

Heparin-benzyl alcohol enhancement of biofilms formation and antifungal susceptibility of vaginal *Candida* species isolated from pregnant and non-pregnant Saudi women

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Abstract:

Biofilm formation by *Candida* species is a major contribute to their pathogenic potential. The aim of this study was to determine *in vitro* effects of EDTA, cycloheximide, and heparin-benzyl alcohol preservative on *C. albicans* (126) and non-*albicans* (31) vaginal yeast isolates biofilm formations and their susceptibility against three antifungal Etest strips. Results of the crystal violet-assay, indicated that biofilms formation were most commonly observed [100%] for *C. kefyr*, *C. utilis*, *C. famata*, and *Rhodotorula mucilaginosa*, followed by *C. glabrata* [70%], *C. tropicalis* [50%], *C. albicans* [29%], *Saccharomyces cerevisiae* [0.0%]. EDTA (0.3mg/ml) significantly inhibited biofilm formation in both *C. albicans* and non-*albicans* isolates ($P=0.0001$) presumably due to chelation of necessary metal cations for the process-completion. In contrast, heparin (-benzyl alcohol preservative) stimulated biofilm formation in all tested isolates, but not at significant level ($P=0.567$). Conversely, cycloheximide significantly ($P=0.0001$) inhibited biofilm formation in all *C. albicans* strains (126) and its effect was even 3 fold more pronounced than EDTA inhibition, probably due to its attenuation of proteins (enzymes) and/or complex molecules necessary for biofilm formation. Results also showed that all non-*albicans* yeasts isolates were susceptible to 5-flucytosine (MIC₅₀, 0.016 µg/ml; MIC₉₀, 0.064 µg/ml), but 14% of *C. albicans* isolates were resistant (MIC₅₀, 0.064 µg/ml; MIC₉₀ >32 µg/ml). The MIC₅₀ value of amphotericin B for all *C. albicans* and non-*albicans* isolates was at a narrow range of 0.023 µg/ml, and the MIC₉₀ values were 0.047 µg/ml and 0.064 µg/ml respectively, thereby confirming its efficacy as a first line empiric- treatment of *Candida* spp infections.

Key words: *Candida* spp, Biofilm formation, EDTA, Heparin-benzyl alcohol, Cycloheximide, Antifungal agents.

Background:

Species of *Candida* are considered important opportunistic pathogens. Thus, *C. albicans* is the most common cause of genital tract infections, in approximately 85-95% of cases, while other species such as *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parasilosis*, *Saccharomyces cerevisiae*, and *Rhodotorula* spp., have also been increased lately [1]. Positive vaginal cultures for *Candida* spp. can be found in almost 30% of pregnant and 15% of non-pregnant women [2]. *Candida* spp. is now the fourth most common cause of hospital-acquired blood stream

infections. Despite advances in antifungal therapy, the high attributable mortality rate (~40-60%), due to *Candida* infections has not clearly improved in the last 2 decades [3, 4]. Many of these infections are implant-associated infections, where the micro-organisms form adherent biofilms on the surfaces of catheters, joint replacements, prosthetic heart valves, and other medical devices [5]. Biofilm formation on biotic and abiotic surfaces is a key pathogenic attribute of *Candida* spp. that enhances its ability to adhere to surfaces and thereby maintain colonization and/or cause diseases in humans. Therefore,

biofilm formation represents a major challenge for the treatment of biomaterial-related *Candida* infections; and in most cases removal and/or replacement of the infected device is difficult or high risk. Policy-management of biomaterial-related complications includes the use of antibiotic lock therapy, heparin locks, and heparin-coated catheters. Ironically, the use of systemic heparin has been identified as a risk factor for catheter-related sepsis in dialysis patients [6]. Heparin has also been found to stimulate *Staphylococcus aureus* biofilm formation *in vitro* [7]. Also, [8] found, that benzyl alcohol, frequently associated with heparin as preservative, induced the most significant increase in biofilm production in *Staphylococcus epidermidis*. In contrast, Miceli *et al.*, demonstrated the inhibitory effects of heparin, though at high concentration (10,000 IU/ml), with or without methyl paraben and/or propyl paraben as preservatives on *C. albicans* (5 strains) mature-biofilm formation *in vitro*. EDTA (Ethylene di-amine tetra-acetic acid) has also been reported to inhibit *in vitro* biofilm formation by *Candida* spp. [9]. Additionally, several studies indicated that tetracycline [10]; inhibited the prokaryotic *Staph. epidermidis* biofilm formation and also affects *Candida*-colonization and/or virulence factors [11]. Although the toxicity of cycloheximide, a potent protein synthesis inhibitor in eukaryotic cells, precluded its clinical use, the drug is being extensively used in mycological culture media to inhibit saprophytic fungi including yeasts. However, as a valuable research tool in laboratory, its effects on the biofilm formation in *Candida albicans* strains have not been previously explored. Therefore, the present study deals with the investigation of *in vitro* effects of EDTA, cycloheximide, and heparin (-benzyl alcohol preservative) on biofilms formation by *C. albicans* (126 strains) and non-*albicans* (31) yeast isolates previously isolated from vaginal specimens of pregnant and non-pregnant Saudi women and recently reported from our laboratory, as well as their susceptibility patterns against amphotericin B, 5-flucytosine and itraconazole using Etest strips method.

