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Evaluation of antifungal activity of blended cinnamon oil and usnic acid nanoemulsion using candidiasis and dermatophytosis models

Peeyush kumar[a,*], P.W. Ramteke[b], Avinash C. Pandey[c], Himanshu Pandey[a]

[a] Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, 211007, India
[b] Department of Biological Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, 211007, India
[c] Nanotechnology Application Centre, University of Allahabad, Allahabad, 211002, India

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ABSTRACT

Fungal infections are one of the most important health issues facing by people since a long time. The objective of the present study was to evaluate antifungal activity of a prepared blended cinnamon oil and usnic acid nanoemulsion (CUN) using candidiasis and dermatophytosis models both *in vitro* and *in vivo*. *In vitro* activity was performed using cup plate method; minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were determined using brain heart infusion (BHI). *In vivo* antifungal activity was determined on cutaneous candidiasis in rats and on dermatophytosis guinea pig model. *In vitro* study of CUN exhibited maximum antifungal activity after 48 h. The maximum zone of inhibition was 1.19 cm for CUN (10 mg/mL), which was near to the fluconazole (1 mg/mL) i.e. 1.82 cm. The MIC of CUN for *C. albicans* and *T. mentagrophytes* were 60 and 55 μg/mL, respectively, which was more than cinnamon oil and usnic acid solution (CUS). *In vivo* study of CUN in cutaneous candidiasis model showed significant antifungal activity. The log colony forming unit per infected site of CUN was 1.36 in comparison to untreated control group (3.91). However, the clinical efficacy profile of CUN on dermatophytosis guinea pig model was more than the standard drug (41.3%) and CUS (35.6%). Its mycological efficacy was 81.4%, which was greater than standard drug (79.6%) and CUS (74.5%). Thus, the present study substantiated that CUN can be used as a feasible formulation for topical application against fungal infections.

1. Introduction

Fungal infections of the skin, hair, and nails are a common public health problem worldwide. Fungal infections are now emerging as major skin disease (Kim SH et al., 2015). These infections are mainly associated with *Candida, Cryptococcus* and *Aspergillus* species. However, *Candida* species covers most of the fungal infections worldwide (Dzoyem JP et al., 2014). For last few decades, *Candida albicans* are the most prevalent pathogens in fungal infections. Dermatophytosis is a type of fungal infection affects hair, nail and skin principally caused by *Trichophyton, Epidermophyton* and *Microsporum* fungal genera. However, these infections are not life threatening but may affect the comfort and quality of patient's life (Long L et al., 2016).

Cinnamon oil is an essential oil obtained from bark, leaves, twigs of *Cinnamomum* genera belongs to family *Lauraceae*. The active constituents of cinnamon oil responsible for antifungal (MIC, 0.40 mg/mL) and antimicrobial (MIC, 0.20 mg/mL) activities are cinnamaldehyde, cinnmyl cinnamate and benzyl cinnamate (Boniface Y et al., 2012). The mechanisms involved in antifungal effect of cinnamon oil are cytoplasm granulation, cytoplasmic membrane rupture and inactivation of intracellular and extracellular enzymes (Gupta C et al., 2008). Usnic acid is secondary lichen metabolite originates from cyanobacteria possess antifungal and antibacterial activity (Toby S et al., 2016). It is obtained from *Cladonia foliacea* of family Cladoniaceae. Usnic acid is used in several topical preparations and skin care agents because of its biological property and its absorption capacity in the ultraviolet region (Russo A et al., 2008).

Nanoemulsions (NEs) are colloidal particulate systems for carrying drug molecules. These are of submicron size, range varies from 10–1000 nm. NEs are solid spheres and their surface is amorphous and lipophilic with negative charge (Jaiswal M et al., 2015). Nanoemulsion based system find significant improvement in topical delivery of antifungal, antiviral, anti-inflammatory, antioxidant, local anesthetics, etc. NEs are small-sized droplets offers greater surface area for better

* Corresponding author. Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, Uttar Pradesh, India.
E-mail address: peeyushkumar037@gmail.com (P. kumar).

