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Effect of Eugenol in presence and absence of nutrients (glucose and Xylose) on H^+ATPase of *C. albicans.*

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Abstract

Candida albicans is an Opportunistic human pathogen causes both superficial and life threatening mycoses fatal for immuno comprised patients. Many essential oils of spices possess antimycotic properties. The electrochemical gradient generated by PM-H^+ATPase of *C. albicans* drives nutrient transport. H^+ ATPase is associated with yeast to hyphal transition, later form being more pathogenic. It is thus important to identify spice extracts which apart from having low MIC also have a profound effect on H^+ ATPase. With this in view we have investigated Eugenol alone and along with nutrients (xylose and glucose) on H^+ ATPase functioning through H^+ extrusion measurement. The antifungal activity of Eugenol alone and along with nutrients (xylose and glucose)was investigated by studying their effect on PM-ATPase mediated H^+-Extrusion activity (H^+ efflux) and growth of *C. albicans* (Disc diffusion and MIC).Eugenol inhibited the H^+ ATPase as observed by H^+ efflux monitored for 30 min. The oil has clear inhibitory effect on H^+ efflux in the concentration range of 500µg/ml to 2000µg/ml. Eugenol has an inhibitory effect on growth and H^+-Extrusion of H^+ ATPase of *C. albicans*. Both glucose and xylose along with Eugenol have inhibitory effect on Candida growth but no such effect has been observed on H^+-ATPase pump of the fungus. Eugenol has a potential to be exploited as future antifungal drug to target plasma membrane H^+ ATPase of *C. albicans* and other pathogenic microbes.

Keywords: H+ATPase, MIC and C. albicans.

Introduction

The dimorphic yeast *Candida albicans* is an obligate associate of warm-blooded animals. It is frequently encountered as a harmless commensal of digestive system and vaginal tract and less frequently as an opportunistic pathogen. Mycelial form of *Candida albicans* is considered more invasive, difficult to phagocytose and therefore more pathogenic [1]. In addition to its dimorphism, *C. albicans* possess well-organized phenotypic switching system that probably helps the pathogen to evade the host immune defense [2].

Patients that become severely immunocompromised because of underlying diseases such as leukemia, acquired immunodeficiency syndrome or patients who undergo cancer therapy, organ transplantation are particularly susceptible to opportunistic fungal infections [3]. A number of antifungal agents are available for the treatment of candidal infection [4]

Recent studies have indicated *C. albicans* resitance to azoles or hepatotoxicity and nephrotoxicity linked to polyene use, particularly amphotericin B [5].

The search for new molecular targets for antifungals has generated considerable research. Na, K-ATPase and H, K-ATPase are well established molecular targets for several clinically important therapeutics [6; 7]. Fungal plasma membrane H^+ ATPase has well defined properties that make it a highly desirable target. It plays a critical role in fungal physiology. It is essential for generating and maintaining an electrochemical gradient that is required for nutrient uptake. PM-ATPase is also associated with regulation of intracellular pH, dimorphism and pathogenicity of the fungus [8]. Several reports suggest H^+ ATPase to be a desirable antifungal target [9-13].

Spices and herbs have now long been recognized for their medicinal values. Essential oils have been used as home remedies for the treatment of vaginal candidiasis [14]. Eugenol, a phenolic compound is the main component of clove (Eugenia caryophillis) oil the compound are present in essential oils of

many plants and are proved to be active against many pathogenic bacteria, fungi and viruses [15]. There is a dearth of information on the effects of these bioactive compounds on *C. albicans*.
Also there are no reports of using these compounds as inhibitory agents against PM-ATPase of *C. albicans*. Therefore, the present study was undertaken to evaluate the efficacy of eugenol as anti-*Candida* agent targeting its plasma membrane ATPase.

