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Original Article

ANTIFUNGAL EFFECT OF *HYPTIS SUAVEOLENS* OIL MICROEMULSION BASED CARBOXYMETHYL MUNGBEAN GEL FOR TOPICAL DELIVERY

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ABSTRACT

Objective: Conventional topical antifungal formulations limit the effectiveness of antifungal therapy. The aim of this study was to formulate effective antifungal microemulsion of *H. suaveolens* oil based carboxymethyl mungbean (CMMS) gel.

Methods: *H. suaveolens* oil was obtained by steam distillation. Standard of *H. suaveolens* oil was performed by gas chromatography mass spectrometry (GC/MS). A high-viscosity CMMS was prepared and its mucoadhesive property was determined using modified USP dissolution test apparatus. *H. suaveolens* oil microemulsion based CMMS gel as transdermal drug carrier was then developed. Finally, *in vitro* drug release study and antifungal activity were determined.

Results: GC/MS analysis exhibited that β-Caryophyllene, Sabinene and Limonene are the major components of *H. suaveolens* oil. CMMS gel revealed good mucoadhesive potential which depended on pH of the medium. A higher retention time in pH 4.5 medium than pH 10 medium was observed. Clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel was successful prepared and *in vitro* sustained release of clotrimazole was determined. Clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel had potent antifungal activity against all studied dermatophytes and *Candida albican* with higher inhibition zone than *H. suaveolens* oil microemulsions based CMMS gel, *H. suaveolens* oil and commercial clotrimazole cream.

Conclusion: *H. suaveolens* oil microemulsions based CMMS gel present promising as an effective alternative for topical delivery of antifungal agents.

Keywords: *H. suaveolens*, Carboxymethyl mungbean, Microemulsion, Antifungal effect.

INTRODUCTION

Superficial fungal infections of the skin and mucous membranes are common fungal diseases in humans. Dermatophytes and several species of *Candida* are the primary causes of these infections [1]. The most frequently prescribed treatment of superficial fungal infection is local treatment with an antifungal agent, especially the thiazole family. The local delivery not only allows the drugs to be absorbed directly to a specific site but also avoids or minimizes the systemic side effects of the drugs that are encountered on oral administration. However, fungal infections are difficult to treat because of increasing resistance to current antifungal agents such as amphotericin B-and fluconazole-resistant *Candida* strains [2, 3]. Besides, conventional topical formulations such as cream, gel and ointment, limit the effective antifungal therapy. These conventional dosage forms cannot sufficiently penetrate to the target site and do not offer prolong duration of action [4]. Thus, the development of more effective antifungal formulation is necessary.

Hyptis suaveolens (Lamiaceae) is a medium, aromatic annual shrub commonly grown in the tropical and subtropical regions. It has been used in traditional medicine for cancer treatment. The essential oil from the leaves of *H. suaveolens* exhibited gastroprotective, neuroprotective, hepatoprotective, antioxidant and antibacterial activities. This oil also showed antifungal potential against *Trichophyton mentagrophytes.* The 20% ethanolic solution of *H. Suaveolens* oil had antifungal power similar to 6% boric acid, 2% benzoic acid or 5% salicylic acid, but higher than 4% phenol [5-8].

Microemulsions are optical, transparent systems composed of oil, water and surfactant frequently in combination with a co-surfactant. They are ease of formulation prepared by spontaneous emulsification method and optimized using phase diagram [9, 10]. Recently, transdermal permeability was improved due to the use of microemulsion as topical delivery vehicle [11]. Permeation enhancement mechanisms of microemulsions include thermodynamic activity toward the skin and increasing in concentration gradient [12]. Microemulsions have other advantages such as increasing drug solubility, good stability without any apparent coalescence during long term storage and low cost preparation [13, 14]. However, low viscosity of microemulsions makes them inconvenient for topical application. Gelling of microemulsion, using bioadhesive agent can increase the viscosity of this formulation as well as enhance resident time to skin or mucous membranes.

The development of new gelling agent from natural sources has regained the attention due to concerns over the biocompatible property. Carboxymethyl mungbean starch (CMMS) is a modified mungbean starch, prepared using a specific condition to yield a biodegradable, high-viscosity polymer product [15]. CMMS gel was suitable for pharmaceutical topical application with a good visual appearance, greaseless, washable gel, spreadable and stable preparation. CMMS gel also exhibited a pseudoplastic flow with thixotropic behavior at low concentrations and a plastic flow with yield value at higher concentrations [16]. The objective of this study was to evaluate mucoadhesive performance of CMMS gel. CMMS gel based *H. suaveolens* oil microemulsion as transdermal drug carrier was then developed. *In vitro* drug release study and antifungal activity were also studied.

