytic leukemia cells (24,25). As transdermal delivery bypasses first-pass metabolism and improves bioavailability, it could be

an attractive route for delivery of honokiol. Zheng *et al.* loaded honokiol into Pluronic F127 micelles and incorporated them into a thermosensitive F127 hydrogel (26). Wang *et al.* formulated PEGylated honokiol liposomes to improve solubility (27). In our study, PG was used as a delivery vehicle

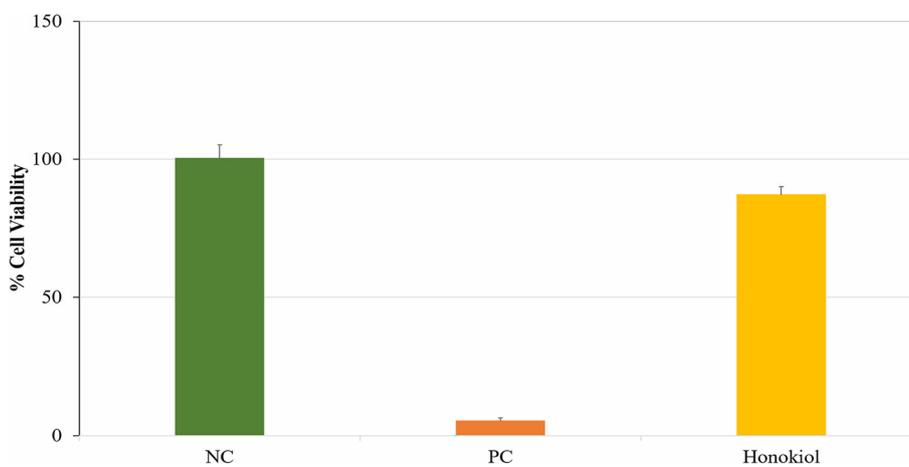


Fig. 6. The relative cell viability in *in vitro* 3D cell culture model for skin irritation test ($n = 3$)

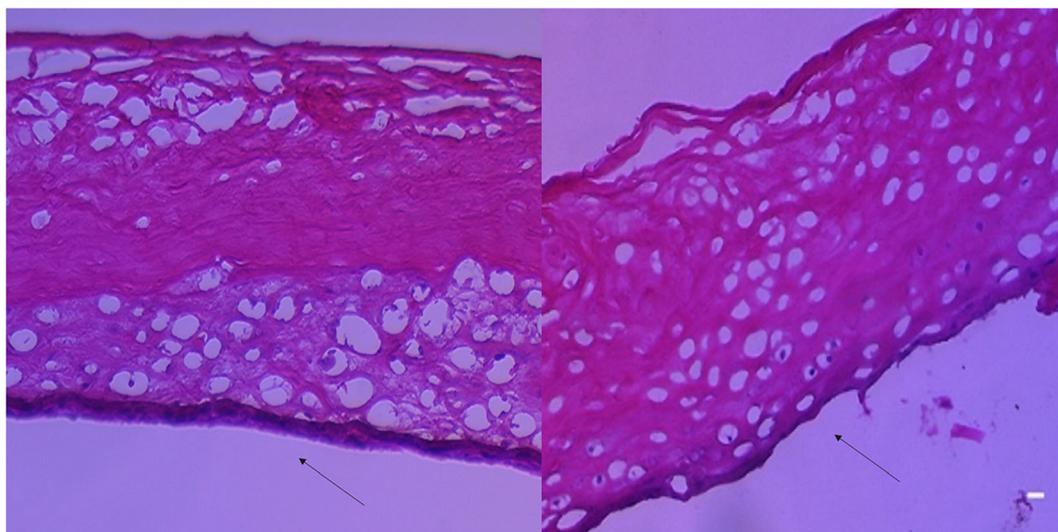


Fig. 7. Representative histological section of human epidermal tissue with hematoxylin and eosin staining. **a** Negative control (untreated skin); **b** honokiol (drug-treated skin)

because honokiol is a lipophilic drug ($\text{LogP} = 5.2$) with poor aqueous solubility. Microneedles provide a physical enhancement technology for transdermal delivery and enhance delivery of drugs by creating micron-sized channels in the skin, and thus allows drug molecules transport into the skin or systemic circulation. This technique is minimally invasive and painless, as microneedles only disrupt the stratum corneum or epidermis, without reaching deep dermal nerves to stimulate pain. The results of scanning electron microscopy showed that after being inserted into the skin, the microneedle tips are dissolved with the length decreasing from 500 to 200 μm . The successful piercing of the stratum corneum by microneedles and creation of the channels which is about 100 μm deep into the underlying layers of skin were confirmed by PPI and confocal microscopy studies. Honokiol permeation through dermatomed human skin was found to be significantly improved with the creation of micropores by microneedles. However, due to the high lipophilicity of the drug, the amount of honokiol retained in the epidermis and dermis layers is comparable with passive delivery.

