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Research Article

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Control of Oral Candidiasis using Clotrimazole and Quercitin Impregnated Mucoadhesive Drug Delivery Films

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ABSTRACT

Oral candidiasis is an opportunistic infection seen in debilitated and immunocompromised patients. In the current study, an innovative drug delivery system comprising of mucoadhesive buccal films to reduce the drug dosing frequencyand improvebioavailability was employed. An unconventional preparation of quercetin and theantimycoticdrug, clotrimazole was prepared and impregnated in buccal mucosal films prepared by the casting method. The physical properties of the filmswere studied. Minimum inhibitory concentration of quercetin and clotrimazole was determined by the broth macrodilution method and was found to be 50 mg/ml and 15 µg/ml respectively. The combined action of the two drugs was assessed by the checkerboard assay. The fractional inhibitory concentration index obtained was 0.5, which indicated synergistic action between the two compounds. Fourier transformed infrared spectroscopy (FTIR) studies revealed no change in the properties of flavonoid quercetin and drug clotrimazole post impregnation in mucoadhesive patches. Ex vivo experiments were performed using goat skin and significant reduction of 97.7 percent was observed in the viability of Candida albicans using a combination of the two antimicrobial compounds. This current study helps to establish a novel treatment regimen for oral candidiasis using an innovative drug delivery system which can provide better patient compliance and therapeutic efficacy.

Keywords: *Candida;* Quercetin; Clotrimazole; Mucoadhesive; Drug delivery

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INTRODUCTION

Oral thrush is an opportunistic infection caused by *Candida albicans*. Although *C. albicans* is a commensalist fungus, comprising the normal flora of the mouth, it frequently causes infection in people wearing dentures and in bottle–fed infants. Severe oropharyngeal candidiasis is reported with increasing frequency in immunosuppressed patients and in individuals receiving anti-cancer radiotherapy [1]. Oral candidiasis is very common among HIV patients and with lower CD4 cell counts and recurrent infections; it can signify HIV disease progression. Oral candidiasis is a major cause of morbidity and mortality in cancer patients. Currently available drugs for the treatment of oral thrush include amphotericin B,nystatin, miconazole, fluconazoleand clotrimazole. These antimycotic drugs are usually administered in the form of tablets, ointments or suspensions. Their side effects include nausea, vomiting as well as renal, cardiovascular and gastrointestinal effects.

Clotrimazoleis administered in the form of a gel or tablet, with repeat doses around three to five times a day. Topical and oral clotrimazole can be used in both adult and pediatric populations. It has been reported that that for dermatophytosis,miconazole is more effective than clotrimazole but for candidiasis, clotrimazole is more effective and faster than miconazole [2] and henceconsidered as the drug of choice for thetreatment of oral thrush.

Currently available treatments for oral thrush are difficult to administer to patients in intensive care units and to the terminally ill. Chronic antimycotic therapy in high doses is undesirable for treatment of oral infections due to potential side effects. Therefore, to minimize these side effects and prevent the ominous risk of drug resistance, topical therapy should be considered as the first line measure for the treatment of oral and pharyngeal candidiasis.

Additionally, patients might face difficulties in swallowing drugs and time to time administration of drugs, even in the form of a gel or an ointment, is difficult to monitor.

Buccal drug delivery is an innovative drug delivery system which releases the drug to buccal mucosa by avoiding first pass metabolism in the liver and pre systemic elimination in the gastrointestinal tract. The buccal mucosa is relatively permeable and provides affluent blood supply and permits prolonged retention of a dosage form,especially with the use of mucoadhesive polymers without much interference in processes such as mastication unlike the sublingual route. Various bioadhesive mucosal dosage forms have been developed, which include adhesive tablets,gels,ointments, patches and more recently films. Buccal films can circumvent the relatively short residence time of oral gels on the mucosa, which is easily washed away and removed by the saliva [3].

In order to avoid the potential side effects associated with high dosages of antifungal agents and to reduce the risk of developing drug resistance; novel therapeutic combinations can be developed by using a natural antimicrobial substance along with an antimycotic drug. Flavonoids are an example of such naturally occurring compounds. They are biologically active, water-soluble and obtained from plants. They include plant pigments ranging in color from yellow to red to blue and occur especially in fruits, vegetables, and herbs. Quercetin is a flavonoid which is an antioxidant and can neutralize free radicals and believed to possess an anti-inflammatory and antihistamine effect.

