

MODERNIZING A THAI TRADITIONAL REMEDY: *IN VITRO*-EX VIVO AND BIOACTIVITY PERFORMANCE OF CLOTRIMAZOLE-EMBEDDED *IN SITU* FORMING PAINT

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Abstract

“Ya-Pai,” a traditional Thai powdered medicine applied as an oral paint, presents application challenges and has seen limited formulation development. This study aims to modernize Ya-Pai by developing it into an advanced dosage form, an *in situ* forming system, for the treatment of oropharyngeal candidiasis (OPC). Clotrimazole-embedded *in situ* forming paints (ISPs) were developed using lauric acid and palmitic acid as ISP formers for the treatment of OPC. Their formation of matrix layer, transformation at interface, buccal mucosa permeation, and antifungal activities investigated to prove the concept. The formulation with a high concentration of ISP former (F2) demonstrated superior matrix formation and a rapid transition at the interface within 0.5 minutes, along with slower matrix growth compared to other formulations (5 minutes). In contrast, the formulation with a low concentration of ISP former (F1) exhibited faster drug permeation through the buccal mucosa and a lower percentage of drug remaining in the buccal mucosa ($28.64 \pm 2.80\%$ and $20.27 \pm 1.27\%$ for F2 and F1, respectively). The drug permeation rates of F1 and F2 were $4.0363\%/hour^{1/2}$ and $3.492\%/hour^{1/2}$, respectively, following Higuchi’s model. The antifungal test using the agar diffusion method demonstrated that the prepared ISPs effectively inhibited the growth of *Candida albicans* (32.3 ± 0.6 and 29.3 ± 1.2 mm for F1 and F2, respectively) and others. Thereby, the developed clotrimazole-embedded ISP shows a promising concept as a modernized Ya-Pai for the OPC treatment. However, further study such as *in vivo* efficacy, mucosal safety, and pharmacokinetic evaluation should be conducted.

Keywords: Bioactivity; Clotrimazole; *In situ* Forming Paint; Performance; Permeation

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Key Contribution

This research presents a novel approach to modernizing a traditional Thai medicine formulation. Our work was inspired by a classic Thai oral paint, a topical remedy applied directly to the oral mucosa. We have successfully re-engineered this traditional concept into a contemporary and user-friendly dosage form, an *in situ* forming paints. The prepared *in situ* forming paint efficiently inhibited the growth of *Candida albicans* ATCC 17110, *Candida krusei* TISTR 5259, *Candida lusitanae* TISTR 5156, and *Candida tropicalis* TISTR 5306. Therefore, the clotrimazole-loaded ISPs oral spray has the potential to effectively treat oropharyngeal candidiasis by providing delayed release of the drug.

Introduction

Oropharyngeal candidiasis (OPC) is a fungal infection of different *Candida* species, such as *C. glabrata*, *C. tropicalis*, *C. krusei*, and especially *C. albicans*. In healthy individuals, these *Candida* organisms can be present in up to 65% of cases without causing infection. However, individuals with an altered immune response, such as those undergoing immunosuppression, individuals with diabetes, and those using broad-spectrum antibiotics or corticosteroids, are at risk of developing symptomatic candidiasis of the oral mucosa (Stegeman & Boyd, 2008; Pankhurst, 2009). Practically, the antifungal agents are used to treat OPC, with clotrimazole troches, miconazole buccal tablet, and nystatin suspension being recommended for mild cases (Pappas *et al.*, 2016; Almangour *et al.*, 2021). Clotrimazole is a synthetic azole compound with broad-spectrum antifungal activity widely used to treat various fungal infections, including dermatophytoses, cutaneous candidiasis, tinea pedis (athlete's foot), vulvovaginal infections, and OPC (Crowley & Gallagher, 2014; Gupta *et al.*, 2021). However, the drug is poorly absorbed. Local application is preferred due to its ability to minimize drug interactions and adverse effects related to systemic antifungal agents. Nevertheless, this approach has limitations, including local irritation, an unpleasant taste, high sugar content (troublesome for patients with dental caries or uncontrolled diabetes), and difficulties with compliance due to frequent application (Sherman *et al.*, 2002; Thompson *et al.*, 2010). Compared to nystatin suspension, the sprays provide superior spreadability with non-excess consumption and are easier to use. Sprays also require less application time than troches, which leads to higher patient compliance. Therefore, clotrimazole-

