Dermatology Practical & Conceptual

Investigation on Utility of Some Novel Terpenes on Transungual Delivery of Terbinafine for the Management of Onychomycosis

Isha Gupta[1], Syeda Nashvia Adin[1], Mohd. Aqil[2], Mohd. Mujeeb[1], Yasmin Sultana[2]

1 Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India
2 Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India

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Corresponding author: Mohd. Aqil, Associate Professor, Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard (Deemed University), New Delhi, India-110062, Contact: +91-9811798725, E-mail: aqilmalik@yahoo.com

ABSTRACT

Introduction: Onychomycosis is a fungal disorder of the nail which afflicts 5% of the population worldwide. The disease is strenuous to cure as it is chronic, hard to eliminate and tends to recur. Topical therapy is at the forefront for the treatment of many disorders of nail. However, the success rate of topical therapy has been halted owing to the poor permeation of topical therapeutics across densely keratinized nail barrier. Therefore, ungual drug permeation must be improved for an effective topical therapy. An approach to achieve this goal would be the use of terpenes from natural sources as potential penetration enhancers.

Objective: This study is aimed to explore the effectiveness of some novel terpenes as potential penetration enhancers on transungual delivery of terbinafine.

Methods: Ex-vivo permeation studies were performed by sopping the nail clippings of healthy human volunteers in control and working solutions containing terbinafine (5mg/ml) per se and terbinafine (5mg/ml) with 6% of each terpenes including lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool respectively for 48 hours. The terbinafine concentration in nail samples was determined using a HPLC (High Performance Liquid Chromatography method.

Results: Statistical analysis showed that studied terpenes increase transungual penetration of terbinafine in the following order: linalool > rose oxide > 3-methyl-2-butene-1-ol > safranal > limonene > lavandulol acetate. Accordingly, linalool was found to be the most effective penetration enhancer for the transungual delivery of terbinafine.

Conclusions: It is concluded that linalool can be used as safe and potential penetration enhancer for enhancing the transungual delivery of terbinafine for onychomycosis.

Introduction

Onychomycosis is the most prevalent fungal disorder of the nails attributable to yeasts (Candida albicans and other candida species), non-dermatophyte molds (Scytalidium hyalinum, Scopularicpsis brevicaulis, Acremonium sp., Aspergillus Sp., S.dimidiatum), and dermatophytes (Trichophyton rubrum, T. krajdenii, T. mentagrophytes, Epidermophyton floccosum) [1]. Due to this infection, the patients experience a prodrome of nail plate thickening, onycholysis, and nail discoloration. The afflicted patients experience paresthesia, local pain, and reduced quality of life. The abnormal appearance of the nails may plague the daily activities and stigmatize social life of affected persons [2]. Onychomycosis progression increases with simultaneous inhabitation of other conditions like HIV, poor peripheral circulation, diabetes, and immunosuppression [1].

The onychomycosis treatment always remains challenging due to the thick impermeable property of the nail and profound infection [3]. However, oral treatment therapy remains the priority for the affected patients due to the accessibility and efficacy of oral therapeutics [2]. The oral treatment includes anti-fungal drugs from allylamine and azole classes. The azole class includes ketoconazole, fluconazole and itraconazole and the allylamine class includes terbinafine wherein itraconazole and terbinafine are US FDA approved medications for onychomycosis treatment [4-6]. Terbinafine excels over itraconazole due to higher cure rate and fewer drug interactions. However, their effectiveness is defined/constrained due to their limited availability at the site of action, which further increases treatment duration, treatment cost, side effects like cardiac disturbances and hepatic toxicity, and drug interactions. The topical treatment provides an alternative approach to evade the drawbacks associated with oral medicines and improves the adherence and localized effect [7-12].

The topical treatments approved by the FDA for effective treatment of onychomycosis include tavaborole 5% solution, ciclopirox 8% nail lacquer, and efinaconazole 10% solution [13-14]. However, their effectiveness is limited due to the minimal permeability of the drug across the nail plate. The nail impermeability can be accredited to the highly stable hydrogen bonds and disulfide linkages in the keratin network. Moreover, globular proteins and keratin fibres make a complex structure that makes the nail plate the most challenging biological barrier [3]. Therefore, extensive research has been focused on altering the nail plate barrier by employing various approaches, which include physical methods, mechanical methods, chemical treatments, and penetration enhancers; wherein one of the most commonly used approaches is the use of penetration enhancers, which act by disrupting the keratinized structure of nail plate thereby increasing the diffusion gradient and penetration capacity of the active through the nail plate [15-18].