MATERIALS AND METHODS

Materials

Mungbean starch (100%) was a gift from Sitthinan Co. Ltd. (Bangkok, Thailand (Pine brand, Thai Industrial Standard, TIS 948–2533). Chemicals and solvents used in the preparation and analysis of modified starches were of AR grade or equivalent. Standard commercial gelling polymers included carbopol 940 (CP), hydroxypropyl methycellulose (HPMC), methycellulose (MC) and an antifungal drug (clotrimazole) were obtained from S. Tong Chemical Co. Ltd. (Bangkok, Thailand). Chemicals used to prepare formulations were of pharmaceutical grade or equivalent. Sabouraud dextrose agar was purchased from Bacto-Difco Lab Co., Ltd (Detroit, MI, USA). All other chemicals were of the highest grade available.

Sample preparation

Aerial part of *H. suaveolens* was collected from Chiang Mai province, Thailand. The voucher specimen was deposited in the Herbarium of Faculty of Pharmacy, Chiang Mai Univerity, Thailand. The essential oil was obtained by steam distillation for 3 h in a simultaneous steam distillation–extraction apparatus. Yield of essential oil was 0.3%.

Essential oil analysis

Essential oil of *H. suaveolens* was analyzed by the Hewlett-Packard 6890 gas chromatography and Agilent Technologies HP 5973N mass spectrometry detector (EI Mode, 70 eV) with HP-5MS column (5% phenyl dimethylsiloxane, 30 m × 0.25 mm × 0.25 µm). Carrier gas was helium at a flow rate of 1 ml/min. The injector and detector temperature was 260 and 280 °C, respectively. The oven temperature was adjusted to 70 °C for 3 min, then increased to 188 °C at the rate of 3 °C/min and increased to 280 °C at the rate of 20 °C/min and held for 5 min.

Preparations of carboxymethyl mungbean starch (CMMS)

A high-viscosity CMMS was prepared and the physicochemical properties were determined according to the procedures previously described by Kittipongpatana *et al.* [15]. In brief, 40g of monochloroacetic acid was dissolved in 254 g of methanol, and then, while stirring, 138g of native mungbean starch powder was added into the solution, followed by 80 ml of 50% sodium hydroxide solution. The mixture was heated to 70 °C, where it was maintained for 60 min, with continuous stirring. At the end, the reaction was stopped by neutralization with glacial acetic acid. The liquid supernatant was decanted and the powder product was washed several times with 80% methanol and a final wash with 100% methanol. The modified starch was oven-dried at 50 °C for 24 h and was passed through sieve no. 60.

Determination of *in vitro* retention time of mucoadhesive gel

Mucoadhesive gels of CMMS, HPMC and MC were used at concentrations of 5 and 10% (wt/wt) whereas CP was used at concentrations of 1 and 2% (wt/wt). The polymer gels were prepared by mixing the polymer powder at specified concentrations with distilled water, and then allowed to fully swell overnight before use. In the case of CP, an addition of TEA was required to adjust pH to 7.2 for maximum swelling.

The retention time can be measured using modified USP dissolution test apparatus which has been modified from a previously published method [17]. Small intestinal tissue of porcine was instantly fixed with glass slab. Mucoadhesive gels were then added onto mucosa and 5 minutes time of contact was given. The obtained sample was immersed in a basket of the dissolution apparatus containing 800 ml of various pH buffers (citrate buffer pH 4.5, phosphate buffer pH 7.4 and carbonate buffer pH 10) at 37 °C. The paddle of the dissolution apparatus was adjusted the distance of 5 cm from the gel and rotated at 25 rpm. The retention time was recorded as the time used for the gel to completely detached or eroded from mucosa.

Preparations of clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel (CH-MBG)

Pseudo-ternary phase diagrams were produced in order to determine the concentration range of oil, water and the mixture of surfactant and co-surfactant for forming *H. suaveolens* oil microemulsions using water titration method. Tween (surfactant) and Propylene glycol (co-surfactant) were mixed (Smix) at weight ratios as 1:1. Weight Smix to oil (*H. suaveolens* oil and Capryol) was varied in 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 10:0. Each Smix-oil mixture was titrated with distilled water under a vortex mixer at room temperature. At the end point, the mixer changed from transparent to cloudy or opaque. The percentage of each component was recorded. Microemulsion region was marked in pseudo-ternary phase diagrams using Axio Vision Rel 4.8 software.