Materials and Methods

Candida albicans 10261 strain was collected from Enzyme Kinetics Lab, dept of Biosciences JMI New Delhi. Cells were grown in yeast extract, peptone and dextrose (1:2:2) medium.
To prepare the yeast culture (YEPD) medium, yeast extracts peptones and dextrose are mixed in the ratio 1:2:2. For preparing solid agar medium (Nutrient agar 28g/L) was also added to the YEPD medium. YEPD Agar plate was streaked and streaking was done in the laminar flow to prevent dust and microbe contamination and incubated at 30°C for 24 hours. It was covered with aluminum foil and was kept inverted in the fridge the culture was considered as stock culture.
A loop full from the YEPD agar plate was introduced into 25 ml of autoclaved medium. This procedure was done in laminar flow. The medium was kept in incubated shaker at 150 r.p.m. till the culture reaches the stationary phase.

Above prepared culture was considered as primary culture.

Cells from primary culture was re-inoculated into a fresh YEPD medium and grown for 8-10 hrs i.e. up to mid log phase (secondary culture). The cells for experimental purpose were withdrawn in mid log phase. Mid log phase cells harvested from YEPD medium were washed thrice with distilled water and routinely 100 mg cells were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. 100 mg cells were taken with 0.1 M KCl and 0.1 $CaCl_2$ mM and different concentration of Eugenol. The solution was adjusted to pH 7 and pH was monitored for 30-minutes, recording noted after every one minute.

Concentrations:

Proton extrusion measurement with eugenol

Group1: contains cells only (control)
Group2: contains 500µg/ml eugenol
Group3: contains 625ml eugenol
Group4: contains 1 mg/ml eugenol
Group5: contains 2 mg/ml eugenol

Proton extrusion measurement with eugenol & glucose

Group1: contains cells only (control)
Group2: contains 500µg/ml eugenol
Group3: contains 625ml eugenol
Group4: contains 1 mg/ml eugenol
Group5: contains 2 mg/ml eugenol

Proton extrusion measurement with eugenol & xylose

Group1: contains cells only (control)
Group2: contains 500µg/ml eugenol
Group3: contains 625ml eugenol
Group4: contains 1 mg/ml eugenol
Group5: contains 2 mg/ml eugenol

Results and Discussion

Natural plant products have been used since antiquity and their use is increasing nowadays. Some essential oils are known to have various health benefit properties. The antimicrobial and antifungal effect of plant essential oils has been described in several studies [5, 16, 17] Antifungal [18], antibacterial [19], and antiparasitic properties of cinnamon have been repoted earlier. It has been found to be active against *Candida albicans* [20], *Helicobacter pylori* (the bacteria that causes stomach ulcers), and even head lice. Various studies have shown that oil extracted from cloves (Eugenol) is believed to account for their medicinal effects [21]. Preliminary human evidence confirms this effect in a clinical trial with AIDS patients suffering from oral *Candida* (thrush) infections that improved with topical application of cinnamon oil [20].

Effect of Eugenol on H^+ efflux of *C. albicans*

H^+ efflux is a consequence of H^+ ATPase activity and inhibition of enzyme activity correlates with cessation of cell growth. The effect of Eugenol on H^+ ATPase activity was investigated by following H^+ efflux as a function of time. Figure 1 shows the inhibitory effect of different concentrations (500ug/ml to 2000ug/ml) of Eugenol on H^+ efflux by *Candida albicans*. *Candida* cells were present in 0.1 mM $CaCl_2$ and 5mM KCl. As expected the untreated cells (control) showed acidification starting from pH 7.0. The acidification decreased in the presence of Eugenol. The inhibition of H^+ efflux increased with their increasing concentrations. A low concentration of 500ug/ml showed slightly less inhibition as compared to higher concentrations. All the other concentrations (625ug/ml, 1000ug/ml, and 2000ug/ml) showed a drastic decrease in efflux (Table1). For all concentrations, the pH showed a tendency towards alkalination. The above results show that Eugenol inhibit H^+ efflux and hence H^+ ATPase of *C. albicans*.

