Comparison of in vitro Antifungal Studies of Different Bifonazole Formulations with Marketed Bifonazole Formulation

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ABSTRACT
Clinical efficacy of topical antifungal therapy depends on the drug ability to penetrate into the stratum corneum (SC) and the duration of treatment. The mechanism of action for antifungal activity of the bifonazole (BFZ) is the inhibition of ergo sterol biosynthesis, an essential step in the membrane formation of fungal cells. BFZ is indicated in the treatment of superficial fungal infections of the skin such as dermatophytoses, cutaneous candidiasis and pityriasis versicolor. Thus, topical gel of bifonazole with increased bioavailability will be favorable for the treatment of fungal infections and symptomatic relief. The objective of the present work is to compare the antifungal activity of BFZ gel formulated using two drug delivery system; micro emulsion and micro sponge drug delivery system. Microemulsion was prepared by oil titration method. Quassi emulsion solvent diffusion-evaporation technique was used for the preparation of micro sponges. The formed micro emulsion and micro sponges are then incorporated into gel. *In vitro* antifungal activity of micro sponge based gel and micro emulsion based gel was compared using cup plate method.

Key words: Topical antifungal, micro sponge, micro emulsion, bifonazole, cup-plate method.

INTRODUCTION
Fungal infections: Fungal infections pose a continuous and serious threat to human health and life. These fungal infections in humans can be classified into, a) Allergic reactions to fungal proteins. b) Toxic reactions to toxins present in certain fungi. c) Infections (mycoses). Healthy individuals are susceptible to a host of superficial, cutaneous, subcutaneous and in certain instances, systemic infections that cause a variety of conditions ranging from Athlete's foot and nail infections to severe life-threatening disseminated disease (e.g., histoplasmosis). Fungal infections of the skin and nails form the most numerous and widespread group of all mycoses. The high prevalence of superficial mycotic infections has risen to such a level in the last decades that skin mycoses now affect more than 20–25% of the world's population, making them one of the most frequent forms of infections. The distribution of the dermatomycoses, their aetiological agents and the predominating anatomical infection patterns vary with geographical location and a wide range of environmental and cultural factors. Dermatophytes thrive at surface temperatures of 25–28ºC and infection of human skin is supported by warm and humid conditions. For these reasons, despite regional characteristics and predispositions for dermatophyte infections, the spectrum of dermatophytes is not static. Booming mass tourism, international sports activities and increasing migration mean that less common or forgotten species are being imported and disseminated. Local socio-economic conditions and cultural practices can also influence the prevalence of a particular infection in a given area. For example, tinea pedis (athlete's foot) is more prevalent in developed countries than in emerging economies and is likely to be caused by the anthropophilic germ *T. rubrum*. In poorer countries, scalp infections (tinea capitis) caused by *T. soudanense* or *M. audouinii* aremore prevalent.[1]

Micro sponges: Micro sponges are tiny true sponge like spherical polymeric particles that consists of myriad of interconnecting voids within a non-collapsible structure with large porous surface. The size of these micro sponges can be varied, usually from 5-300µm in diameter depending on the degree of smoothness, or after feel required for the end formulation. A typical micro sponge bead is a 25µm sized sphere which can have up to 250,000 pores and an average internal pore structure equivalent to 10 feet in length with average pore volume of about 1ml/gm. The surface can be varied from 20-500 m^2/gm and

pore volume range from 0.1-0.3 cc/gm. This results in a large reservoir within each micro sponge, which can be loaded with up to its own weight of active agents.[2]

The scanning electron microscopy of the ruptured bead reveals its internal structure as a 'Bag of Marbles', The porosity is due to the interstitial spaces between the 'Marbles'. The pores in the micro particles form a continuous arrangement open to the exterior surface of particles which permits the outward diffusion of the entrapped drug molecule at a controlled rate depending on the pore size. The high degree of polymeric cross-linking in these micro particles results in insoluble, inert particles with satisfactory strength to stand up to the high shear. These porous microspheres can entrap a wide range of active ingredients such as emollients, fragrances, essential oils, sunscreens, anti-infective and anti-inflammatory agents. These entrapped active agents can be incorporated into many product forms, such as creams, lotions, powders, gels and soaps. After the product is applied, the entrapped material are then delivered to the skin in a controlled time release pattern or a pre- programmed manner through the use of several different 'triggers', rubbing or pressing the micro sponge after it has been applied to the skin, elevates skin surface temperature introducing solvents for the entrapped materials such as water, alcohol or even perspiration and controlling the rate of evaporation. Active ingredients entrapped in the porous polymeric structure display altered behavior, with respect to their release, which is restricted and prolonged.[3]

Micro emulsion: It was not until 1943 that the transparent fluids were identified in literature as distinct colloidal systems by Hoar and Schulman and further 15 years was to lapse before the "micro emulsion" was used to describe such systems. The term "micro emulsion" was introduced by Schulman and co-worker in 1959 to describe the clear, fluid systems obtained by titration to the point of clarity of an ordinary milky emulsion (micro emulsion) by the addition of a medium- chain alcohol such as pentanol or hexanol. At the point of clarification no stirring was required and transparent dispersions were spontaneously formed.