Clotrimazole, a model antifungal drug for mucosal delivery, was loaded into *H. suaveolens* oil at a concentration of 1% w/w. Clotrimazole loaded *H. suaveolens* oil microemulsion was prepared by dissolving drug powder into the oil-Smix mixture. The weighted quantity of distilled water was then mixed. The mixture was blended together adequately to form a clear and transparent dispersion. The whole process was performed at room temperature. To prolong its retention time when applied to the mucous membrane, gel was prepared to enhance the adhesion of *H. suaveolens* oil microemulsion. CMMS gel was selected because of its good mucoadhesive property as well as good spreadability, good visual appearance and greaseless. Clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel was prepared using 5% of CMMS. Plain CMMS was swelled with some water. Clotrimazole loaded *H. suaveolens* oil microemulsion was slowly added to highly-viscous CMMS gel. The viscosity and clarity of formulations were evaluated in comparison with 5% plain CMMS gel.

Dynamic light scattering

Globule size of *H. suaveolens* oil microemulsions was determined by dynamic light scattering (DLS), using a Malvern system (Zetasizer, version 5.00, Malvern Instruments Ltd., Malvern, UK) consisting of computerized auto-titrate and DLS software. Sizing measurements were taken at a fixed angle of 173°.

The *in vitro* release

The *in vitro* release of clotrimazole from clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel was determined by a dialysis method. An aliquot of 1 g of a freshly prepared clotrimazole microemulsions based CMMS gel was carefully filled, to avoid any air bubbles, into a pre-swollen dialysis bag with a molecular weight cutoff for 3,500 Da (Cellu Sep® T4 regenerated cellulose tubular membrane, Membrane Filtration Products, Inc.). The dialysis bag was tightly closed and submerged into 100 ml of 50%v/v Dioxan of Citrate Buffer (pH4.5) with gentle stirring at 200 rpm and temperature at 37 °C. The receiving medium containing the released clotrimazole (5 ml) were withdrawn periodically and 5 ml of fresh buffer containing 50% Dioxan was added to maintain the original volume. The concentration of clotrimazole in the different samples was measured by HPLC at 250 nm UV detector based on a standard curve of clotrimazole. The release profiles and the amounts of clotrimazole released were compared with clotrimazole solution.

***In vitro* antifungal activity**

Antifungal activity of clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel (CH-MBG), clotrimazole-loaded microemulsions based CMMS gel (C-MBG), *H. suaveolens* oil microemulsions based CMMS gel (H-MBG), *H. suaveolens* oil (Hyptis oil) and commercial clotrimazole cream (Com-C) was evaluated against dermatophytes *(Trichophyton mentagrophyte, Trichophyton rubrum, Microsporum gypseum*) and *Candida albican* using agar disc diffusion method. To reach stationary phase of growth, C. Albican was cultured in Sabouraud dextrose agar at 37 °C overnight whereas dermatophytes were cultured at 25 °C for 3 nights. Samples (100 g each) were then added to agar plates and incubated at 37 °C for 24 h and at 25 °C for 72 h of agar plates containing C. Albican and dermatophytes, respectively. Finally, the mean zone of inhibition was recorded after incubation.

RESULTS AND DISCUSSION

Essential oil analysis

The main compositions of essential oil were identified by comparison their chromatographic retention indices and mass spectra with Wiley and NIST library data and standards. The composition of *H. suaveolens* oil derived from steam distillation was identified by GC/MS as shown in fig. 1 and table 1. *H. suaveolens* oil contained mainly β-Caryophyllene (25%), Sabinene (15%) and Limonene (6%). β-Caryophyllene is natural bicyclic sesquiterpene representing in a major component in the oils of *Copaiba balsam*, *Eugenia caryophyllata* L., *Cinnamomum* spp. and *Piper nigrum* L. β-Caryophyllene exhibited anti-inflammatory, antioxidant, hepatoprotective properties and had beneficial effects on glucose homeostasis. β-Caryophyllene rich oils of *Zingiber nimmonii*, *Salvia sclarea* L. and *Lippia multiflora* were also the effective antimicrobials against pathogenic bacteria and fungi [18]. Moreover, oils from *Juniperus communis* subsp. alpine and Schinus species with the main compounds being sabinene and limonene present interesting antifungal activity [19, 20].