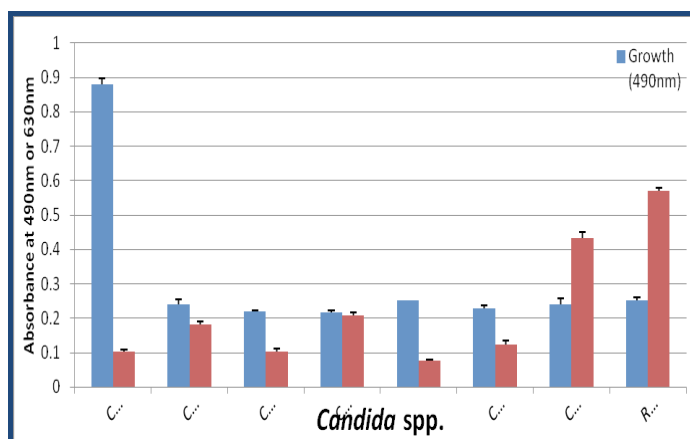


Figure 1: Distribution of *Candida* spp. isolates on the basis of their capability for growth and biofilm-formation at 37 c for 48h.

Methodology:

Microorganisms

One hundred and fifty seven isolates belonged to eight species of yeasts were recently recovered from vaginal specimens (707 HVS) of pregnant and non-pregnant Saudi women, and used in this study. These comprises *C. albicans* (126); *C. glabrata* (20); *C.*

tropicalis (4); *C. kefyr* (2); *S. cerevisiae* (2); and one strain of *C. famata*; *C. utilis* ; or *R. mucilaginosa*. The collected *Candida* species strains were stored in 10% glycerinated water at -20°C until experiments were performed. The reference strain *C. albicans* (ATCC 10231) was used as control in biofilm assay, and antifungal susceptibility tests [12].

Inoculum-preparation and Biofilm quantification

Biofilm formation was assisted as previously described [13, 14]. Briefly, fresh yeast-inoculum-pellets were collected by centrifugation (5 minutes at 4000 rpm) from static logarithmic growth in Sabouraud Dextrose Broth (SDB) at 37°C for 6 h, washed and re-suspended in sterile saline to match 3 McFarland turbidity scale. To each well of the microtiter plates (flat bottom) 180 μl of SDB (supplemented with extra 8% glucose) were added, then inoculated with 20 μl of the above yeast cell suspension. *C. albicans* (ATCC 10231) reference strain and yeast-free medium controls were also included. Four wells for each yeast isolate were performed: i-saline (50 μl), ii- EDTA 50 μl (to give final concentration 0.3 mg/ml; iii- heparin from its appropriate dilution in sterile saline, 50 μl (to give final concentration of heparin 1000 IU/ml and 0.4 mg/ml benzyl alcohol; and cyclohexamide 50 μl (only for *C. albicans* strains), to give final concentration of 100 μM . The plates were then incubated for 48 h at 37°C [15]. After incubation, plate growth was assessed by measuring the absorbance at 490nm (A_{490}). Then for biofilm quantification, the planktonic cells were aspirated, and the wells were washed thrice with sterile saline. The plates were then inverted on filter paper and were allowed to dry for 1 h at room temperature, 200 μl of 1% crystal violet (Sigma, USA) was added to each well, and the plate was allowed to stand for 20 min at room temperature. The wells were subsequently washed thrice with sterile saline to wash off the excess crystal violet. A volume of 200 μl of ethanol was added to each well and absorbance was measured at 630nm (A_{630}). Testing of these isolates was performed thrice for each yeast strain. As control the background crystal violet binding was measured for wells exposed only to the medium with no *Candida* spp. Biofilm positive strains were considered those wells where the A_{630} of which was higher than mean A_{630} of negative control plus 3X SD.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed using Etest strips (ETS). Amphotericin B, 5-flucytosine, and itraconazole, ETS were provided by AB BIODISK (Solna, Sweden), with a concentration gradient range from 0.002 $\mu\text{g}/\text{ml}$ to 32 $\mu\text{g}/\text{ml}$. The strips were stored at -20°C until use. The medium used was RPMI-1640 agar (Sigma Aldrich Ltd., st. Quentin Fallavires, France) at 10.4g/l supplemented with MOPS (3-[N-morpholino]-propanesulfonic acid at 165mM), glucose at 20g/l, agar at 15g/l, and L-glutamine at 200mM (added after autoclaving). The tested *Candida* spp. isolate colony previously grown on Sabouraud Dextrose Agar (SDA) plate for 24 h at 37°C , was picked up, suspended in 0.85% sterile saline, and adjusted to match (0.5) McFarland turbidity. A swab of the cell suspension was then spread in three directions on entire surface of a RPMI plate, and left for 15 min to allow moisture absorption at room temperature before applying the ETS on the agar. The agar plates were then incubated with bottom up at 37°C for 24 h except for *C. glabrata*, *R. mucilaginosa*, or *S.*