loaded *in situ* forming film (ISF) and *in situ* forming gel (ISG) oral sprays have been developed to enhance therapeutic effects compared to conventional topical preparations (Lertsuphotvanit *et al.*, 2023; Mahadlek *et al.*, 2021). These film-forming sprays not only ensure uniform distribution and spreading at the desired location but also offer ease of use, promoting patient compliance with the treatment regimen (Ranade *et al.*, 2017).

Lauric acid (LAU) is a saturated fatty acid chain that possesses hydrophobic, biocompatible, and biodegradable properties (Anneken *et al.*, 2006). According to reports, it has demonstrated antimicrobial activity against various microbes (Solano *et al.*, 2020). When added to biocomposite edible films, LAU can reduce water vapor permeability, which tends to be high in such films due to their hydrophobic nature (Rochima *et al.*, 2021). Palmitic acid (PAL) is a 16-carbon saturated fatty acid that has been studied for its gelation properties in various vegetable oils. Oleogels containing PAL and safflower oil have shown potential as ocular delivery vehicles (Mohanty *et al.*, 2020). Furthermore, the addition of PAL to microspheres has been found to affect the release profile of rotigotine-loaded microspheres significantly (Wang *et al.*, 2016). Clove oil (CO) consists of over 90% eugenol, which possesses antimicrobial and analgesic properties, making it useful for treating dental pain and gingivitis (Maypole & Woolf, 2007; Bai *et al.*, 2023). Therefore, CO is interesting to be used as an antimicrobial enhancer in oral products. In a previous study, clotrimazole-embedded *in situ* forming system as an oral sprays was formulated by employing N-methyl pyrrolidone (NMP) as the solvent, CO as an additive, and LAU and PAL as agents responsible for forming the film. The physical properties, spray angle, and spray pattern of these formulations were investigated, and they exhibited suitable properties that make them potentially effective as oral sprays (Mahadlek *et al.*, 2022). In Thai traditional medicine, 'Ya-Pai' (paints) refers to a category of solid or semi-solid topical preparations, such as powder or pastes, designed for direct application onto a specific lesion, particularly within the oral cavity, to deliver a high concentration of medicinal herbs for localized effects. However, the efficacy of these conventional forms is often compromised by significant challenges including inconvenience of use and an uncontrolled release of active compounds. Since it is a powder, it is quite difficult to apply at the target which brings poor patient

compliance. Moreover, the traditional Ya-Pai showed uncontrolled drug release pattern. To address these limitations, this research investigates the development of an '*in situ* forming paint', which is an advanced *in situ* forming system applied as a low-viscosity liquid that transforms into a thin, adherent paint on the target site. *In situ* forming systems have been widely recognized for their ability to provide sustained and controlled drug release through mechanisms such as solvent exchange, phase separation, and gelation (Madan *et al.*, 2009). Incorporating this theoretical foundation would strengthen the discussion by highlighting how the LAU-PAL matrix in the present study exemplifies these well-established principles of ISFS. This modern approach offers numerous advantages over traditional topical formulations including ease of use, enhanced adhesion for prolonged contact time, the formation of a protective barrier against external irritants, and the creation of a drug reservoir for sustained drug release. Therefore, the objective of this study is not merely to formulate a single product but to establish a proof-of-concept for this versatile technology. The goal is to investigate the '*in situ* forming' system as a platform that can be adapted in the future to modernize other Thai traditional medicine formulations, thereby enhancing their therapeutic efficacy and patient compliance by overcoming the inherent limitations of their traditional dosage forms. Therefore, this study aims to examine the matrix formation, matrix transformation at the interface, release profiles, and antifungal activities of these formulations.