Researches from past decades have shown that chemical penetration enhancers yield higher permeation rates than other approaches for effective transungual delivery, but irritancy at the site of application always remains a challenge [19,20]. Therefore, research has been oriented towards finding safe and effective penetration enhancers from natural sources. Terpenes acquired from natural sources have emerged as promising candidates and are considered as clinically acceptable penetration enhancers. In addition, many terpenes appear in the list of generally recognized as safe (GRAS) agents published by the USFDA. Terpenes are used for permeation enhancements of both lipophilic and hydrophilic drugs. Their activity depends on their chemical structure and physicochemical properties such as degree of unsaturation, boiling point, size and chirality, the energy of vaporization, and lipophilicity [21].

In the present study, we chose terbinafine, an allylamine fungicidal drug that acts by inhibiting squalene epoxidase in the ergosterol biosynthesis pathway and shows higher efficacy against dermatophytes and many non-dermatophytes [22]. Topical therapy can eminently treat onychomycosis. The route is highly advantageous attributable to its localised effect and minimal adverse effect profile. However, its potential is hampered by the rigid keratinous structure of the nail plate which is hard to breach [23].

Objectives

Therefore, our research has been centered on improvement of transungual delivery of terbinafine with the assistance of natural permeation enhancers ie terpenes viz. lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool. The salient features of the studied terpenes have been highlighted in Table 1.

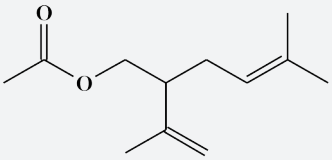
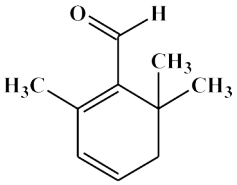
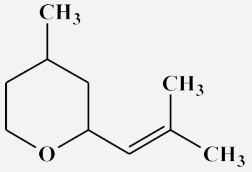
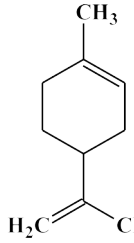
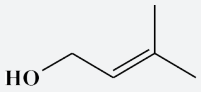
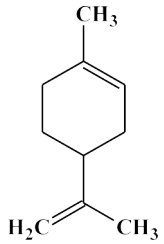
Methods

Terbinafine was purchased from Virupaksha Organics Limited. Lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool were purchased from Sigma-Aldrich. Absolute ethanol, methanol, hydrochloric acid, sodium hydroxide, propylene glycol, Tween 80, and HPLC grade methanol were purchased from S.D. Fine Chemicals. All chemicals were of Analytical Reagent grade.

Liquid Chromatographic Conditions

HPLC Quaternary System consisting of lichrospher C18 reverse-phase column of 25 x 4.6 mm with particle size of 5 µm was used for performing HPLC analysis. All analyses were carried out under isocratic conditions at a flow

Table 1. Some Important Properties of Studied Terpenes.

Name	Molecular Formula	Structure	Type	Log P	Boiling Point
Lavandulol acetate	$C_{12}H_{20}O_2$		Monoterpene	3.1	90 °C
Safranal	$C_{10}H_{14}O$		Aldehydic terpene	2.1	70 °C
Rose oxide	$C_{10}H_{18}O$		Monoterpene	2.9	71-73 °C
Limonene	$C_{10}H_{16}$		Monoterpene	3.4	175 °C
3-methyl-2-buten-1-ol or prenol	$C_5H_{10}O$		Monoterpene	0.91	140 °C
Linalool	$C_{10}H_{18}O$		Monoterpene	2.7	198.5 °C

rate of 1ml/min using mobile phase methanol: acetonitrile: 0.2% triethylamine (55:35:10, %v/v) with U.V. detection at 280 nm [24].

Preparation of Stock and Working Solutions

In propylene glycol: tween 80: ethanol: dimethyl sulfoxide (30: 3: 62: 5), the stock solution of terbinafine (25 mg/ml) was prepared and stored at -20°C. By diluting the stock solution in water: ethanol (50:50, v/v) five times, terpene-free working solution (control) was prepared. The concentration was selected based on the method sensitivity used in the experiment and the terbinafine solubility. Further, 6 working solutions were prepared, each containing 5 mg/ml of terbinafine stock solution and 6% of terpenes (solution B, C, D, E, F, and G labelled for lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool respectively). To assess the optimal effect of terpenes on transungual delivery of terbinafine, terpenes concentration was chosen to be more than 4.7%, which emulated the highest concentration of terpene in market formulation viz. Vicks Vaporub® (camphor, 4.7%). The prepared solutions were subjected to nail penetration studies [25].

