International Journal of Multidisciplinary Research and Development
Online ISSN: 2349-4182, Print ISSN: 2349-5979, Impact Factor: RJIF 5.72
www.allsubjectjournal.com
Volume 4; Issue 6; June 2017; Page No. 177-179

Anti-biofilm activity of clotrimazole in combination with quercitin against *Candida albicans*

***Suparna Dugal, Farheen Rashid**

Dept. of Microbiology, Sophia College, University of Mumbai, Maharashtra, India.

Abstract
Candidiasis is an opportunistic infection seen in debilitated and immunocompromised patients. It has been well documented that *Candida* biofilms once formed, exhibit high resistance to antimycotic drugs. Additionally, high doses of these drugs over a prolonged period of time can induce serious side-effects. Hence, the present study was designed to explore a novel approach to control colonization by these yeasts. *C.albicans* biofilms were exposed to the antimycotic drug, clotrimazole alone and in combination with the flavanoid, quercitin. Additional tests were performed to check the antimicrobial activity of quercitin alone. The reduction in biofilm formation was verified by the viable count technique. Exopolysaccharide formation, indicative of the amount of biofilm formed, was determined by the Anthrone method. Biofilms formed on glass slides were observed by Allison and Sutherland staining. Minimum inhibitory concentration of quercetin and clotrimazole was determined by the broth macrodilution method and was found to be 50 mg/ml and 15 ug/ml respectively. Both antimicrobial compounds when used in combination caused reduction in the viable count and exopolysaccharide formation by *Candida*. These reductions were more than those induced by clotrimazole and quercetin separately. Thus quercitin, a nutraceutical, could represent an important adjunct to conventional antifungal therapy, employing clotrimazole.

Keywords: *Candida*, quercetin, clotrimazole, biofilm, exoploysaccharide

1. Introduction
Candida infections range from non-life threatening superficial mucocutaneous disorders to invasive disseminated disease involving multiple organs. *Candida* species present both as commensalistic and opportunistic pathogens of the oral cavity. With the increase in number of AIDS cases, there is a resurgence of less common forms of oral *Candida* infections. Oral thrush is a major cause of morbidity and mortality in cancer patients. Candidal cells grow slowly to form a biofilm on the tongue or inner cheeks. It is well known that *C.albicans* uses cell to cell chatting or quorum sensing signaling for biofilm formation and to produce a wide array of virulence factors. These virulence factors are primary sources of drug resistance and development of invasive infections because they are difficult or impossible to eradicate with conventional anti candidal agents. Hence, it seems logical to target quorum sensing signaling to overcome therapy resistance. Treatment with systemic antifungal agents such as amphotericin B or fluconazole in high doses is undesirable for treatment of oral infections or infections nvolving yeast biofilms, due to potential side effects. Therefore to minimize these side effects and the ominous risk of drug resistance, novel therapeutic measures should be considered.
Efforts to regulate quorum sensing have enabled the identification of bioactive molecules produced by plants [1]. Recently several studies have shown that dietary phytochemicals inhibit quorum sensing dependent biofilm formation in various human pathogenic bacteria [2-8]
Flavonoids are a large group of phytochemicals typically biologically active, water-soluble compounds that include pigments ranging in color from yellow, red or blue and occur especially in fruits, vegetables, and herbs such as grapes, citrus fruits, peppers, and dill. Quercetin is a flavonoid derived from citrus fruits ubiquitously used in dietary supplements and is known for its antioxidant property. Quercetin is reported to be effective against *Candida albicans* because it can inhibit biofilm formation by regulating quorum sensing.
In the current study the use of a dietary phytochemical like quercitin has been explored in combination with clotrimazole, a common antimycotic drug in order to control *Candida* biofilms.

2. Materials and Methods
2.1. 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 Natrol.

2.2. 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 of clotrimazole 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 ^{0} C for 24 hrs. MIC was recorded as the lowest concentration that showed complete inhibition of visible growth.

2.3. 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 ^{0}c for 24 hrs. MIC was recorded as the lowest that showed complete inhibition of visible growth.

2.4. Susceptibility of *Candida* biofilm to clotrimazole and quercetin

Sterile coplin jars containing Saboraud's broth were used. Sterile slides were placed in these jars. The density of *Candida albicans* culture was adjusted to 0.1O.D. 'Test' jars contained broth along with the drugs clotrimazole and quercetin at a concentration which was four times the MIC of these drugs respectively and culture of *Candida albicans*. These jars were incubated at 37 ^{o}C for 48 hrs for adequate biofilm formation. 'Control' jars did not contain any drug. After incubation slides were washed with phosphate buffer saline (PBS) to remove non-adherent cells. Adherent cells were scraped off in a tube containing PBS and plated on Saboraud's agar to determine the viable count of the cells.