Materials and Methods

The selection of these specific formulations is a strategic decision rooted in both our preliminary screening data and the overarching goal of modernizing Thai traditional medicine. Our previous work identified formulations containing fatty acid blends as promising candidates based on their physicochemical properties (Khaing *et al.*, 2022). The formulations which have no Duro-Tak® were not further investigated since they showed undesirable attributes in previous report. However, it was found that the post-transformation of the unpreferred formulations showed an interesting characteristic. The character of post-transformation was like a Thai traditional paint (Ya-Pai) which is designed for direct, conformal application to oral lesions. Therefore, these formulations were specifically chosen for this in-depth study. This research aims to investigate their performance, establishing a proof-of-concept for a new class of

Applied Thai Traditional Medicine. In the current study, various ratios of PAL and LAU were employed as agents for forming the matrix film without the plasticizer and other polymer. They were dissolved in NMP. Subsequently, a 5% concentration of CO was introduced to the mixture. Eventually, 1% clotrimazole was integrated into the prepared systems, and the mixture was allowed to settle for 24 hours to attain a transparent solution. F1 and F2 were selected from the preliminary study of preparations varying matrix former concentrations. These formulations provided a suitable formation time and showed high consistency in preparation, which could be easily scaled up. The constituents of these ISPs are detailed in Table 1.

Table 1. Composition formula of ISP oral spray solutions

Formula	Components (%w/w)				
	Clotrimazole	LAU	PAL	CO	NMP
F1	1	20	20	5	q.s.100
F2	1	25	25	5	q.s.100

Matrix formation

To simulate buccal tissue, the formulated ISP oral solution was injected into a cavity within an agarose gel that contained phosphate buffer solution (PBS) pH 6.8. The matrix transformation was observed using a stereo microscope (SMZ-171 Series, Motic Inc., Kowloon, Hong Kong).

Transition of matrix at the interface

To study the transformation of the matrix at the interface between the ISP oral solution and agarose gel, drops of the ISP oral solution (20 μ L) were carefully applied to the cut edge of a 2 cm $\times$ 2 cm agarose gel. The subsequent formation of the matrix at various time intervals on the boundary was observed using an inverted microscope (Nikon ECLIPSE TE2000-S, Nikon Instruments Inc., USA).

In vitro drug release and drug remaining study

The release of clotrimazole from the ISPs was examined using a Franz diffusion cell (DHC-6T, Logan Instrument Corp., NJ, USA). The receptor cells of the Franz diffusion cell were filled with PBS (pH 6.8). The experimental setup at 37 °C using water flow through the cells and continuous stirring of the receptor solution at a constant speed. The content of the released drug was determined using HPLC equipped with a UV detector (Agilent 1220, Santa Clara, CA, USA). Analysis was performed using a C18 column (150 $\times$ 4.6 mm), and the samples were measured at 229 nm. The mobile phase was a mixture of methanol and water (90:10),

flowing at a rate of 1.0 mL/min, with an injection volume of 20 μ L. The flux of clotrimazole release from the ISPs was calculated based on the permeation test through a synthetic membrane (n=3). Additionally, the porcine cheek (buccal mucosa) obtained from a general slaughterhouse (Nakhon Pathom, Thailand) was used as a membrane model for studying the remaining drug. After the drug permeation test for 12 hours, the tissue was sonicated in absolute ethanol for 1 hour. The drug content was measured using the same HPLC method mentioned above (n=3).

Antifungal activity

The antifungal activities of the clotrimazole-embedded ISPs formulations were evaluated using the agar cup diffusion method against *Candida tropicalis* TISTR 5306, *Candida lusitanae* TISTR 5156, *Candida krusei* TISTR 5259, and *Candida albicans* ATCC 17110. The fungal inocula, adjusted to a turbidity equivalent to the 0.5 McFarland standard, were spread on Sabouraud Dextrose Agar (SDA) plates. A cylinder cup (6 $\times$ 10 mm) filled with 100 μ L of each ISP formulation was placed on the surface of the inoculated medium. The diameter of the inhibition zone around each cylinder cup was measured in millimeters after incubation at 37°C for 72 hours. For the positive control, a solution of 1% clotrimazole and 5% CO in NMP was used (n=3).