Since the introduction of micro emulsion by Hoar and Schulman, it has been found to be suitable for many applications including: dry cleaning fluids, paints, metal recovery, cosmetics, agriculture, beverages, and drug solubilization. Microemulsion are either o/w or w/o type. Main characteristics of micro emulsion are: low viscosity, transparent, thermodynamically stable, optically isotropic, possible incorporation of large quantities of water or oil, ultralow interfacial tension at the oil/water interface, forms spontaneously, act as super solvents of drug solubilizing both hydrophobic and lipophyllic drugs.[4]

In topical formulations, micro emulsions have been proved to increase the cutaneous absorption of both lipophilic and hydrophilic medicaments when compared to conventional vehicles (emulsion, pure oil, aqueous solutions). Antifungal agents e.g. Miconazole, ketoconazole, being lipophilic in nature have been formulated as micro emulsions to impart the advantage like ease of preparation due to spontaneous formation, thermodynamically stability, transparent, and elegant appearance, increased drug loading, enhanced penetration through the biological membranes and increased bioavailability compared to conventional dosage forms.

Bifonazole (BFZ) is a substituted imidazole antifungal agent structurally related to other drugs in this group. It possesses a broad spectrum of activity *in vitro* against dermatophytes, moulds, yeasts, dimorphic fungi and some Gram-positive bacteria. The mechanism of action for antifungal activity of the bifonazole is the inhibition of ergosterol biosynthesis, an essential step in the membrane formation of fungal cells. Bifonazole is indicated in the treatment of superficial fungal infections of the skin such as dermatophytoses, cutaneous candidiasis and pityriasis versicolor. [5]

Thus, topical micro sponge based gel of bifonazole with increased bioavailability by controlling release of drug through micro sponge with prolonged residence time will be much favorable for the treatment of fungal infections and symptomatic relief. Therefore, the aim of the present research work was to formulate micro sponge based gel of bifonazole for topical application.

MATERIALS AND METHODS

Bifonazole was kindly gifted by Amoli Organics Pvt. Ltd. Micro emulsion was prepared by oil titration method and micros pong was prepared using aqueous emulsion diffusion evaporation method. *In- vitro* antifungal was tested using Sabouraud dextrose agar medium, on *Candida albicans* ATCC 10231 fungal

species. Antifungal activity was tested using cup plate method of micro emulsion based gel and microsponge based gel of BFZ.

Preparation of bifonazole loaded micro sponge based gel:
Microsponges were prepared using aqueous emulsion diffusion evaporation method.[6] Gel of BFZ micro sponge was prepared by adding a clear dispersion of Carbopol, prepared in water using moderate agitation (before dispersion Carbopol was presoaked in sufficient quantity of water for 24 h). Exactly weighed BFZ microsponges equivalent to 1% of BFZ were mixed in propylene glycol. Various ingredients viz. parabens, were dissolved in water and added to the drug solvent system. In this mixture pre-soaked carbopol was added by gentle stirring taking into consideration that air bubbles were should not form. Finally volume was made up with water. Triethanolamine was used to neutralize micro sponge based gel. Gel prepared was degassed by ultra-sonication. [7]

Table 1: Composition of bifonazole loaded micro sponge based gel

Ingredients	Quantity (%w/w)
BFZ (entrapped micro sponges, equivalent to)	1
Propylene glycol	40
Carbopol 940	1
Methyl Paraben	0.18
Propyl Paraben	0.02
Triethanol amine	q.s
Water	q.s. 100

Preparation of microemulsion based gel of BFZ
Microemulsion was prepared using oil titration method. Gelling agent was slowly mixed with the formed microemulsion under stirring. Xanthan gum was used as gelling agent in the concentration of 1%.

Table 2: Composition of BFZ microemulsion based gel

Ingredients	Quantity
Oil%	3
Smix %	24
Water %	71
Drug %	1
Xanthan gum %	1

***In vitro* antifungal susceptibility test** [8, 9]
The micro sponge and micro emulsion based gel of BFZ was subjected to an *in vitro* antifungal susceptibility test to determine the antifungal activity against pathogenic fungal organism - *Candida albicans.* Antifungal activity of bifonazole micro sponge and micro emulsion gel was compared.

The *in - vitro* antifungal activity was carried out under the following conditions:

- Technique/Method: Ditch - Plate diffusion technique
- Fungal Species: *Candida albicans* ATCC 10231
- Medium: Sabouraud - Chloramphenicol Agar
- Turbidity Standard: 0.5 McFarland standards
- Incubation Period: 48 hrs
- Incubation Temperature: 37°C
- Test Sample: Optimized micro sponge based gel
- Positive Control: Standard BFZ (Stock Solution)

- Negative Control: Placebo micro sponge/micro emulsion based gel

Specimen: Few isolated colonies of candida albicans of similar colony morphology at least 1 mm in diameter grown for 24-48 hrs. on Sabouraud dextrose agar medium.