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absorption (Singh Y et al., 2017). However, due to poor water solubility of cinnamon oil and instability of cinnamic aldehyde in the blood, it has been proved that topical nanoemulsion preparation can significantly improve its bioavailability. Therefore, blended nanoemulsion of cinnamon oil and usnic was used to determine its antifungal activity for superficial skin and hair roots infections.

2. Material and methods

2.1. Drugs and chemicals

Brain heart infusion (BHI) broth obtained from HiMedia lab (Mumbai, India), Amphotericin B was purchased from Sigma-Aldrich (St. Louis, MO, USA). 0.5% Fluconazole cream (Fuconal), Boryung Co., Seoul, Korea and 8% ciclopirox nail lacquer (Penlac), Bausch Health Companies Inc., Canada was used. All other chemicals and solvents were of analytical grade.

2.2. Preparation of blended cinnamon oil and usnic acid nanoemulsion (CUN) nanoemulsion

The blended Cinnamon oil and Usnic acid nanoemulsion (CUN) was prepared using Cinnamon oil (6%) as an essential oil, Tween 20 (24%) as a surfactant, ethanol (12%) as co-surfactant, Usnic acid (0.5%) as drug and deionized water (57.5%) as continuous phase.

2.3. Preparation of cinnamon oil and usnic acid drug solution (CUS)

The cinnamon oil and usnic acid solution (CUS) meant for comparative evaluation was prepared by dissolving cinnamon oil (50 mg) and usnic acid (12.5 g) in a beaker and stored in a vial at room temperature.

3. Evaluation of antifungal activity

3.1. Fungal strains and culture media

The fungal strains *C. albicans* (ATCC 18804) and *T. mentagrophytes* (TIMM 1189) were used in the study. The fungal strains were cultured and maintained using brain heart infusion (BHI) agar or BHI broth supplemented with 5% sucrose.

3.2. In vitro experimental study

In-vitro experimental study is performed to investigate the potency of test compound (CUN) in covering the most relevant pathogens of dermal mycoses in comparison to standard drug.

3.2.1. In vitro antifungal activity using cup-plate method

This experimental model depends upon diffusion of drug from a vertical cylinder through a solidified agar layer in a petridish or plate to an extent such that growth of added microorganism is prevented entirely in a zone around the cylinder containing antifungal agent. The overnight grown culture of *Candida albicans* was seeded with 100 µL of the fungal inoculation into the sterilized agar media plates. The concentration of the final inoculum was 5×10^3 cfu/mL. After solidification, wells (6 mm) were cut into the media and fixed with 50 µL each of blank nanoemulsion, fluconazole and CUN. The concentration of fluconazole was 1 mg/mL. The plates were incubated at room temperature and the widths of zone of inhibitions resulting after drug diffusion into media were measure (Carrilo-Muñoz AJ et al., 1996).

3.2.2. Determination of minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Microdilution method using 96-well microplates was used to determine MIC and MFC of CUN. Amphotericin B (AmB) was used as standard at concentration from 0.015 to 2 µg/mL. To prepare inoculum, microbial suspensions prepared in BHI broth were standardized in tubes containing 5 mL of 0.9% sterile saline solution and adjusted spectrophotometrically to 90% transmittance at 530 nm. It corresponds to a concentration of 5×10^3 cfu/mL. The samples (CUS and CUN) were serially diluted in the microdilution wells containing 100 µL of BHI broth supplemented with 5% sucrose. The plates were incubated at 35 ± 1 °C and subcultures were performed after 24 h, 48 h and 72 h up to 7 days until the drug-free control well was fully occupied by fungal growth and visually evaluated after addition of inoculum (100 µL) in separate plates for each test organisms (*C. albicans,* ATCC 18804 and *T. mentagrophytes,* TIMM 1189). MIC was corresponded to the lowest sample dilution capable of providing growth inhibition of organisms. MFC was obtained by taking 100 µL of each serial dilution from each well and spread on BHI agar then plates were incubated at 28 °C for 3–7 days. The MFC was defined as the lowest drug concentration that ≥1 colony visible on agar plate (i.e. 99% of the inoculum was killed). All tests were performed in duplicate (Guimaraes GP et al., 2014) (Guarro J et al., 1999).