Statistical analysis

Statistical analysis was conducted using an independent t-test using SPSS for Windows (version 11.5). A significance level of $p < 0.05$ was established for statistical significance.

Results and Discussion

Matrix formation

All ISPs oral spray solutions exhibited clear, colorless, and low-viscosity properties. Upon insertion into the agarose well, an opaque ring formed within 0.5 minutes. The formulation with a higher concentration of fatty acid (F2) resulted in a larger opaque ring compared to the F1 formulation. However, the matrix growth of F2 was slower than that of F1 (see Figure 1).

Transition of matrix at the interface

The initiation of matrix transition at the boundary between the ISP solution and agarose gel was detected within 0.5 minutes as a result of solvent influx and water efflux. F2, which contained a higher concentration of fatty acid, exhibited a more significant film transition at the initial stage compared to F1, consistent with the matrix formation findings. However, as time progressed, an increase in film sizes was observed for both F1 and F2 formulations (see Figure 2).

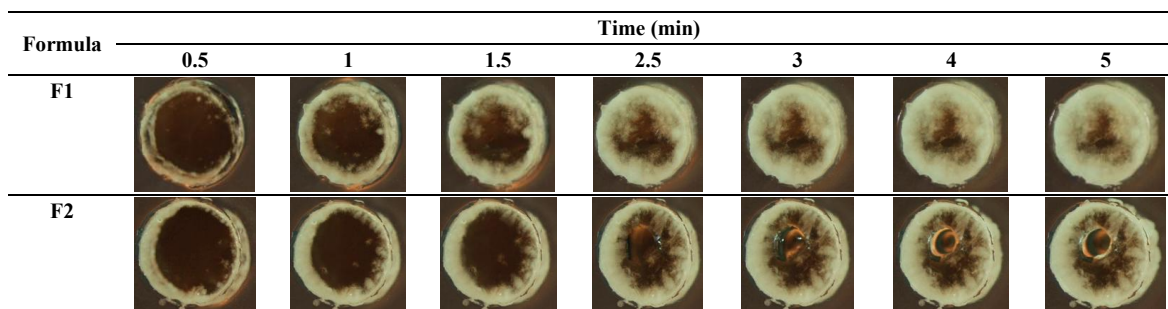


Figure 1. Matrix formation of ISP oral spray solutions at different time points

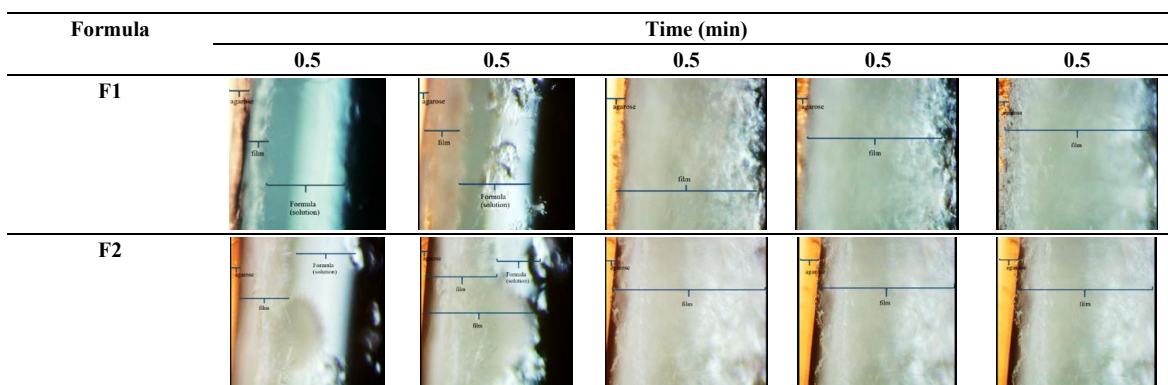


Figure 2. Interfacial interaction of ISP oral spray solutions at different time points