Preparation of Slant: 6.5 g of on Sabouraud dextrose agar media was taken and dissolved in 100 ml distilled water in conical flask with the help of heat till the uniform transparent solution was obtained. After heating, medium was transferred in sterile test tube. In each test tube 10-15 ml media was transferred and then sterilized by autoclave at 15 lbs pressure (121^0C) for 15 min. After sterilization, test tubes were placed in slants position and cooled at room temperature. Slants were then inoculated with one loop full of fungi (*Candida albicans* ATCC 10231) and incubated for 24 hr, slants were observed for fungal growth.

Preparation of 0.5 McFarland standards: McFarland standard were prepared by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dehydrate with 9.95ml of 1% sulfuric acid.

Preparation and standardization of fungal suspension: Saline was prepared and transferred in test tubes. Saline was then sterilized by autoclave at 15 lbs pressure (121^o C) for 15min. Test tubes were cooled to room temperature. Loop full culture of fungi from prepared slant was transferred into saline to obtain fungal suspension. This suspension was standardized by comparing with McFarland standard for % transmittance.

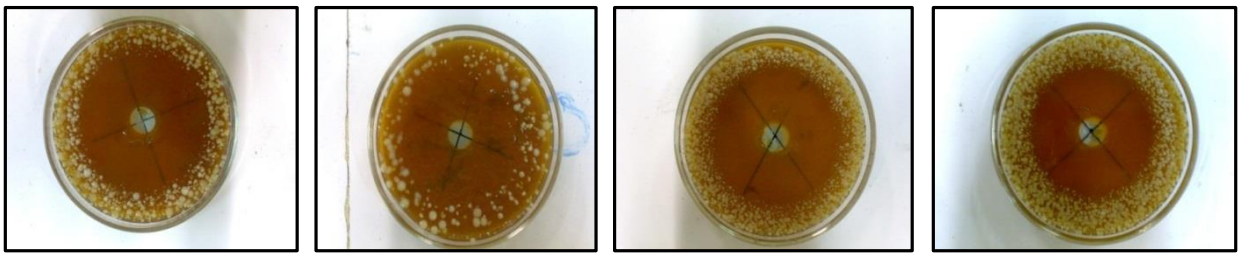
Cup plate method: Cup plate method was used for testing of anti fungal activity of formulated gel. Sabouraud dextrose agar media was prepared in conical flask as mentioned above and sterilized by autoclaving at 15 lbs pressure (121^oC) for 15 min. After sterilization, media was poured into sterile Petri plates in sterile environment and allowed to solidify. On the surface of solidified media, fungal suspension was spread with the help of sterile glass slide. Presterilized stainless steel borer was used to bore the well in the media. 100mg of micro emulsion based gel, micro sponge based gel and 0.1 ml of BFZ standard solution (10 mg/ml of drug in DSMO) was placed in each bore well. Positive control and negative control plates were also maintained to validate the method. Plate seeded with culture was used as positive control to confirm the suitability of the medium to support the growth of the microorganism under the specific conditions. Plate with sterile medium was used as negative control to confirm the sterile condition. Petri plates were then incubated at 37^0Cfor 24 hr. Zone of inhibition observed around the well was measured by recording diameter with the help of measuring scale. The entire operation was carried out under aseptic conditions.

RESULTS AND DISCUSSION

The results of the *in vitro* antifungal susceptibility test are given in the Table 3. Zone of inhibition was measured in cm.

Table 3: Results of *in vitro* antifungal susceptibility test (n = 3)

S. No.	Micro sponge based gel (Test)	Micro emulsion based gel(Test)	Standard Bifonazole (Positive Control)	Marketed Formulation
Zone of Inhibition ± SD (cm)	4.10±0.07	4.3 ±0.45	3.9±0.00	3.5±0.45



A. Micro sponge based gel | B. Micro emulsion based gel | C. Standard | D. Marketed

Fig. 1: Photographs showing zones of inhibitions

It was evident from the results that the micro sponge based gel of Bifonazole and micro emulsion based gel of Bifonazole showed an optimum antifungal activity against *Candida albicans.* It has been seen that the micro sponge based gel of BFZ and micro emulsion based gel of bifonazole had shown higher zone of inhibition than the standard bifonazole and marketed formulation. So it was concluded that the micro sponge based gel of bifonazole and micro emulsion based gel of Bifonazole had shown a significantly higher antifungal activity than standard (positive control) and Marketed formulation as can be seen from the diameter of inhibition zone in Table 3. Photographs of the clearly observable zones of inhibition of micro sponge based gel of bifonazole, micro emulsion based gel of bifonazole**,** standard bifonazole, and marketed formulation are shown in the Fig. 1.

CONCLUSION

From above discussion it was concluded that the micro sponge based gel of bifonazole and micro emulsion based gel of bifonazole had shown a significantly higher antifungal activity than standard (positive control) and Marketed formulation as can be seen from the diameter of inhibition zone with good in vitro antifungal activity. Gel based bifonazole micro sponge and micro emulsion can be used for effective topical drug delivery.

ACKNOWLEDGMENT

The authors are grateful to the Amoli Organics Pvt. Ltd. India for providing gift sample of bifonazole.

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