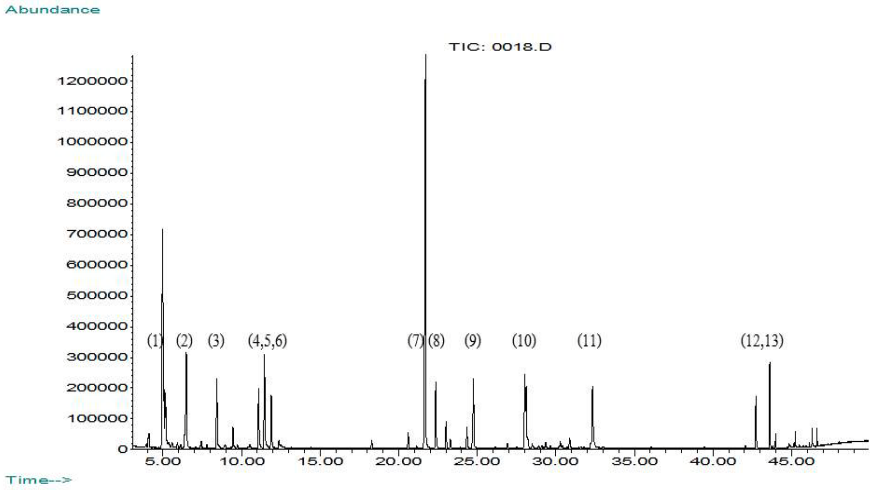

Fig. 1: Gas chromatograph mass spectrometry (GC/MS) chromatogram of *H. suaveolens* oil

However, the quantification of compounds of currently used *H. suaveolens* oil was different from our previous report, in which the sample was collected from the same area but at different harvest time. This result suggests that various time of harvest affects compound quantification of essential oil.

Determination of *in vitro* retention time of mucoadhesive gel

The recommended concentration of CMMS as a gelling agent was 3% (wt/wt) or higher [16]. CMMS gel concentrations used in the mucoadhesive study was 5% and 10%. In the comparison of mucoadhesive properties between CMMS gel and standard mucoadhesive gels (5% and 10% HPMC, 5% and 10% MC, 1% and 2% CP), the retention time of CMMS gel (202-232 min) was around the same as that of MC and CP (36-109 min) but significantly higher than that of HPMC (30–62 min) as shown in fig. 2. These results present that CMMS gel has interesting mucoadhesive property as comparable mucoadhesive potential with MC and CP gels but higher mucoadhesive time than HPMC.

This study also determined pH-dependent experiment of mucoadhesion on CMMS gel. Retention times of mucoadhesive CMMS gel at pH 4.5, 7.4 and 10 were carried out as shown in fig. 3. Retention times of 5% CMMS at pH 4.5, 7.4 and 10 were 202±6, 152±9 and 73±18 min, respectively. Retention of 10% CMMS also gradually decreased in pH (232±4, 166±10 and 112±16 min at pH 4.5, 7.4 and 10 respectively).

The retention time of 10% HPMC decreased from 109 min at pH 4.5 to 47 min at pH 10 while retention time of 5% HPMC was constant in pH with value around 30 min. fig. 3 revealed that mucoadhesice time of 5% MC, 10% MC and 2% CP was gradually decreased in pH as the same as 5% CMMS, 10% CMMS and 10% HPMC with retention time around 159–243 min at pH 4.5, 122–192 min at pH 7.4 and 30–148 min at pH 10. Beside, the mucoadhesive times of 1% CP at pH 4.5 and pH 10 were 122 min and 93 min, respectively.

However, the highest retention time of 1% CP was found at pH 7.4 (160 min). The results show that CMMS and standard mucoadhesive gels had pH dependent mucoadhesive potential. The highest mucoadhesive property was found at low pH (pH 4.5) whereas the lowest mucoadhesive property was detected at high pH (pH 10). These results were clearly shown at high conconcentration of studied gels.