2.5. Staining of exopolysaccharide using Alison Sutherland method

Candida biofilms similarly formed on glass slides were covered with 10 millimolar cetylpyridinium chloride and air dried for 20 minutes. The slides were then heat fixed and stained for 15 minutes with saturated Congo red solution and 10 % v/v Tween 80, washed with water, air dried and observed.

2.6. Determination of exopolysaccharide content by Anthrone test

The growth scraped off from the slide was used to perform Anthrone test for determination of exopolysaccharide content. For preparation Anthrone reagent, 200 mg of Anthrone reagent was added to 100 ml of concentrated sulphuric acid. Standard tubes were prepared using glucose solution ranging in concentration from 0.2 to 1mg/ml. Distilled water was used as the diluent. 4ml Anthrone reagent was added to all tubes and heated in a boiling water bath for 10 minutes. The optical density of all the tubes was read at 600nm.

All experiments were performed in triplicate and repeated three times.

3. Results and Discussion

Oral thrush is seen commonly in bottle-fed infants and in the elderly people who use dentures. Also any disequilibrium of oral microbiota favours the growth of this opportunistic yeast and they are increasingly seen in tumour patients receiving chemotherapy or radiotherapy. Oral thrush can be painful and can bleed on scraping the growth. In certain cases, the infection can spread into the esophagus and cause pain or difficulty swallowing. In addition to oral thrush, *Candida* can also infect other parts of the body and cause intestinal candidiasis, vaginal thrush, pulmonary candidiasis, cutaneous candidiasis and systemic candidiasis.

In the current investigation, MIC of clotrimazole and quercetin was determined by the broth macrodilution method. The MIC of clotrimazole was found to be 15 ug/ml and that of quercetin was found to be 50 mg/ml. the effect of combined chemotherapy of clotrimazole and quercitin on controlling *Candida* biofilms was studied by allowing biofilm formation on glass slides kept in coplin jar and supplemented with growth medium and the drugs of interest. After 48 hours of exposure to the combined action of the drugs, the viable count of biofilm yeast cells was determined. The mean results are depicted in Table 1.

Table 1: Study of the effect of clotrimazole and quercitin alone and in combination with each other on *C. albicans* biofilm. Data obtained after performing viable count of cells present within the biofilm and estimating the amount of exopolysaccharide (EPS) formed by Anthrone test.

	Biofilm-Viable Count	**EPS**
Control	12.9 x 10^{16} cfu/ml	0.85 mg/ml
Quercetin	2.32 x 10^{14} cfu/ml	0.4mg/ml
Clotrimazole	4.66 x 10^{8} cfu/ml	0.3 mg/ml
Combination Clotrimazole+ Quercetin	1.39 x 10^{5} cfu/ml	0.2 mg/ml

Biofilms play an important role in many chronic microbial infections. Production of an extracellular mixture of sugar polymers called exopolysaccharide (EPS) is characteristic and critical for biofilm formation. Exopolysaccharide is a key component of the biofilm matrix in many biofilm forming bacteria and is composed of various sugar polymers. It has an important role in immune evasion and tolerance toward antibacterial agents. By far the most known EPS molecules are neutral or polyanionic. Enzymatic alteration of EPS is believed to significantly change its physico chemical properties and thus the biofilm structure [9]. Significant reduction in EPS content was observed after treating the biofilms with clotrimazole and quercetin as compared to control.

Previously, Gao *et al* [10] reported that quercetin in combination with fluconazole could significantly inhibit the initial adherence and biofilm formation in *Candida albicans* ($p<0.05$), while the effects of quercetin and fluconazole alone on the initial adherence were negligible. These results correspond to the results obtained in the present study. There was a significant reduction in biofilm mass which was evident by the reduction in the viability of *Candida albicans* when a combination of clotrimazole and quercetin was used as compared to the individual effect.

The significant reduction in the EPS formed on glass slides when treated with a combination of clotrimazole and quercetin was evident after staining the slides by Alison Sutherland method [Figure 1]. Since greater the activity of the drug, lesser the EPS content of biofilms, it indicates that a combination of clotrimazole and quercetin can be used as an effective method to prevent infections due to *Candida* biofilm formation.