In the current investigation, a novel preparation of quercitin along with clotrimazole was made and the synergistic activity of these compounds tested against *C.albicans*. This therapeutic preparation was then impregnated into mucosadhesive buccal films and the unique drug delivery system tested *ex vivo*. Hence, the present study not only explores a new therapeutic combination of a drug with a nutraceutical, but also attempts to establish a novel technique of drug delivery for critically ill patients suffering from oral thrush.

MATERIALS AND METHODS

Culture and Growth Conditions

Culture of *Candida albicans* was obtained from a local hospital. The antifungal agent, clotrimazole used in this study was procured from Intas Pharmaceuticals. Pvt. Ltd. (India). Quercetin was purchased from Natroland sodium alginate from S.D.Fine Chemicals, Mumbai. All other chemicals used were of analytical grade.

Minimum Inhibitory Concentration (MIC) of Clotrimazole

Minimum inhibitory concentration of the antifungal drug clotrimazole was determined by the broth macro-dilution method. To determine the MIC of clotrimazole, a stock solution containing 100 micrograms/ml ofclotrimazole was prepared. Briefly, 24 hr old culture of *Candida albicans* adjusted to 0.1 OD was added to Saboraud's broth supplemented with clotrimazole at different concentrations ranging from 1-100 microgram/ml. Positive and negative controls were maintained. Tubes were incubated at 37°C for 24 hrs. The MIC was recorded as the lowest concentration that showed complete inhibition of visible growth.

Minimum Inhibitory Concentration of Quercetin

Minimum inhibitory concentration of quercetin was determined by the broth macrodilution method. To determine the MIC of quercetin, a stock solution containing 500 milligrams of quercetin was prepared. Briefly, 24 hr old culture of *Candida albicans* adjusted to 0.1 OD was added to Saboraud's broth supplemented with quercetin at different concentrations ranging from 50-500 mg/ml. The tubes were then incubated at 37°C for 24 hrs. The MIC was recorded as the lowest that showed complete inhibition of visible growth.

Determination of Minimum Inhibitory Concentration of Clotrimazole and Quercetin in Combination by Checkerboard Assay

In order to test the synergy of thetwo antimicrobial compounds, clotrimazole and quercetin, a checkerboard assay was performed. Serial two fold dilutions of each drug was prepared in a microtitre plate, to obtain concentrations from one sixteenth to at least double the MIC. Clotrimazolewas serially dilutedalong the ordinate while quercetin is dilutedalong the abscissa. The resulting checkerboard yielded every combination of the two test drugs. Culture and growth medium was added in each well and the plate incubated at 37°C for 24 hrs. To determine whether the two compounds exhibited synergy, the FIC (fractional inhibitory concentration) Index was determined.

FIC index was calculated by the following formula: -

$$FIC\ index = \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone}$$

An FIC index of less than 0.5 is considered evidence for synergism; an index of greater than 2.0 is an evidence for antagonism.

Preparation of Mucosadhesive Buccal Films

The mucosadhesive films were prepared by casting method (Figure 1). Sodium alginate 2% w/w was dissolved in acetic acid solution 1% w/v at room temperature. The mixture was stirred using magnetic stirrer for 1 hr until viscous gel like solution was formed. Clotrimazole and quercetin were added individually and in combination at their MIC concentrations. The viscous solution was left overnight at room temperature to ensure clear bubble free gel. Drug solubilizing agents like propylene glycol, polyethylene glycol, Tween 80 and oleic acid were mixed with the prepared gels at different concentrations of the total dry weight of sodium alginate. The bubble free liquid was then spread on a clean, dry glass plateand dried at 50°C for 24 hrs. The dry films were then peeled off, cut into circles of 13 mm diameter, packed in aluminum foil and stored at room temperature.