In vitro drug release and drug remaining study

The formulation with a higher fatty acid concentration (F2) exhibited slower drug release kinetics (see Figure 3A). However, after a 12-hour drug permeation test, the remaining drug content in the buccal mucosa indicated that F2 had a higher percentage of the remaining drug compared to F1 ($28.64 \pm 2.80\%$ and $20.27 \pm 1.27\%$ for F2 and F1, respectively). The drug release showed a linear correlation with the square root of time ($\sqrt{t}$), indicating conformity to the Higuchi model, with R^2 values of 0.9941 and 0.9954 for F1 and F2, respectively (see Figure 3B). The drug release rates were calculated as 4.0363% per hour^{1/2} for F1 and 3.492% per hour^{1/2} for F2.

Antifungal activity

The antifungal activities of the clotrimazole - embedded ISPs oral spray solutions against *C. albicans* ATCC 17110, *C. krusei* TISTR 5259, *C. lusitanae* TISTR 5156, and *C. tropicalis* TISTR 5306 were assessed by measuring the diameter of the inhibition zone (see Table 2). The clotrimazole-embedded ISPs oral spray solutions exhibited smaller inhibition zones compared to the positive control for all strains, primarily due to the slower drug release resulting from the hydrophobic nature of the fatty acid matrix, indicating the sustained

release of developed systems. F1 formulation showed significantly larger inhibition zones against *C. albicans* ATCC 17110, *C. lusitanae* TISTR 5156, and *C. tropicalis* TISTR 5306 compared to F2. Consistent with the drug release study, the faster drug release of F1 resulted in greater antifungal activity. However, there was no significant difference in antifungal activity against *C. krusei* TISTR 5259 between F1 and F2.

Discussion

The observed matrix formation directly reflects the successful modernization of the traditional 'Ya-Pai' concept into an *in situ* forming paint (ISP). Since *in situ* forming systems have been widely recognized for their ability to provide sustained and controlled drug release through mechanisms such as solvent exchange, phase separation, and gelation (Madan *et al.*, 2009). The matrix formation followed a solvent exchange mechanism, where the exchange of the NMP solvent with aqueous PBS induced the insolubility and precipitation of the fatty acids, initiating the formation of a dense matrix (Khaing *et al.*, 2022). This process is critical as it creates an adherent, protective layer analogous to a medicinal paint, but with significantly improved properties. As a result, the fatty matrix growth was easier for F1 compared

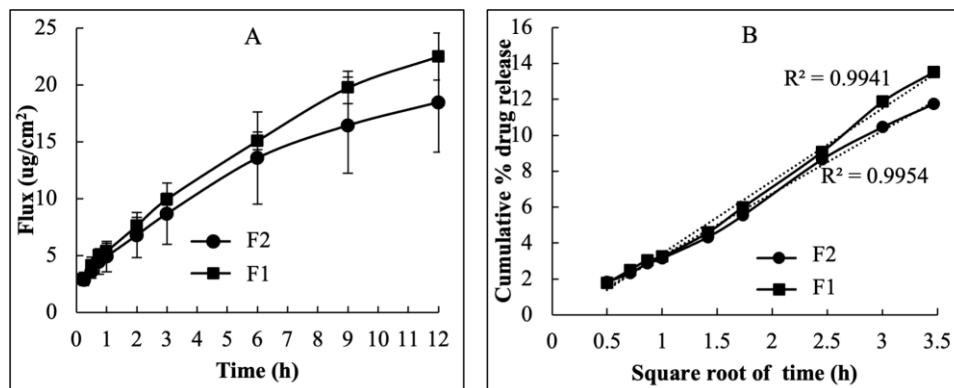


Figure 3. (A) Flux release and (B) Higuchi release model of drug from clotrimazole-embedded ISPs oral spray (n=3)

Table 2. Antifungal activity against *Candida* spp. of clotrimazole-embedded ISP oral spray (n=3)