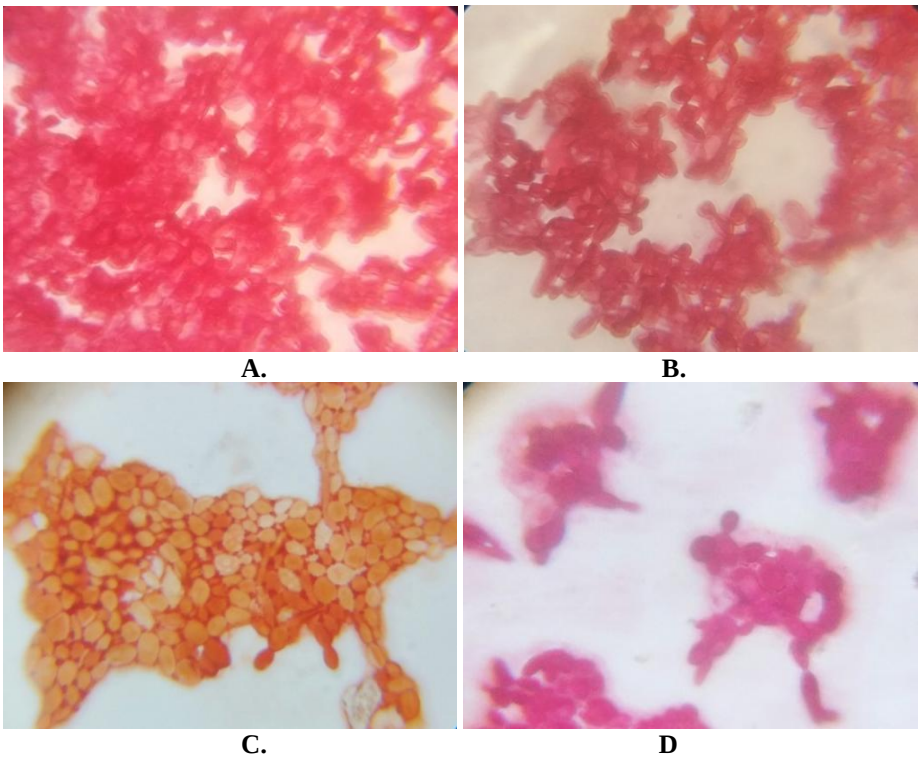

Fig 1: Exopolysaccharide Staining of *Candida* biofilm (a) control (b) after treating with clotrimazole (c) after treating with quercetin (d) after treating with a combination of clotrimazole and quercetin.

4. Conclusion

The antimycotic drug, clotrimazole and quercetin were capable of inhibiting both planktonic and biofilm forms of *C.albicans* Clotrimazole and quercetin showed significant reduction in biofilm formation when used in combination, as compared to them used seperately. This was evident from the reduction in viable count of yeast cells as well by the Anthrone test and Alison and Sutherland staining.

5. References

1. Cowen LE. The Evolution of Fungal Drug Resistance: modulating the trajectory from genotype to phenotype. Nat Rev Microbiol. 2008; 6:187-198.
2. Borges A, Serra S, Cristina Abreu A, Saavedra MJ, Salgado A, Simoes M. Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and *in vitro* cytotoxicity. Biofouling. 2014; 30:183-95.
3. Kalia VC. Quorum sensing inhibitors: an overview. Biotechnol Adv. 2013; 31:224-245.
4. Singh BN, Singh HB, Singh A, Singh BR, Mishra A, Nautiyal CS. Lagerstroemia speciosa fruit extract modulates quorum sensing-controlled virulence factor production and biofilm formation in Pseudomonas aeruginosa. Microbiology. 2012; 158:529-538.
5. Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G *et al*. Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of 544 Moringaoleifera. Food ChemToxicol. 2009; 47:1109-1116.
6. Singh BN, Singh BR, Singh RL, Prakash D, Sarma BK, Singh HB. Antioxidant and 546 anti-quorum sensing activities of green pod of Acacia nilotica L. Food Chem Toxicol 2009; 47:778-547, 786.
7. Adonizio A, Kong KF, Mathee K. Inhibition of quorum sensing-controlled virulence factor production in Pseudomonas aeruginosa by south Florida plant extracts. Antimicrob Agents Ch. 2008; 52:198-203.
8. Adonizio A, Dawlaty J, Ausubel FM, Clardy J, Mathee K. Ellagitannins from Conocarpus erectus exhibit anti-quorum sensing activity against Pseudomonas aeruginosa. Planta Med. 2008; 74:1035-1035.
9. Vuong C, Kocianova S, Voyich JM, Yao Y, Fischert ER, DeLeo FR *et al*. A crucial role for exopoysaccharide modification in bacterial biofilm formation, immune evasion and virulence. J Biol Chem. 2004; 279(52):54881-6.
10. Gao M, Wang H, Zhu L. Quercetin Assists Fluconazole to Inhibit Biofilm formation of Fluconazole Resistant *Candida albicans* in *In Vitro* & *In Vivo* Antifungal Management of Candidiasis. Cellular Physiology and Biochemistry. 2016; 40(3-4):727-742.