Effect of Eugenol + glucose on H^+ efflux of *C. albicans*

Figure 2 shows the inhibitory effect of different concentrations (500ug/ml to 2000ug/ml) of Eugenol + glucose on H^+ efflux by *Candida albicans*. For all concentrations, the pH showed a tendency towards alkalination. The above results show that Eugenol inhibits H^+ efflux and hence H^+ ATPase of *C. albicans* but glucose doesn't show synergy with Eugenol (table2).

Effect of Eugenol + xylose on H^+ efflux of *C. albicans*

Figure 3 shows the inhibitory effect of different concentrations (500ug/ml to 2000ug/ml) of Eugenol + xylose on H^+ efflux by *Candida albicans.* For all concentrations, the pH showed a tendency towards alkalination. The above results show that Eugenol inhibits H^+ efflux and hence H^+ ATPase of *C. albicans* but xylose doesn't show synergy with Eugenol (Table3).

The dimorphic yeast *Candida albicans* is an obligate associate of warm-blooded animals. It is frequently encountered as a harmless commensal of digestive system and vaginal tract and less frequently as an opportunistic pathogen. Mycelial form of *Candida albicans* is considered more invasive, difficult to phagocytose and therefore more pathogenic [1]. In addition to its dimorphism, *C. albicans* possess well-organized phenotypic switching system that probably helps the pathogen to evade the host immune defense [2].

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Recent studies have indicated *C. albicans* resitance to azoles or hepatotoxicity and nephrotoxicity linked to polyene use, particularly amphotericin B [5].

The search for new molecular targets for antifungals has generated considerable research. Na, K-ATPase and H, K-ATPase are well established molecular targets for several clinically important therapeutics [6,7]. Fungal plasma membrane H^+ ATPase has well defined properties that make it a highly desirable target. It plays a critical role in fungal physiology. It is essential for generating and maintaining an electrochemical gradient that is required for nutrient uptake. PM-ATPase is also associated with regulation of intracellular pH, dimorphism and pathogenicity of the fungus [8]. Several reports suggest H^+ ATPase to be a desirable antifungal target [9-13].

Spices and herbs have now long been recognized for their medicinal values. Essential oils have been used as home remedies for the treatment of vaginal candidiasis [14]. Eugenol, a phenolic compound is the main component of clove (Eugenia caryophillis) oil the compound are present in essential oils of many plants and are proved to be active against many pathogenic bacteria, fungi and viruses [15]. There is a dearth of information on the effects of these bioactive compounds on *C. albicans*.

Also there are no reports of using these compounds as inhibitory agents against PM-ATPase of *C. albicans*. Therefore, the present study was undertaken to evaluate the efficacy of eugenol as anti-*Candida*l agent targeting its plasma membrane ATPase.

Conclusion

Eugenol has inhibitory effects on growth and H^+-Extrusion of H^+ ATPase of *C. albicans*. Both glucose and xylose along with Eugenol have inhibitory effect on *Candida* growth but no such effect has been observed on H^+-ATPase pump of the fungus. Therefore Eugenol is a potential candidate to be exploited as future antifungal drug to target plasma membrane H^+ ATPase of *C. albicans* and other pathogenic microbes.

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Table.1- Effect of Eugenol on H^+ efflux of *C. albicans*

Time	control	500ug/ml	625ug/ml	1000ug/ml	2000ug/ml
0	7	7	7	7	7
1	6.76	6.67	6.73	6.93	6.96
2	6.74	6.57	6.63	6.89	6.92
3	6.57	6.5	6.57	6.87	6.87
4	6.51	6.46	6.52	6.85	6.83
5	6.46	6.42	6.48	6.81	6.79
6	6.43	6.38	6.45	6.78	6.75
7	6.4	6.37	6.42	6.75	6.72
8	6.38	6.34	6.4	6.71	6.7
9	6.37	6.33	6.38	6.68	6.69
10	6.36	6.31	6.37	6.66	6.68
11	6.34	6.29	6.37	6.65	6.68
12	6.33	6.27	6.36	6.65	6.68
13	6.32	6.26	6.34	6.65	6.68
14	6.31	6.26	6.33	6.66	6.68
15	6.31	6.26	6.32	6.67	6.69
16	6.31	6.26	6.32	6.68	6.7
17	6.3	6.27	6.32	6.69	6.71
18	6.29	6.27	6.32	6.7	6.72
19	6.27	6.28	6.33	6.71	6.73
20	6.26	6.29	6.33	6.71	6.73
21	6.25	6.3	6.33	6.72	6.74
22	6.24	6.3	6.33	6.72	6.75
23	6.24	6.3	6.34	6.73	6.76
24	6.23	6.3	6.35	6.74	6.77
25	6.22	6.31	6.35	6.75	6.77
26	6.21	6.32	6.36	6.76	6.78
27	6.21	6.33	6.37	6.76	6.79
28	6.2	6.34	6.38	6.77	6.8
29	6.2	6.35	6.39	6.77	6.81
30	6.19	6.36	6.4	6.77	6.82