3.3. In vivo experimental study

3.3.1. Experimental animals

Animals used in the study were obtained from the animal house of United Institute of Pharmacy, Allahabad, approved by Institutional Animal Ethical Committee (IAEC) of United Institute of Pharmacy, Allahabad and Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) reference no. 1451/PO/Re/S/11/CPCSEA. Animals were kept in 12 h light and 12 h dark laboratory condition at 25 ± 2 °C temperature and humidity 47 ± 5%. All animals were given standard ad libitum diet and were immunosuppressed with dexamethasone (1 mg/L of their drinking water up to 72 h before the experimental procedure).

3.3.2. In vivo antifungal effect of CUN on cutaneous candidiasis in rats

Candidiasis was induced by using fungal strain *C. albicans* (ATCC 18804) to study the *in vivo* performance of the prepared nanoemulsion formulation based on method described by Maebashi K et al., 1995. Animals (Male albino rats weighing 100–150 g) hairs were removed using depilatory cream, marked the skin (3×3 cm^2) and *C. albicans* inoculum (5×10^4 cells/mL) was applied to marked area on skin. A cell suspension of 100 µL was applied to the marked area using a sterile pipette-tip and rubbed thoroughly. Animals were divided into three groups each containing six animals i.e. control group, CUN and CUS treated groups. The different formulations, i.e. CUN and CUS, were applied topically in gel form by mixing with polymer carbopol 980. Treatment was continued for once daily for six consecutive days, starting on the day of post-infection to albino rats of respective groups, excluding the animals of the control group. The skin was excised from treated sites, minced with scissors and homogenized in saline then streaked on the solidified growth medium. Sabouraud dextrose agar (SDA) media supplemented with sisomicin (50 µg/mL) and mezlocillin (100 µg/mL) was used. After incubation for 48 h at 37 °C, the number of Candida colonies was counted and the logarithm of the number of CFUs per infected site was calculated. The performance of animals of various groups to alleviate mycosis (candidiasis) was compared with that of control after 6 days then skin texture was evaluated, and recovery time was measured.

3.3.3. In vivo activity of CUN in the Guinea pig trichophytosis model

Infection in guinea pig model was induced by *T. mentagrophytes* (TIMM 1189), it resulted in inflammatory reactions of the skin and hair root invasion. *T. mentagrophytes* inocula was prepared in a petridish containing potato dextrose agar (PDA) and incubated at 30 °C for 5–7 days. A freshly prepared suspension of 1×10^7 conidia/100 µL was applied on shaved skin of animals (Male albino guinea pigs weighing

Table 1
In vitro antifungal activity of CUN.

Time (h)	Zone of inhibition (cm)		
	Blank NE	Fluconazole (1 mg/mL)	CUN (10 mg/mL)
24	0.11	0.78	0.95
48	0.12	1.19	1.82

CUN = Cinnamon oil and usnic acid nanoemulsion, NE = nanoemulsion.

450–550 g) (Long L et al., 2016). Animals were divided into four different groups each containing five animals i.e. untreated normal control (NC), cinnamon oil and usnic acid drug solution (CUS) gel, 8% ciclopirox (CX), and cinnamon oil and usnic acid blended nanoemulsion (CUN) gel. Treatment was started at 72 h post challenge and continued once daily for the next 7 days (Ghannoum M et al., 2009).

3.3.3.1. Clinical and mycological evaluation. Treated animals were examined daily throughout the study and clinical and mycological evaluations were performed on 13th day. The clinical assessment of local changes occurred on guinea pig skin was scored on a scale of from 0 to 5 in which 0 indicated no lesions, 1 indicated few slightly erythematous places on the skin, 2 indicated well-defined redness, 3 indicated large areas of marked redness, incrustation, scaling and ulceration in places, 4 indicated partial damage to the integument and the loss of hair, and 5 indicated extensive damage to the integument and a complete loss of hair at the infection site (Long L et al., 2016).