Table 1: Composition of *H. suaveolens* oil determined by Gas chromatograph mass spectrometry (GC/MS)

	RT	Compounds	Percent
1	4.99	Sabinene	14.68
2	6.49	Limonene	6.29
3	8.43	α-Terpinolene	3.88
4	10.52	Camphor	0.19
5	11.47	endo-Borneol	5.61
6	12.38	P-Cymen-8-OL	0.41
7	21.7	β-Caryophyllene	25.18
8	22.36	α-Farnesene	3.72
9	24.76	1,5-Heptadiene,2,5-dimethyl-3 methylene	4.45
10	28.02	Spathulenol	4.48
11	28.11	Caryophyllene oxide	3.67
12	32.33	Bergamotol	4.64
13	42.73	Rimuene	2.62
14		others	20.18

Vaginal lumen is the site for traditionally local delivery and a possible route for systemic administration in order to avoid hepatic or gastrointestinal degradation or reduce side effects in the gastrointestinal tract. However, physiological removal mechanisms are a drawback of the vaginal route. The contact time of administered drugs with vaginal mucosa is limited that leads to a short duration of treatment, causing a necessary frequent dosing regimen [21]. Potential mucoadhesive based gel formulation is one interesting vaginal delivery system for prolonged contact period between administered drug and vaginal mucosa [4, 22]. According to vaginal pH is usually in the range 4–4.5 which CMMS gel represented the highest mucoadhesive activity. In this respect CMMS gel has a high potential for using as a base for vaginal formulation.

Various polymers have been used as mucoadhesive drug carriers. Possible mucoadhesive mechanisms of polymer and mucin

interactions are hydrogen bonding and van der Waals forces. The basic component of all mucous is the mucin glycoprotein. Mucin contains a large number of hydroxyl, carboxylate and sulfonate groups in the branched sugar chains.

Polymer with significant numbers of H-bonding group is good candidates for mucoadhesion [23]. Hydrogen bonds formed between the carboxylic group of CMMS and the glycoprotein component of mucous may play a significant role in mucoadhesion.

The mucoadhesive retention time is strongly dependent on pH. CMMS gel adhere mucin via H-bonds at pH 4.5.

However, at pH 7.4 and pH 10, the carboxylic acid groups become deprotonated. This would decrease H-bonding between carboxylic groups of CMMS and proton acceptors in mucin, accounting, in part, for the decreased mucin adsorption with increasing pH.

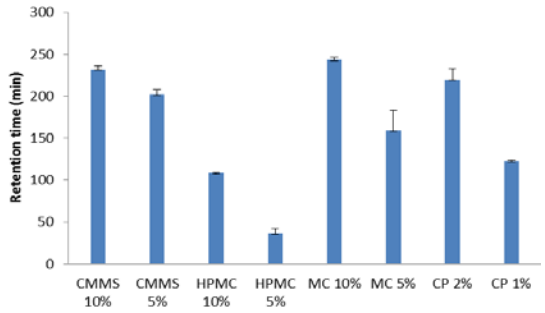

Fig. 2: *In vitro* retention time of carboxymethyl mungbean starch (CMMS), hydroxypropyl methycellulose (HPMC), methycellulose (MC) and carbopol 940 (CP) using modified USP dissolution test apparatus at pH 4.5