Table.2- Effect of Eugenol + glucose on H^+ efflux of C. albicans

Time	control	500ug/ml	625ug/ml	1000ug/ml	2000ug/ml
0	7	7	7	7	7
1	6.76	6.72	6.88	6.89	6.9
2	6.74	6.56	6.77	6.78	6.81
3	6.57	6.46	6.69	6.71	6.74
4	6.51	6.37	6.62	6.67	6.69
5	6.46	6.32	6.56	6.59	6.68
6	6.43	6.28	6.52	6.57	6.63
7	6.4	6.24	6.49	6.55	6.59
8	6.38	6.19	6.44	6.49	6.57
9	6.37	6.17	6.41	6.48	6.53
10	6.36	6.17	6.4	6.47	6.51
11	6.34	6.18	6.38	6.47	6.54
12	6.33	6.18	6.39	6.46	6.54
13	6.32	6.18	6.4	6.46	6.55
14	6.31	6.19	6.4	6.46	6.56
15	6.31	6.19	6.4	6.47	6.57
16	6.31	6.19	6.4	6.47	6.58
17	6.3	6.2	6.41	6.48	6.6
18	6.29	6.21	6.42	6.49	6.61
19	6.27	6.24	6.43	6.49	6.62
20	6.26	6.25	6.43	6.5	6.64
21	6.25	6.25	6.44	6.51	6.65
22	6.24	6.26	6.45	6.52	6.67
23	6.24	6.27	6.47	6.53	6.68
24	6.23	6.28	6.5	6.53	6.69
25	6.22	6.31	6.52	6.55	6.7
26	6.21	6.33	6.54	6.56	6.72
27	6.21	6.36	6.56	6.58	6.73
28	6.2	6.37	6.56	6.6	6.75
29	6.2	6.39	6.57	6.62	6.76
30	6.19	6.4	6.6	6.63	6.79

Table.3- Effect of Eugenol + xylose on H^+ efflux of *C. albicans*

Time	Control	500µg/ml	625µg/ml	1000µg/ml	2000µg/ml
0	7	7	7	7	7
1	6.71	6.77	6.73	6.89	6.9
2	6.6	6.65	6.63	6.78	6.83
3	6.53	6.56	6.57	6.71	6.79
4	6.43	6.49	6.52	6.67	6.74
5	6.39	6.44	6.48	6.59	6.7
6	6.34	6.4	6.45	6.57	6.67
7	6.32	6.37	6.44	6.55	6.64
8	6.3	6.35	6.42	6.53	6.62
9	6.28	6.32	6.4	6.49	6.6
10	6.27	6.3	6.39	6.47	6.59
11	6.26	6.28	6.37	6.47	6.58
12	6.25	6.26	6.36	6.47	6.57
13	6.24	6.24	6.34	6.46	6.56
14	6.23	6.23	6.33	6.46	6.54
15	6.23	6.21	6.32	6.47	6.54
16	6.22	6.2	6.32	6.47	6.56
17	6.21	6.19	6.32	6.47	6.56
18	6.21	6.19	6.32	6.47	6.56
19	6.21	6.2	6.32	6.48	6.56
20	6.2	6.21	6.32	6.49	6.57
21	6.19	6.2	6.33	6.51	6.58
22	6.18	6.19	6.33	6.52	6.59
23	6.18	6.19	6.34	6.53	6.62
24	6.18	6.18	6.34	6.54	6.63
25	6.18	6.18	6.34	6.55	6.64
26	6.17	6.18	6.35	6.56	6.65
27	6.16	6.19	6.37	6.56	6.66
28	6.16	6.19	6.37	6.57	6.66
29	6.15	6.2	6.39	6.59	6.67
30	6.14	6.2	6.39	6.6	6.67