Mycological evaluation was performed by hair root invasion test in which hair samples of guinea pigs were planted onto the surface of PDA plates. The plates were incubated at 30 °C for 2–4 days and counted number of hairs exhibiting fungal growth using a stereomicroscope. The clinical and mycological efficacies for each treatment group was expressed as a percentage relative to the result for the infected untreated control group using the following formula:

$$100 - [T \times (100/K)]$$

Where, T is the total score for the test group and K is the total score for the infected untreated control group.

3.4. Statistical analysis

Statistical analysis of experimental data was performed using Graphpad prism software 5.0 and Microsoft Office Excel 2013. The results were analyzed statistically using one-way analysis of variance test followed by post-hoc Dunnet test for a level of significance at $p < 0.05$. Values are expressed as mean ± SD.

4. Result

4.1. In vitro antifungal activity in cup plate method

CUN exhibited maximum antifungal activity at concentration 10 mg/mL after 48 h comparable to fluconazole cream. In first 24 h, the zone of inhibition for CUN was 0.78 cm, which extended to 1.19 cm after 48 h. However, this zone of inhibition was near to fluconazole cream i.e. 0.95 and 1.82 cm after 24 and 48 h respectively. Table 1 shows the antifungal activity of CUN.

Table 3
Animals showing positive culture with *C. albicans* in skin after treatment with different formulations.

Treatment	No. of animals with positive culture
Control	6/6
CUS gel	4/6
CUN gel	1/6

CUN = Cinnamon oil and usnic acid blended nanoemulsion, CUS = Cinnamon oil and usnic acid drug solution.

4.2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of prepared CUN

Both the microorganisms studied proved sensitive to cinnamon oil and usnic acid solution, which showed low activity against *C. albicans* (MIC = 260 and MFC = 270 μg/mL) and activity more than one order of magnitude greater against *T. mentagrophytes* (MIC = 240 and MFC = 245 μg/mL), while the MIC and MFC values for the positive control (AmB) was only 0.5 μg/mL. In contrast, CUN proved moderate activity against microorganism, mainly *C. albicans* (MIC = 60 and MFC = 70 μg/mL), and *T. mentagrophytes* (MIC = 55 and MFC = 60 μg/mL). However, CUS also exhibited inhibitory activity against fungal strains but it was not significant as much as CUN and positive control. The MIC and MFC of CUS were 260 and 270 μg/mL for *C. albicans* and 240 and 245 μg/mL for *T. mentagrophytes*, respectively. The tests of MIC and MFC of the CUN are shown in Table 2.

4.3. In vivo efficacy of formulations on cutaneous candidiasis in rats

Topical application of CUN gel on albino rats showed significant efficacy against cutaneous candidiasis in comparison to control group animals. Results exhibited that only one animal out of six exhibited a positive culture test (*C. albicans* infection); in contrast, CUS gel treatment showed four animals out of six (Table 3). The untreated control group animals were all infected with fungi, its infection site exhibited 3.91 log CFU. However, CUS gel treated group showed 3.21 and CUN treated group animals showed 1.36 log CFU per infected sites (Fig. 1). The recovery was fast in the case of CUN gel. The effectiveness of CUN gel was possibly due to better occlusive and bioadhesive properties.

4.4. In vivo efficacy of prepared formulations in the Guinea pig trichophytosis model

Animals were observed daily for sings of infection. The animals started showing scaling and redness at the infected area. The clinical efficacy of CUN gel was found to be highest 41.3% in comparison to ciclopirox (38.0%). and CUS gel (35.6%) as shown in Table 4. The mean clinical score for CUN gel was 2.54 indicating good antifungal activity in comparison to ciclopirox (3.01) and CUS (3.78) (Fig. 2). Untreated infected group (control) exhibited hair loss, scaly skin and ulcerated at

Table 2
Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC).