Chemical permeation enhancers increase drug absorption by reversibly reducing the barrier properties of the skin. Enhancers interact with stratum corneum lipids and/or proteins and affect the partitioning behavior of drug molecules (28). Several investigations performed with different vehicles report that inclusion of penetration enhancers in the formulation significantly enhanced the permeation of different drugs through skin (29–31). PG can act as an enhancer by itself and also has a synergistic action with other penetration enhancers. In this study, IPM, oleyl alcohol, and oleic acid were added to a PG vehicle to investigate any enhancement effect on absorption of honokiol. IPM is an aliphatic ester which penetrates between the lipid bilayers of stratum corneum and disrupts their order and arrangement (32). Oleyl alcohol can increase fluidization in the stratum corneum by interacting with phospholipids at the lipid layer, and oleic acid is an enhancer which has the greatest synergistic effect in the presence of PG by decompressing stratum corneum and its resistance (16). However, in our study, IPM should have a much less enhancement effect on the permeation of honokiol through skin compared with oleyl

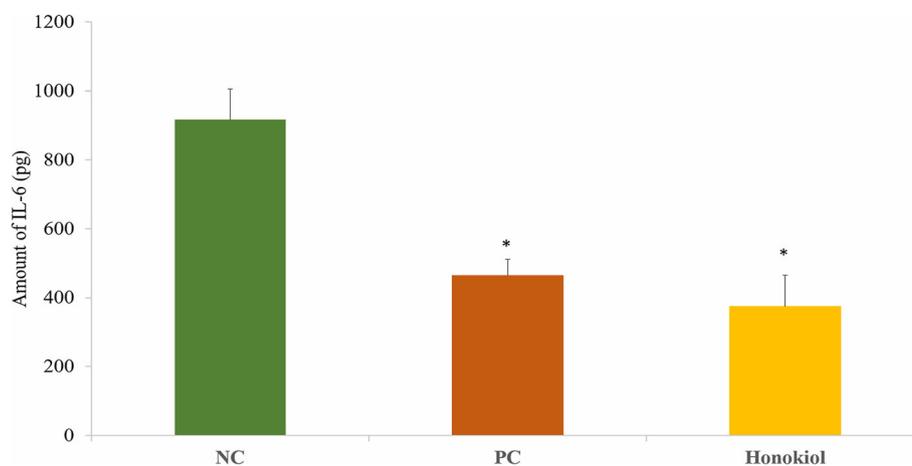


Fig. 8. Percent decrease in amount of IL-6 levels with honokiol treatment compared with untreated skin (NC) ($n = 4$). * $p \leq 0.05$, significant difference between the test groups, ANOVA Turkey's HSD test