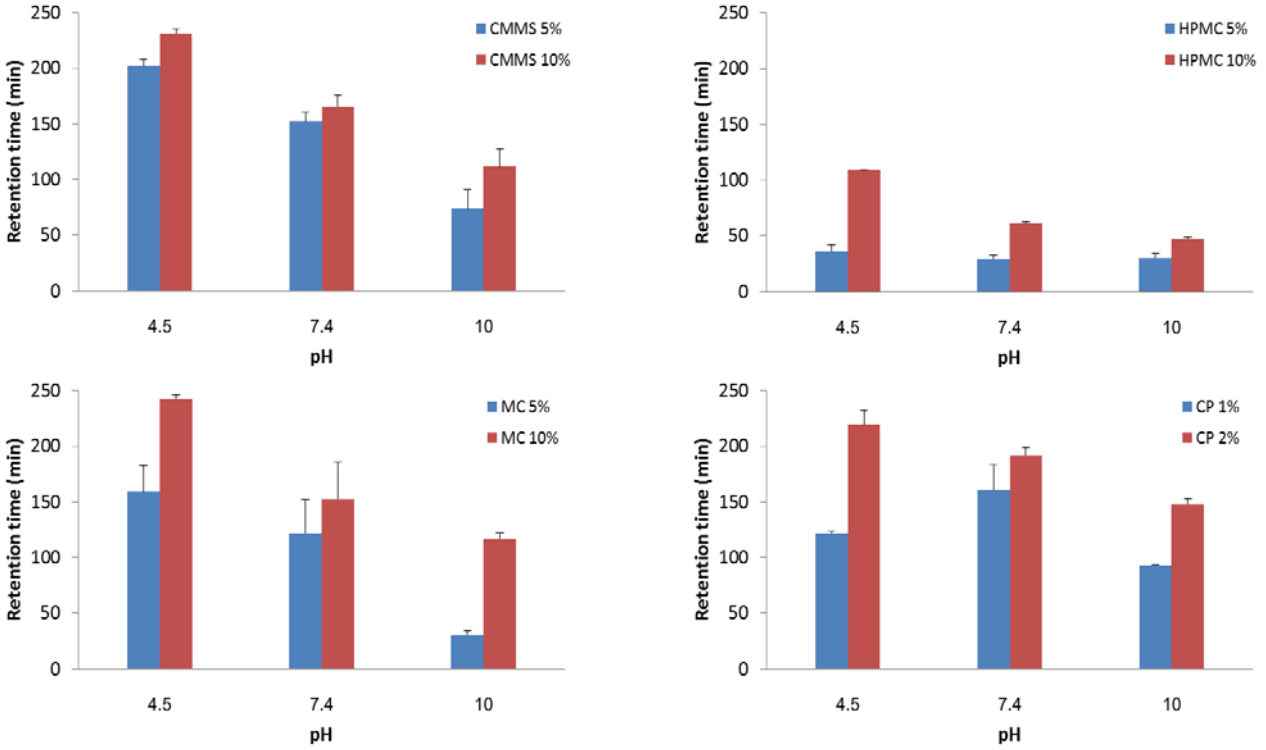

Fig. 3: *In vitro* retention time of carboxymethyl mungbean starch (CMMS), hydroxypropyl methycellulose (HPMC), methycellulose (MC) and carbopol 940 (CP) using modified USP dissolution test apparatus at pH 4.5, 7.4 and 10

Preparations of clotrimazole-loaded *H. suaveolens* oil micro emulsions based CMMS gel

Fig. 4 shows the phase diagram of *H. suaveolens* oil microemulsions. Red region in the fig. 4 is the microemulsion region (42.3%) while the white region is non microemulsion region. The selection of *H. suaveolens* oil microemulsion composition for clotrimazole entrapment was based on solubility of clotrimazole in the oil phase as shown in table 2.

The results demonstrate that *H. suaveolens* oil microemulsion for further study had droplet diameter of 242.1±17.1 nm and PDI of 0.30±0.03 which was slightly higher than the usual microemulsion droplet size range of 20–200 nm [24]. The incorporation of clotrimazole did not have considerable influence on the globule size of the microemulsion. Clotrimazole was found to undergo acidic pH-catalyzed degradation [4].

The pH value of *H. suaveolens* oil microemulsion was 6.42 and the pH value of clotrimazole-loaded H. suaveolens oil microemulsions based CMMS gel was 6.37 which close to a commercial formulation of CMZ (Candid-V® Gel) has pH value of 6.81 and it claims stability of up to 1.5 years.

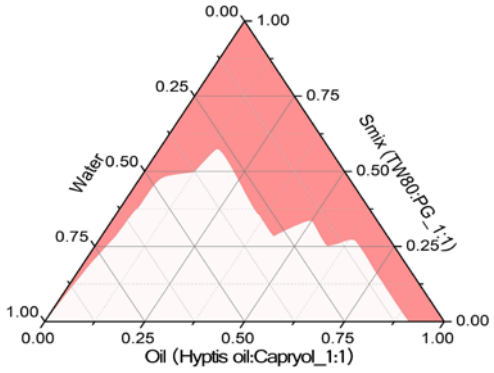

Fig. 4: Pseudo-ternary phase diagram composed of water, oil (hyptis oil and capryol 90 in ratio 1:1) and Smix (tween 80 and propylene glycol in ratio 1:1). The red area represents the microemulsion region whereas white area represents non microemulsion region

Table 2: *H. suaveolens* oil microemulsion and clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel (CH-MBG) composition

Formulation composition	*H. suaveolens* oil Microemulsion (percentage)	CH-MBG (percentage)
Clotrimazole	-	1.0
H. suaveolens oil	12.5	12.5
Capryol	12.5	12.5
Tween 80	25.0	25.0
Propylene glycol	25.0	25.0
Water	25.0	14.0
CMMS	-	10.0

The *in vitro* release

The *in vitro* release study of clotrimazole from *H. suaveolens* oil microemulsions based CMMS gel is demonstrated using a dialysis membrane. Fig. 5 presents that the initial burst of release clotrimazole was not observed; whereas 28 % of loaded clotrimazole were gradually released from *H. suaveolens* oil microemulsions based CMMS gel in the first 44 h of incubation. Clotrimazole was subsequently slowly released the following 6 days and reaching 40% from *H. suaveolens* oil microemulsions based CMMS gel.