Microorganism	MIC (μg/ml)			MFC (μg/ml)		
	Amphotericin B	CUS	CUN	Amphotericin B	CUS	CUN
Candida albicans ATCC 18804	0.5	260	60	0.5	270	70
Trichophyton mentagrophytes TIMM 1189	0.5	240	55	0.5	245	60

CUS = Cinnamon oil and usnic acid solution, CUN = Cinnamon oil and usnic acid nanoemulsion.

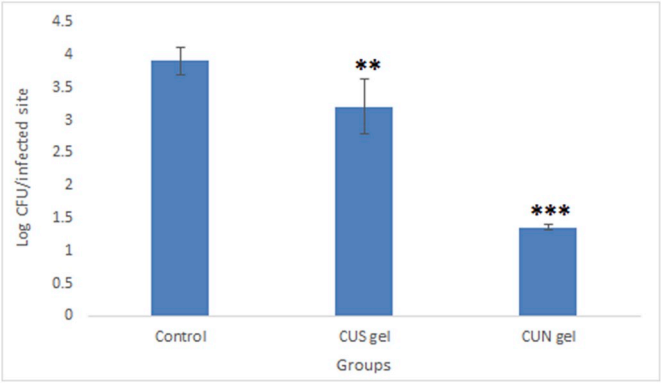

Fig. 1. Effect of CUN on colony forming unit of *C. albicans* in candidiasis rats. CUN = Cinnamon oil and usnic acid blended nanoemulsion, CUS = Cinnamon oil and usnic acid drug solution.

Table 4
Clinical and mycological efficacies of CUN using guinea pig trichophytosis model.

Treatment	Clinical efficacy (%)	Mycological efficacy (%)
NC	–	–
CX	38.0 ± 1.32	79.6 ± 1.44
CUS	35.6 ± 1.14	74.5 ± 0.95
CUN	41.3 ± 1.34	81.4 ± 1.26

NC (Normal control), CX (8% ciclopirox), Cinnamon oil and usnic acid solution (CUS), Cinnamon oil and usnic acid nanoemulsion (CUN).

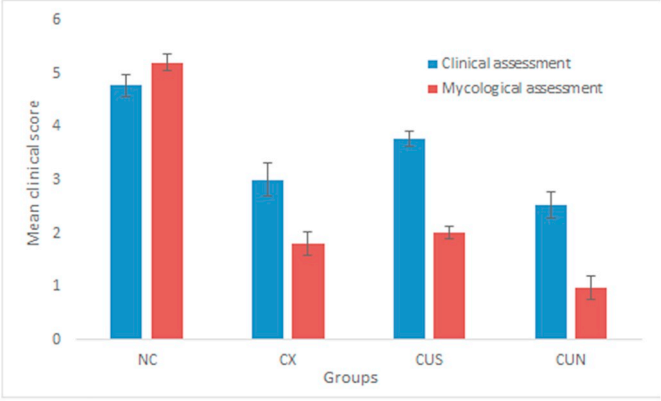

Fig. 2. Mean clinical score of CUN in the guinea pig trichophytosis model
Data are represented as Mean ± SD (n = 5), Where, NC (Normal control), CX (8% ciclopirox), Cinnamon oil and Usnic acid drug solution (CUS), Cinnamon oil and usnic acid blended nanoemulsion (CUN).

the day 13th. However, the CUN treated test group showed healing of inflamed area. Table 4 shows the clinical efficacy of each treatment groups.

Mycological assessment exhibited that CUN may be an effective formulation against fungal infections (Table 4). The percentage efficacy for CUN was 81.4%, which was slightly greater than ciclopirox (79.6%) and CUS gel (74.5%) as shown in Table 4. The mean clinical score of CUN was found to be 0.98 indicating a good mycological efficacy in comparison to ciclopirox (1.81) and CUS (2.02) (Fig. 2).