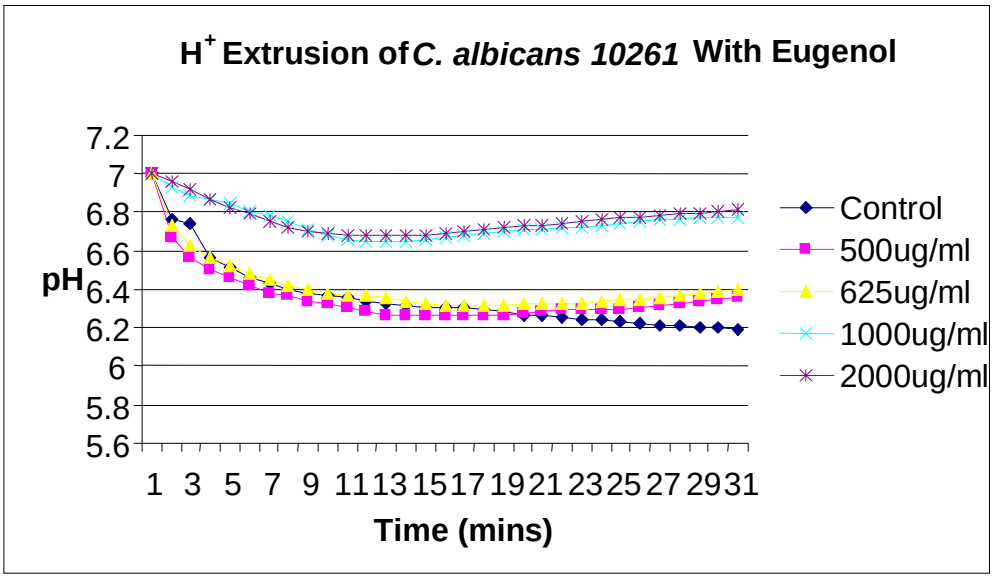

Figure.1- Effect of Eugenol on H^+ efflux of *C. albicans*

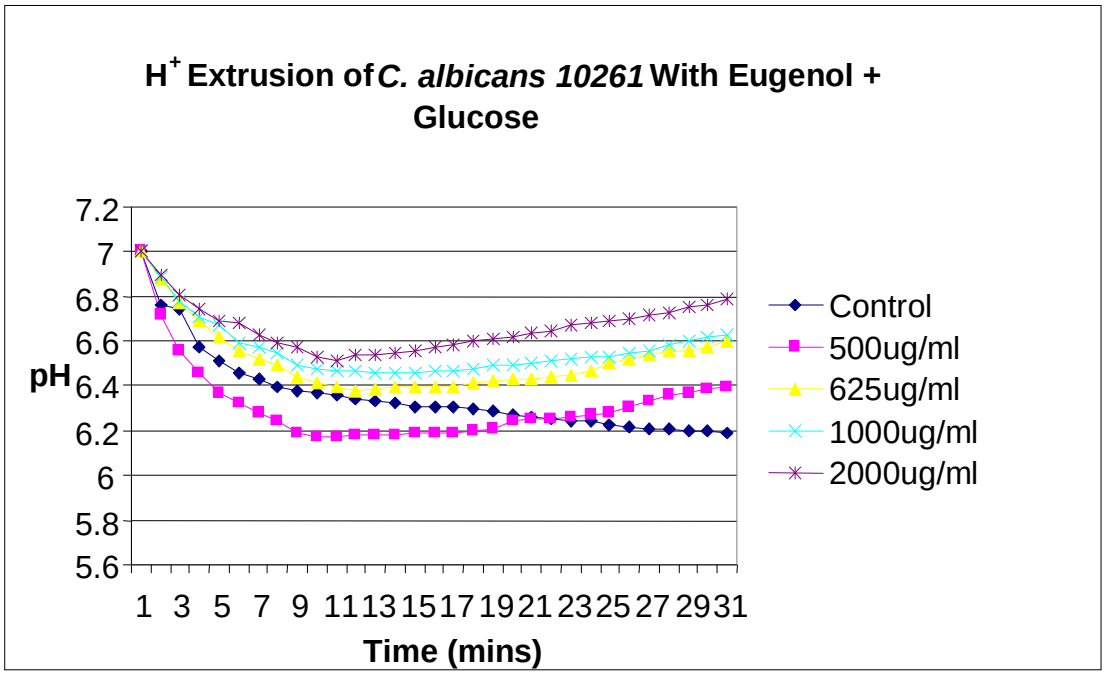