Formula	Inhibition zone (mm) (Mean $\pm$ SD)			
	<i>Candida albicans</i>	<i>Candida krusei</i>	<i>Candida lusitanae</i>	<i>Candida tropicalis</i>
F1	$32.3 \pm 0.6^{a,c,d}$	$19.7 \pm 3.5^{f,h}$	30.0 ± 1.0^j	$24.3 \pm 0.6^{m,o}$
F2	$29.3 \pm 1.2^{b,c,e}$	$18.0 \pm 1.7^{g,i}$	$26.3 \pm 1.2^{k,l}$	$21.3 \pm 1.2^{n,p}$
Control	$40.3 \pm 1.5^{a,b}$	$34.7 \pm 2.3^{e,g}$	$43.0 \pm 1.7^{j,k}$	$37.3 \pm 1.2^{m,n}$
NMP	$28.7 \pm 0.6^{d,c}$	$28.0 \pm 1.0^{h,i}$	30.7 ± 0.6^l	$28.0 \pm 1.0^{o,p}$

Note: Control = 1% Clotrimazole and 5% CO in NMP solution, Superscript indicate p value < 0.05

to F2. The results of *in vitro* drug release and drug remaining study can be attributed to the hydrophobic nature of saturated fatty acids (Lents *et al.*, 2014), a characteristic desirable for creating a durable and long-lasting therapeutic paint on the oral mucosa.

This diffusion-controlled release, effectively modeled by the Higuchi equation, represents a significant functional improvement over traditional 'Ya-Pai' formulations, which typically exhibit rapid and uncontrolled release of active compounds. The controlled release of drugs from hydrophobic matrices generally follows a diffusion-based mechanism described by Fick's law, particularly when the initial drug concentration exceeds its solubility in the matrix (Talevi, 2022). This process is effectively modeled by the Higuchi equation, which correlates drug release linearly with the square root of time. Comparable drug delivery systems have demonstrated similar release profiles. For instance, methotrexate-loaded vesicles containing oleic acid exhibited slower, more consistent release compared to free drug solutions, underscoring the influence of hydrophobic matrices on controlled drug delivery (Sharma & Arora, 2012). Additionally, eutectic fatty acid-based nanocarriers showed delayed release of doxorubicin relative to aqueous formulations, further supporting the diffusion-limiting properties of fatty acid matrices (Parveen *et al.*, 2022). Similarly, findings have been reported for various hydrophobic drug delivery systems. Heterocyclic drugs such as cefazolin sodium, gemcitabine hydrochloride, and metronidazole exhibited prolonged release from medium-chain saturated fatty acid vesicles, emphasizing the role of the fatty matrix in delaying drug diffusion (Wang *et al.*, 2023). Conversely, the incorporation of hydrophilic excipients, such as lactose, disrupted the matrix structure, enhancing drug release and facilitating more complete diffusion (Killen & Corrigan, 2006). These findings highlight the critical importance of matrix composition, particularly the selection and combination of fatty acids, in optimizing drug release profiles. The drug release rates demonstrate the ability of the clotrimazole-embedded ISFs to retard drug diffusion and penetration across the buccal membrane. Therefore, the ability of the clotrimazole-embedded ISFs to retard drug diffusion and penetration across the buccal mucosa transforms the traditional, short-acting 'Ya-Pai' into a modern sustained-release system enhancing patient compliance throughout the treatment process.

The therapeutic efficacy of this advanced 'Ya-Pai' is further amplified by the synergistic antifungal contributions of its components. Unlike