5. Discussion

Usnic acid and Cinnamon as an essential oil having inhibitory effects on the proliferation and growth of a wide range of microorganisms. On the other hand, the blended cinnamon oil and usnic acid formulation could have stronger antifungal effects because of the potential synergic effects from two different sources. Nanoemulsions are a sub-group of emulsions, which have nanosized droplets (Zhang S et al., 2017). Nanoemulsion preparation of above drugs showed effective antifungal effect, it may be due to their unique subcellular size can effectively increase the distribution of antifungal agents in fungal cells, and therefore greater antifungal activity was achieved (Kumar P et al., 2018). *In vitro* antifungal evaluation of CUN was found effective against *C. albicans* and *T. mentagrophytes*. *In vitro* cup plate method was used to determine efficacy of CUN against *C. albicans*. The results showed significant zone of inhibition of by CUN after 48 h that was near to standard drug fluconazole. Similar results was reported by Ansari MJ et al., 2016, for evaluation of antifungal activity of olive oil based nanoemulsion. The agar well plate or cup plate method is the only suitable diffusion technique as antimicrobial evaluation of test compound as the presence of suspended particulate matter in the sample being tested is much less likely to interfere with the diffusion of the drug into the agar medium than in the filter paperdisc.

MIC and MFC results of CUN demonstrated that the formulation was effective in inhibiting fungal growth on skin. In contrast, CUS was less effective against both the strains in comparison to CUN. The inhibitory concentration of CUN was small in comparison to CUS. The antifungal effects of nanoemulsion formulations using different essential oil and incorporating with natural drugs have been proved effective against selected fungus strains (Bajerski L et al., 2016). The antifungal activity of tea tree oil *(Melaleuca alternifolia)*, incorporated into nanoemulsion was investigated for the growth of dermatophyte fungus caused by *T. rubrum* (Flores FC et al., 2013).

In vivo study for confirming the antifungal efficacy of CUN against *C. albicans* and *T. mentagrophytes* was performed on animal models naming cutaneous candidiasis on rats and dermatophytosis on guinea pigs, respectively. CUN gel was applied on rats infected with *C. albicans* in cutaneous candidiasis animal mode. The formulation treated group showed significant reduction in colony forming units in comparison to untreated control group. Only one animal out of six was detected positive fungal culture those treated with CUN. However, the number of positive culture animals was 4 and 6 for CUS and control group animals, respectively. The log CFU units for nanoemulsion treated group was very less i.e. 1.36 ± 0.04 in comparison to control group (3.91 ± 0.21). Recently, one study reported greater antibacterial activity of cinnamon oil nanoemulsion in comparison to cinnamon oil microemulsion (Valizadeh A et al., 2018). Similarly, Topuz OK et al., 2016, showed that cinnamon oil nanoemulsion has higher antimicrobial activity compared to bulk anise oil. The clinical efficacy of CUN against *T. mentagrophytes* was evaluated in guinea pigs. Guinea pig was used as an experimental model for dermatophytosis, this animal species is naturally susceptible to zoophilic *T. mentagrophytes* infection. The clinical features exhibit by this animal are comparable to those observed in human dermatophytoses (Ghannoum M et al., 2009). Results showing that nanoemulsion formulation reduced fungal growth, which was greater than standard ciclopirox (8%). On the other hand, the effectiveness of CUS was smaller than the standard but more than untreated normal control group. The data showed that all the treated groups demonstrated significant clinical and mycological efficacies; however, CUN showed clinical efficacy more than that of ciclopirox (41.3% and 38.0%, respectively). Likewise, mycological efficacy for all treatment groups was significantly better than that for the infected untreated control group. Furthermore, the mycological efficacy of CUN (81.4%) was more than that of the other treated groups (CX, 79.6% and CUS, 74.5%).

6. Conclusion

The blended nanoemulsion of cinnamon oil and usnic acid was found effective in treating skin infections. . CUN demonstrated high antifungal efficacy both *in vitro* and *in vivo*. Therefore, clinical

evaluation of CUN in the topical treatment of candidiasis and dermatophytosis in man are warranted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101062.

Disclosure

The author reports no conflicts of interest in this work.

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