Ex-vivo Nail Penetration Studies

Healthy adult volunteers (5 women, 5 men, 18-50-years-old, N = 10) who were not on any prescribed drug were chosen for sample collection. Nail samples were collected using nail clippers and scissors. After giving a brief description about the experiment, written consent was taken from all participating individuals. No ethical approval was needed since nails are waste materials and voluntarily donated by the participants. After collecting nail samples, each nail was cut into

small pieces; 25 mg of nail sample was used for each experiment [25]. To remove the adhered dirt from the surface and prevent any interference in the study, 20 ml of water was added to the nail samples to digest for 20 minutes. Then, nail samples were washed with methanol three times and then air-dried. Further, the nail samples were stored at a room temperature in a sealed plastic bags till analysis.

Eight propylene tubes of 2 ml capacity were taken and eight aliquots (each 25 mg) of nail samples of each volunteer were transferred into it. One ml of a drug-free working solution was taken in the first tube as blank sample (solution A). To determine the effect of terpenes on the penetration of terbinafine across the nail samples, 1 ml each of blank solution A and working solutions B, C, D, E, F, and G was added to the remaining tubes and kept at room temperature for 48 hours. Further, nail samples were sonicated for 15 minutes with 10 ml methanol thrice to remove the adhered drug residue from the surface.

Further, the samples were transferred to the centrifuge tubes. Then, the tubes were incubated after addition of 1ml of 0.5 M NaOH for 12 hours to digest the samples. The samples were neutralized by adding 0.5 M HCl solution. The samples were then vortexed for 2 minutes and centrifuged at 6000 rpm for 20 minutes. The supernatant was collected and the concentration of terbinafine in 20 uL of each solution was determined by HPLC analysis.

Stability of Terbinafine in the Digestion Procedure

In propylene glycol: tween 80: ethanol: dimethyl sulfoxide (30: 3: 62: 5 v/v) vehicle, 1 mg/ml stock solution of terbinafine was prepared. The 100ul/ml solution of terbinafine was prepared by diluting the stock solution 10 times in water: methanol (50:50, v/v). Further, two different solutions were prepared using this solution. First, an aqueous solution of 5 µl/ml of terbinafine was made by diluting the above solution 20 times, and 20 µL of the prepared aqueous solution was subjected into the column. Secondly, a 5 µl/ml solution was made in 0.5 M NaOH and incubated for 12 hours. After incubation, the solution was neutralized by adding 0.5 M HCl, centrifuged; and 20 µL of this solution was subjected for HPLC analysis. To determine the stability of terbinafine in the digestion procedure, the experiment was repeated three times to ascertain the stability of terbinafine in the digestion procedure by taking into consideration the dilution factor and the areas attained after injection of aqueous and basic solutions.

Preparation of the Calibrators

In 0.5 M NaOH, five calibrators of different concentrations of 5, 7.5, 10, 12.5, and 15 µg/ml were prepared from 1mg/ml stock solution of terbinafine. For HPLC analysis of calibrators, the aforementioned procedure was employed.

The concentration of terbinafine in nail samples was determined from the calibration curve plotted using the above five calibrators.

Statistical Analysis

The outcome result of the terbinafine extraction from the nail samples of the control solution (without terpene) was compared individually with the results acquired with each test solution (carrying 6% terpene) by statistical analysis. Shapiro-Wilk test was employed to determine the normal distribution of variables using SigmaPlot v 14.0 software. For comparing the quantitative variables with normally distributed and non-normally distributed quantitative variables, Paired t-test and Mann-Whitney U test were employed, respectively, and a P value < 0.05 was considered statistically significant.

Results

Sample Preparation and Drug Stability

To assess the transungual permeation of the drugs, the nail samples of the human volunteers were digested in various media [25]. Alkaline, acidic and methanolic digestion methods were used to hydrolyze the nail matrix. Various digestion procedures have been reported, including acid digestion by HCl (0.1 – 5.0 M), basic digestion by NaOH (1-10 M), digestion by nitric oxide (5% – 60%), their combination with methanol and hydrogen peroxide at a temperature between 25-60^{0}C and digestion with benzyltrimethyl ammonium hydroxide. Stability of terbinafine was determined with the above methods (data not shown); eventually basic digestion by NaOH (0.5 M) for 16 hours at room temperature was found as the best approach to digest the nail samples. The recovery of terbinafine by applying this method was found to be 30.7%.

Ex-vivo Nail Penetration and Statistical Analysis

The experimental data from ex-vivo nail penetration studies and statistical analysis are presented in Table 2 and HPLC chromatograms of drug-free (blank), calibrator (standard terbinafine) and sample solution (terpene spiked) are shown in Figure 1.