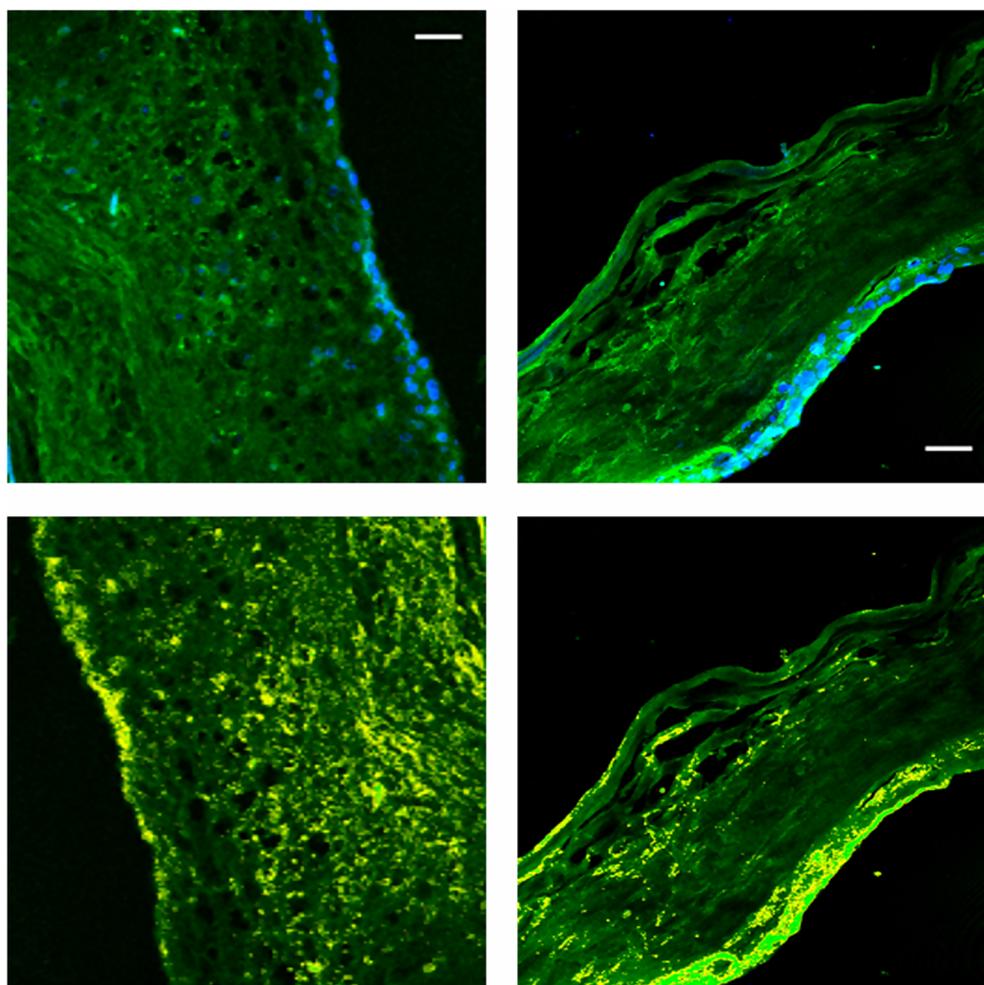


Fig. 9. Immunohistochemical analysis of psoriatic tissues treated with and without honokiol

alcohol and oleic acid, which dramatically increased the delivery of honokiol up to 17- and 27-fold, respectively. Oleic acid and oleyl alcohol also resulted in significantly greater skin absorption of honokiol compared with IPM.

We also investigated the anti-cancer activity of honokiol by using psoriatic cell culture model. Psoriatic skin tissue has similar inflammation as present in cancerous cells. Thus, we wanted to investigate any reduction in levels of cytokines. The skin penetration study allowed us to first determine the feasibility for the molecule to permeate through the skin. The cell culture study was then explored to determine if the molecule could have a biological effect in diseased skin. Psoriatic skin and a cancerous skin tissues are associated with greater secretion of IL-6, a pro-inflammatory cytokine. The cell culture psoriatic model produced by MatTek resembles psoriatic skin with hyperproliferation of epidermal cells and overexpression of IL-6 and IL-8. In this study, we dosed the psoriatic tissue with honokiol for 96 h and measured the levels of IL-6 by ELISA. Honokiol significantly decreased the IL-6 compared with the basal levels in the untreated group (Fig. 8). This suggests that honokiol has a biological effect which may be beneficial in the treatment of cancer.

Topical/transdermal formulations may cause skin irritation due to drug vehicle or enhancers. Since our formulation contained propylene glycol and oleic acid, we wanted to

evaluate the irritation potential of the formulation. In our study, we used three-dimensional human EpiDerm™ model consisting of human epidermal keratinocytes, which has a SC and viable epidermis. As seen in Fig. 7, since the percentage of the cell viability of the honokiol formulation was greater than 50%, the formulation can be classified as non-toxic and non-irritant. Due to the positive results obtained with oleic acid as a chemical enhancer, we further investigated if this enhancer could increase delivery into mammary papillae and we found that oleic acid resulted in significantly greater drug delivery into the local mammary papillae compared with the surrounding breast tissue. This was further confirmed by H&E staining; morphological changes were observed in the stratum corneum for honokiol-treated skin as compared with non-treated skin.

As stated earlier, the psoriatic tissues have similar morphological characteristics as compared with cancerous tissue. Psoriatic skin contains increased number of immune cells that produce high numbers of cytokines, chemokines, and inflammatory molecules similar to cancer cells. Inflammation is marked by the excessive production of numerous proinflammatory cytokines including TNF- α , MCP-1, and IL-6. Honokiol has been shown to regulate inflammation and affect the factors affecting inflammatory pathways. Krueger's group tested an antibody directed against IL-15 that was known to inhibit the production of T lymphocytes as well as the liberation of TNF- α

in vitro cell culture. Anti-cancer drugs have been shown to suppress the production of proinflammatory proteins; Ki-67 is a nuclear protein that serves as a cell proliferation marker and is associated with cell proliferation (33). Similarly, a study reported that honokiol can downregulate the production of proinflammatory cytokines and inflammatory proteins (34). Thus, our findings were similar to previous studies, demonstrating that honokiol caused decrease in the expression of Ki-67 hyperproliferation marker. The suppression of Ki-67 proliferative protein can be observed in Fig. 9, suggesting the anti-cancer activity of honokiol.

CONCLUSION

In conclusion, the transdermal route is a promising approach for delivery of honokiol. Incorporation of oleic acid in the delivery system improved the permeation of honokiol across skin significantly, and moreover, delivery of honokiol through mammary papillae was enhanced, and this could serve as a potential localized treatment for breast cancer. The formulation was found to be non-irritant in the *in vitro* study. The anti-cancer activity of honokiol was further confirmed with decrease in production of IL-6 and suppression of Ki-67 marker. These *in vitro* testing techniques are useful tools in the investigation of skin delivery of drugs with therapeutic efficacy and safety.

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