Figure.2- Effect of Eugenol + glucose on H+ efflux of C. albicans

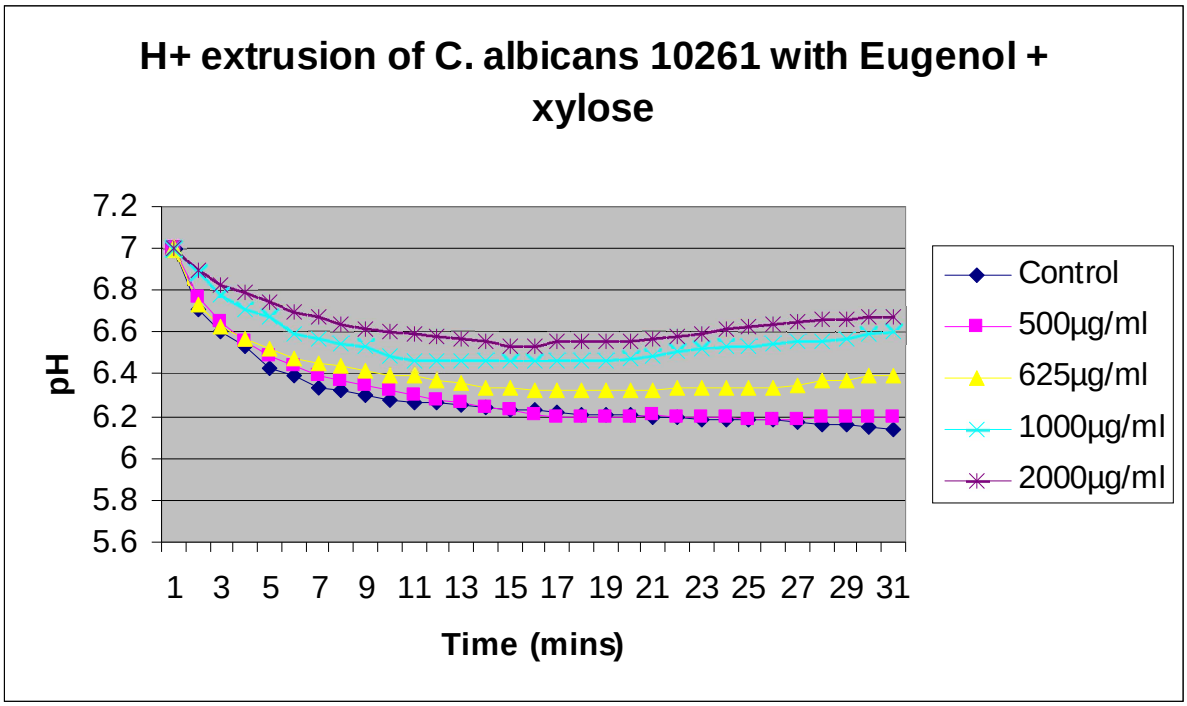

Figure.3- Effect of Eugenol + xylose on H^+ efflux of *C. albicans*