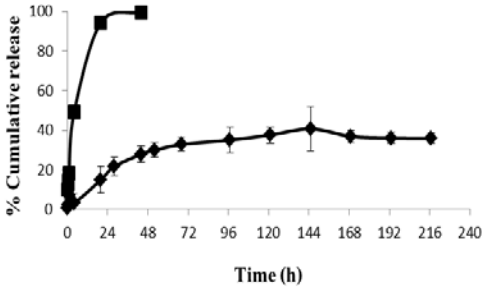

Fig. 5: Release of clotrimazole from solution (▪) and from *H. suaveolens* oil microemulsions based CMMS gel (♦), by a dialysis membrane, pH 4.5, 37 °C

This data suggests that *H. suaveolens* oil microemulsions based CMMS gel could prolong action of clotrimazole by continuously releasing clotrimazole over an extended period of time and frequency of drug administration could be reduced, making an improvement patient compliance.

***In vitro* antifungal activity**

The results of antifungal studies are shown in table 3. It was indicated that *H. suaveolens* oil (hyptis oil) had antifugal activity against all tested dermatophytes *(T. mentagrophyte, T. rubrum, M. gypseum)* and *C. albican* with inhibition zone around 13.3±0.1 to 15.7±0.4 mm. H-MBG showed higher antifungal activity (inhibition zone of 23.9±0.4 to 57.2±0.5 mm) than hyptis oil.

Moreover, C-MBG also presents more potent antifungal activity (inhibition zone around 37.0±1.1 to 55.6±0.5 mm) as compared to market clotrimazole cream (inhibition zone of 24.0±0.2 to 35.4±0.7 mm). The enhanced *in vitro* antifungal activity of both H-MBG and C-MBG may be attributed to enhanced penetration of Hyptis oil and clotrimazole through fungal cell walls to the targeted site of action. CH-MBG had bigger inhibition zone than H-MBG.

However, CH-MBG did not show significantly different from C-MBG. Besides, CH-MBG subsequently slowly released their content. This is considered that synergistic effect between hyptis oil and clotrimazole was not observed.

Table 3: Inhibition zones of *H. suaveolens* oil (Hyptis oil), clotrimazole-loaded microemulsions based CMMS gel (C-MBG), *H. suaveolens* oil microemulsions based CMMS gel (H-MBG), clotrimazole-loaded *H. suaveolens* oil microemulsions based CMMS gel (CH-MBG) and commercial clotrimazole cream (Com-C) against dermatophytes and *Candida albican*

Samples	Inhibition zone (mm)			
	M. gypseum	***T. rubrum***	***T. mentagrophytes***	***C. albican***
Hyptis oil	13.3±0.1	14.7±0.2	15.7±0.4	13.7±0.6
H-MBG	57.2±0.5	23.9±0.4	28.6±0.2	31.3±1.0
C-MBG	55.6±0.5	37.0±1.1	38.4±1.6	38.9±0.9
CH-MBG	61.4±1.0	32.7±0.4	33.7±0.6	39.9±0.5
Com-C	35.4±0.7	24.0±0.2	25.3±1.2	25.3±0.5

All values represent mean±SD, n=3

CONCLUSION

The results presented in this paper show that CMMS appeared to be a potential mucoadhesive polymer for vaginal drug delivery system. Clotrimazole was successfully loaded into *H. suaveolens* oil microemulsions based CMMS gel. Sustained release of incorporated drug was observed. *H. suaveolens* oil microemulsions based CMMS gel formulations showed better penetration and antifungal property as compared to the commercial formulation. It can be concluded that *H. suaveolens* oil microemulsions based CMMS gel could be effectively prepared for topical treatment of dermatophytes and *Candida albican*.

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CONFLICT OF INTERESTS

Declared None.

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