traditional pastes that are quickly diluted, the *in situ* paint ensures that the combined antifungal activities of clotrimazole, clove oil (CO), and N-methyl-2-pyrrolidone (NMP) are sustained at the site of infection. It is worth noting that clotrimazole has been reported to exhibit high *in vitro* susceptibility against *Candida spp.* (Frej-Mądrzak *et al.*, 2021; Meneses *et al.*, 2022). Clotrimazole-loaded ISGs have also demonstrated effective inhibition of growth against *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis* (Lertsuphotvanit *et al.*, 2023). The primary mechanism of action of clotrimazole involves disrupting the permeability barrier of the fungal cytoplasmic membrane (Haller, 1985). Additionally, NMP, the solvent used in the preparation of the ISFs, has been reported to exhibit inhibition against *Candida spp.* and bacteria growth (Phaechamud *et al.*, 2012). CO exhibits various biological activities, including antibacterial, antifungal, insecticidal, and antioxidant properties. Furthermore, it is employed as an antiseptic for oral infections. CO exhibited robust antifungal activity against opportunistic fungal pathogens such as *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Fusarium moniliforme* NCIM 1100, *Fusarium oxysporum* MTCC 284, *Aspergillus sp.*, *Mucor sp.*, *Trichophyton rubrum* and *Microsporum gypseum* (Bai *et al.*, 2023; Nuñez & Aquino, 2012; Panizzi *et al.*, 1993; Rana *et al.*, 2011). It has been suggested that the combination of CO and NMP, both of which have antimicrobial properties, contributes to the antifungal activity of the developed ISFs (Phaechamud *et al.*, 2012; Puyathorn *et al.*, 2023). The antimicrobial mechanism of CO may occur through many pathways. It was probably related to the damage of cell wall and membrane, the inhibition of biofilm formation, oxidative stress-mediated apoptosis, and the disruption of DNA synthesis (Bai *et al.*, 2023). Moreover, NMP, which contains a 5-membered lactam structure, has been shown to possess antimicrobial activity (Mahadlek *et al.*, 2022). Therefore, the combination of clotrimazole, CO, and NMP might enhance the antifungal activities of the developed ISFs. Although the antifungal activity of the formulations (particularly F2) was lower than the positive control in terms of inhibition zone, this outcome may reflect the balance between immediate antifungal effect and sustained drug release. While F1 demonstrated faster release and stronger inhibition in the short term, F2 offers the advantage of prolonged drug retention within buccal tissue, which could improve patient compliance by reducing dosing frequency. Since long-term management and adherence is a critical determinant of therapeutic success of oropharyngeal candidiasis

treatment, F2 which provide sustained release and high remaining of drug is a competitive choice (Pankhurst, 2009). As it is reported a synergistic effect of CO and NMP (Chantadee *et al.*, 2022; Mahadlek *et al.*, 2022), the result of using this combination which provide the effect on candida indicating that not only clotrimazole but also this combination of additives bring an effectiveness on antimicrobial activity.

Conclusion

The clotrimazole-embedded ISPs oral spray was prepared with NMP as the solvent, CO as an additive, and LAU and PAL as paint-forming agents. The ISPs solutions exhibited clear, colorless, and low viscosity liquid. The matrix formation occurred through a solvent exchange approach for sol-gel transformation. The higher fatty acid concentration in F2 produced a larger opaque ring at the beginning but slower matrix growth. The enlargement of paint sizes on the interface between ISPs solution and agarose gel was noticed as time passed in both F1 and F2. The prepared ISPs, which contained a higher concentration of fatty acid, showed slower drug release; however, a higher % remaining drug in the buccal mucosa. The cumulative percentage of drug released fit the Higuchi model; hence, the drug liberation was based on Fickian diffusion. The faster release of F1 significantly exhibited more inhibition zones for almost all of the tested *Candida spp.* than the F2 formula. The combination of clotrimazole, CO, and NMP in the developed ISPs might promote antifungal activities. Thus, the formulated clotrimazole-loaded ISP oral spray could potentially treat OPC. This research presents a novel approach to modernizing a traditional Thai medicine formulation. Our work was inspired by a classic Thai oral paint, a topical remedy applied directly to the oral mucosa. We have successfully re-engineered this traditional concept into a contemporary and user-friendly dosage form, an *in situ* forming paints. This research serves as a conceptual cornerstone, bridging the gap between traditional medicine and advanced pharmaceutical technology. It provides a foundational blueprint for transforming natural bioactive compounds into clinically effective, next-generation therapeutic systems of Thai traditional medicine. However, this study is aim to prove the concept which based on *in vitro* and *ex vivo* study, further study such as *in vivo* efficacy, mucosal safety, and pharmacokinetic evaluation should be investigated.

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Conflict of Interest

The authors declare that they hold no competing interests.

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