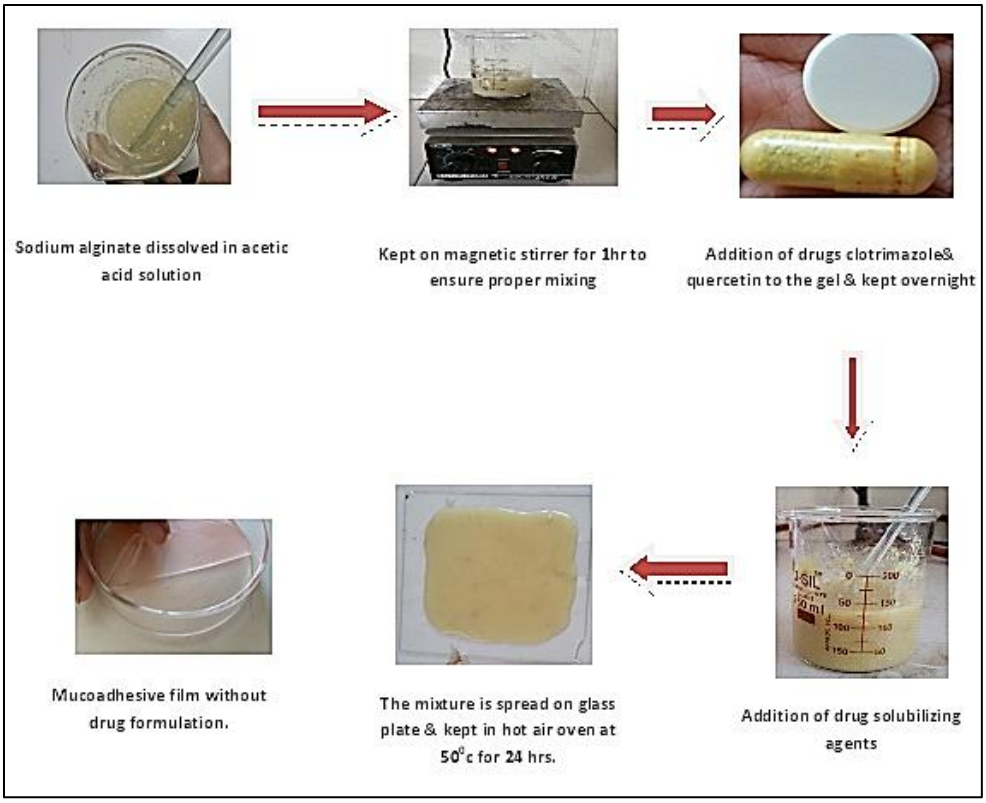

Figure 1: Preparation of drug impregnated mucoadhesive films

Evaluation of Mucoadhesive Buccal Films using Physical Tests

Film weight and thickness:

The weight of each prepared film was measured using a digital balance. The thickness of each film was measured at different points in the film and average was calculated.

Folding endurance:

Folding endurance of the films was determined by repeated folding one film at the same place till it broke. The number of times the film could be folded at the same place without breaking or cracking gave the value of folding endurance.

Surface pH:
Surface pH of the films was determined by allowing three films to swell for 2 hrs on an agar plate surface. pH was measured by means of pH paper positioned on the surface of the swollen film and the average value calculated.

Swelling index:
The prepared films were weighed individually and placed on the surface of an agar plate kept in an incubator and maintained at 37 ± 0.2°C. The film was allowed to swell. An increase in weight of the film was noted at regular time intervals and weight was determined. The percent swelling was calculated using the following equation:

$$\%\ swelling = (X_T - \frac{X_0}{X_0}) \times 100$$

Where, $X_{T=}$ weight of the swollen film after time t; $X_{o=}$ initial film weight at zero time

FTIR (Fourier Transformed Infrared Spectroscopy) Analysis
FTIR analysis was carried out at to ascertain that the drugs had not been inactivated after incorporation into the mucoadhesive film.

***Ex vivo* Experiment to Check Activity of Drug Impregnated Bio Adhesive Films**
To evaluate the gradual release of clotrimazole and quercetin impregnated in the buccalmucosadhesive patch, 0.1 ml of *C.albicans* culture (adjusted to 0.01 O.D) was added to several small pieces of goat buccal mucosal skin (1 cm × 1 cm) and allowed to adhere for 24 hrs. These pieces were then washed with phosphate buffered saline and treated with buccalmucosadhesive patches in for 24 hrs. The tests included goat buccal mucosa treated with buccal mucoadhesive films impregnated with the antifungal drug clotrimazole and quercetin individually and in combination. Goat buccal mucosa along with culture only, was maintained as control. Viable counts of *C. albicans* were determined by suspending and vortexing the mucosal pieces in sterile saline, followed by plating on sterile *Candida* differential agar plates. Plates were incubated at 37°C for 24 hrs and colonies counted.