The concentration of terbinafine in nail samples of control, lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool was found ranging from 3.094 to 3.445 µg/mg (3.280 ± 0.122), 3.372 to 3.548 µg/mg (3.486 ± 0.051), 3.419 to 3.774 µg/mg (3.601 ± 0.115), 4.469 to 4.682 µg/mg (4.554 ± 0.061), 3.502 to 3.605 µg/mg (3.551 ± 0.038), 4.386 to 4.538 µg/mg (4.463 ± 0.041) and 4.708 to 4.796 µg/mg (4.748 ± 0.028) respectively. After employing Mann-Whitney U test, the results showed a significant

Table 2. Concentration of Terbinafine (μg mg^{-1}) Found in Nail Samples from Healthy Individuals and the Results from Statistical Analysis.

Volunteers	Control group A	Group B	Group C	Group D	Group E	Group F	Group G
1	3.445	3.544	3.608	4.599	3.579	4.496	4.785
2	3.345	3.495	3.492	4.682	3.526	4.386	4.742
3	3.125	3.473	3.571	4.564	3.605	4.462	4.721
4	3.320	3.372	3.774	4.469	3.502	4.465	4.746
5	3.395	3.448	3.546	4.532	3.578	4.436	4.796
6	3.142	3.494	3.596	4.486	3.527	4.482	4.746
7	3.365	3.521	3.419	4.516	3.553	4.428	4.771
8	3.094	3.548	3.549	4.542	3.581	4.538	4.708
9	3.325	3.476	3.773	4.569	3.542	4.462	4.727
10	3.239	3.486	3.685	4.576	3.515	4.475	4.739
Mean	3.280	3.486	3.601	4.554	3.551	4.463	4.748
SD	0.122	0.051	0.115	0.061	0.038	0.041	0.028
Normality test		Passed	Passed	Passed	Passed	Passed	Passed
P value		0.437	0.382	0.921	0.517	0.665	0.381

A= terbinafine: B = terbinafine with lavandulol acetate; C = terbinafine with safranal; D = terbinafine with rose oxide; E = terbinafine with limonene; F = terbinafine with 3-methyl-2-butene-1-ol; G = terbinafine with linalool.

difference between the control group and the lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool groups. The above results reveal that terpenes increase transungual penetration of terbinafine in the following order: linalool > rose oxide > 3-methyl-2-butene-1-ol > safranal> limonene > lavandulol acetate.

Conclusions

Topical therapy is at the forefront for the treatment of many disorders of nail and skin due to higher patient compliance and lesser side effects accompanied with systemic therapy. Howbeit, the nail disorders are frequently strenuous to cure and require long term therapy. The nail plate is an aggregate of highly dense keratinized tissue which in turn gives low permeability to the diffusing substances [26]. A number of approaches have been shown to be effective in enhancing transungual permeation such as mechanical methods (nail abrasion and nail avulsion), physical techniques (U.V light, carbon dioxide laser, iontophoresis, occlusion and hydration, sonophoresis, photodynamic therapy, electroporation and acid etching) or use of chemical penetration enhancers (keratolytic agents, water, keratinolytic enzymes, urea, mercaptans, sulfides and hydrogen peroxides) [26].

Literature and patent search divulged that chemical penetration enhancers excel over other strategies for the transungual delivery of drugs. Albeit the concentration required to achieve useful levels of permeation enhancements may cause irritancy at the site of application [19,20]. Hence, the investigations have been diverted towards searching safe and effective permeation enhancers from natural sources. In particular, terpenes have gained significant interest and are considered clinically acceptable permeation enhancers [21]. These promising candidates are considered effective and constitute a very safe class of permeation enhancers acquired from natural sources.

Furthermore, a good number of terpenes is incorporated in the list of generally recognized as safe (GRAS) classified by the US FDA [21]. The penetration enhancement ability of terpenes for hydrophobic and hydrophilic drugs are ascribed to their chemical structure in addition to the physicochemical properties, including size and chirality, degree of unsaturation, lipophilicity, the energy of vaporization, and boiling point [21].

In the past decades, several findings have shown that in vitro transungual studies are used to simulate and characterize the in vivo nail permeability. Permeation studies using Franz diffusion cells, measurement of nail swelling and drug uptake by nails after soaking nail clippings in drug solutions are often used. Though, in the recent works, it was found that keratin guards the analyte present in the nail matrix and hampers the availability of solvent and other reagents [18]. Hence, a digestion step was required to assess the terbinafine concentration in the nail before analysis. In the present study, effect of different terpenes was investigated on the transungual permeation of terbinafine and these were found to increase its permeation in following order: linalool > rose oxide > 3-methyl-2-butene-1-ol > safranal> limonene > lavandulol acetate. Thus, linalool was found to be the most effective penetration enhancer for the transungual delivery of terbinafine.