RESULTS AND DISCUSSIONS

Nowadays, novel drug delivery systems are being developed to serveas easier and non-invasive modes of drug administration. Methods of drug administration like intravenous or intramuscular injections are invasive and quite painful. In the present study, novel mucoadhesive buccal films were developed for efficient drug delivery to treat candidiasis inchildren, in immunosuppressed patients, patients in ICU and the terminally ill. These patients might have difficulty in swallowing the drug due to their physical conditions andregular and frequent administration of the drug is also difficult to supervise. In order to determine the concentration of the drug which needed to be impregnated in the mucoadhesivepatches, the MIC of the two antimicrobial compounds against *C. albicans* was determined. The MIC of clotrimazoleand quercetin against *Candida albicans* by the macrobroth dilution technique was found to be 15 µg/ml and 50 mg/ml respectively after incubation at 37°C for 48 hrs.
In previous studies, Shafaq Z et al. [4] have reported MIC of <0.015 µg/ml of clotrimazole against *Candida albicans* while Rene et al. [5] reported an MIC of 0.25 µg/ml of clotrimazole against *Candida albicans.* Also, Gao et al. [6] reported an MIC range of 128-512 µg/ml for quercetin against *Candida albicans*. These results are different from the results obtained which might be due to the difference in the strain of *Candida albicans* tested.
The activity of clotrimazole and quercetin in combination with each other was tested by the checkerboard assay and the FIC index found to be 0.5. Since an FIC index 0.5 or less than 0.5 is an indicator of synergism, the results show synergy between the drug clotrimazole and the flavonoid quercetin.
In an earlier study, Gao et al. [6] determined the minimum inhibitory concentrations of fluconazole and quercetin in combination, against *Candida albicans*. They found FIC index to be in the range of 0.188-1. These results are similar to the results obtained, hence both drugsclotrimazole and fluconazole, can act synergistically with quercetin to inhibit *Candida albicans*. Buccalmucoadhesive films prepared by the casting method were homogenous, clear and flexible. The prepared formulations were found to provide an acceptable pH range that is compatible with normal buccalmucosal pH (6.78 ± 0.04) in healthy people. Consequently, these films can be considered nonirritant to the buccal cavity [1]. The prepared mucoadhesive films were of appropriate stability and could suitably be used as a drug delivery system. Physical tests were performed to determine the film weight and thickness, folding endurance, surface pH and swelling index [1]. The results obtained are shown in Table 1.

Table 1: Physical properties of the mucoadhesive film

Physical properties	Results obtained
Film weight	159 mg
Film thickness	0.20 mm
Surface pH	6.7 ± 0.04
Swelling index	0.1029

The prepared film showed good folding endurance as it could be folded up to 300 times without any breakage. Maximum swelling capacity was obtained with films containing propylene glycol 10%. This may be due to the fact that propylene glycol can absorb moisture from environment because of its humectant ability resulting in increase of film moisture uptake. The degree of swelling of the bio adhesive polymer is an important factor affecting film bioadhesion. The faster the swelling of the polymer is, the faster the initiation of drug diffusion and formation of adhesive bonds resulting in faster initiation of bioadhesion. Hence, the film showed good physical properties and is suitable for being used as a mucoadhesive polymer. Previously, Bazigha K et al. reported the preparation of buccal mucoadhesive films with the pH of 6.78 ± 0.04 and swelling index of 10 [1]. These values are similar to our results. FTIR analysis was carried out post impregnation of clotrimazole and quercetin in the buccal mucoadhesive film. Data obtained is shown in Figures 2-4. FTIR analysis relies on the fact that most molecules absorb light in the infrared region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency range are measured as wave numbers typically over the range 4000-600 cm^{-1}. The background emission spectrum of the IR source is first recorded, followed by the emission spectrum of the IR source with the sample in place. The ratio of the sample spectrum to the background spectrum is directly related to the sample's absorption spectrum. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample. FTIR is particularly useful for identification of organic molecular groups and compounds due to a range of functional groups, side chains and cross-links involved, all of which have characteristic vibrational frequencies in the infrared range. The wavenumbers obtained for clotrimazole and quercetin after FTIR analysis were corresponding to those obtained post their impregnation into the buccal mucoadhesive film. Hence, the results obtained from FTIR analysis revealed that both the drugs were active, post impregnation into the buccal mucoadhesive film.