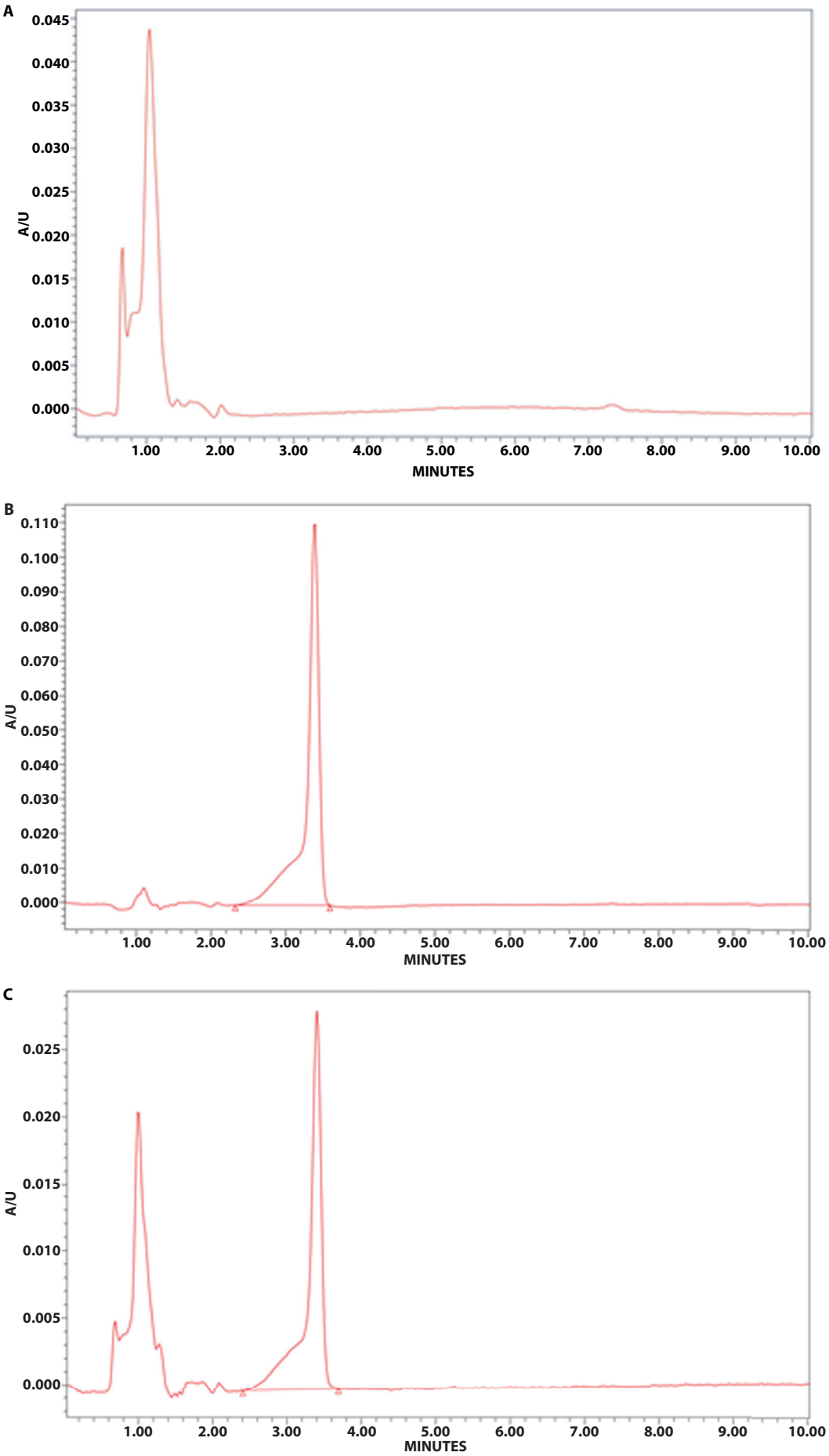


Figure 1. (A) HPLC chromatogram of blank. (B) HPLC chromatogram of standard terbinafine. (C) HPLC chromatogram of sample terbinafine.

The present study concludes that terpenes including lavandulol, safranal, rose oxide, limonene, 3-methyl-2-butene-1-ol, and linalool accentuate the transungual penetration of terbinafine and among these, linalool has been found to be the most effective penetration enhancer for the transungual delivery of terbinafine. By virtue of their enhancing effect, formulation of terbinafine with one or combination of these terpenes could be beneficial in topical dosage form development for treatment of onychomycosis.

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