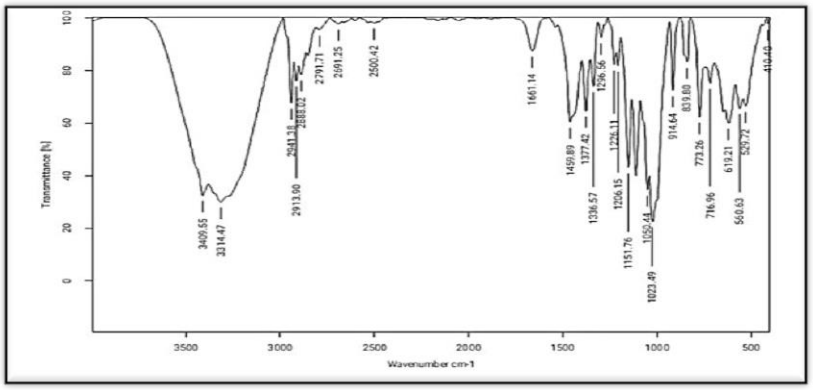

Figure 2: FTIR spectra obtained for clotrimazole

Results obtained on performing the *ex vivo* test to evaluate the gradual release of clotrimazole and quercetin impregnated in the buccalmucosadhesive patch are shown in Table 2. The data obtained revealed a significant reduction in the percentage of viable cells *Candida albicans* when treated with buccal mucoadhesive film impregnated with a combination of clotrimazole and quercetin as compared to when treated with buccal mucoadhesive films impregnated with clotrimazole and quercetin individually.

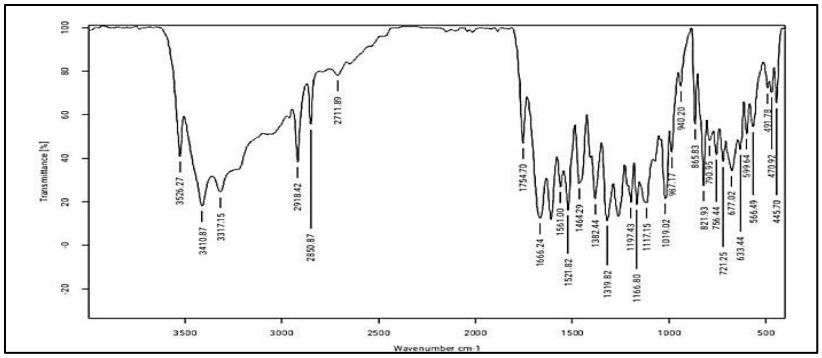

Figure 3: FTIR spectra obtained for quercetin

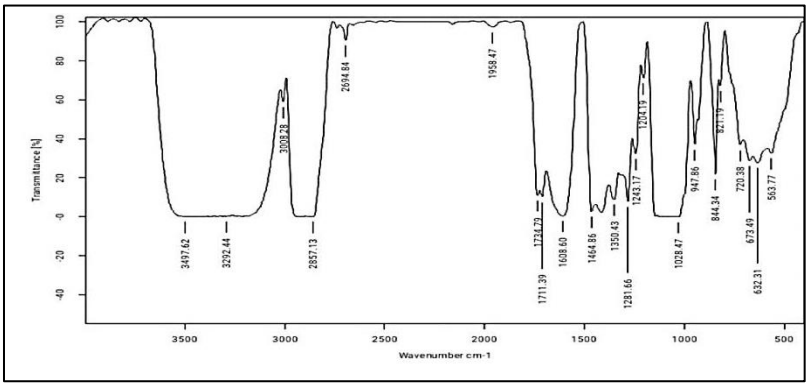

Figure 4: FTIR spectra obtained after impregnation of clotrimazole and quercetin in mucoadhesive film

Table 2: Percentage reduction in the viable count of *Candida albicans* after treating with mucoadhesive film impregnated with the drugs

Drug used	Percent reduction (%)
Clotrimazole	88.04
Quercetin	83.25
Clotrimazole + quercetin	97.7
Control	100

CONCLUSION

The prepared buccalmucoadhesive patches can be used as an efficient mode of drug delivery for the treatment of oral candidiasis. They can act as a suitable alternative over other invasive modes of drug delivery. By sustained drug release, the frequent administration of drug can be avoided, thereby increasing patient compliance. Since emerging drug resistance is a major problem in treating all types of infections, combination of clotrimazole with a dietary flavonoid like quercetin would help in reducing the concentration of the drug needed for treatment and hence control the development of drug resistance and harmful side-effects.

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