cerevisiae the incubation time was 48 h. After incubation, the MIC_s of the tested antifungal agents were determined as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the ETS [16]. The interpretive susceptibility criteria were performed according to Negri *et al.*, (2009) breakpoints: amphotericin B ($\leq 1\mu\text{g/ml}$, susceptible; $\geq 2\mu\text{g/ml}$, resistant); 5-flucytosine ($\leq 4\mu\text{g/ml}$, susceptible; $8\text{--}16\mu\text{g/ml}$, intermediate; $\geq 64\mu\text{g/ml}$, resistant); itraconazole ($\leq 0.125\mu\text{g/ml}$, susceptible; $0.25\text{--}0.5\mu\text{g/ml}$, susceptible-dose dependent; $\geq 1.0\mu\text{g/ml}$, resistant).

Statistical methods

The results were analyzed using SPSS 19 (Statistical Package for Social Science; release 19.0). Chi-square test and Fishers exact test were used to compare association between proportions and a *P*-value < 0.05 was considered as significant.

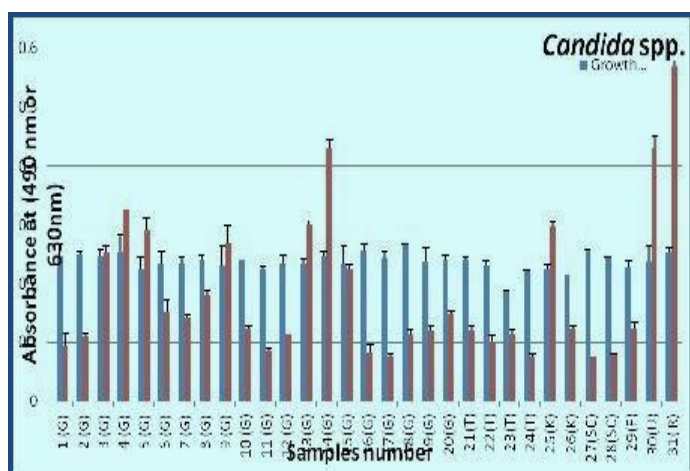


Figure 2: The blue line represent the absorbance of the growth, wells read at (490nm); and the red represent the absorbance of biofilm-formation crystal violet ethanol extracted stained-wells read at (630).

Result:

Results revealed that biofilm formation varied from one strain to another within the same species as well as from species to species. As presented in **Table 1** (see supplementary material), only 29 % of *C. albicans* strains formed biofilms, while as much as 68 % of *non-albicans spp.* strains were capable of biofilm formation. The rank order of biofilm formation was *C. kefyr*, *C. utilis*, *C. famata*, and *R. mucilaginosa* (100%), followed by *C. glabrata* (70%), *C. tropicalis* (50%), *C. albicans* (29%), and *S. cerevisiae* (0%). As illustrated in **Figure (1 & 2)** there was no correlation between intensity of growth and capacity of biofilm formation. EDTA proved efficient in reducing the biofilm formation by *non-albicans spp.* ($P < 0.0001$) almost completely **Table 2** (see supplementary material), and with considerable reduction in the biofilm formation capacity of most *C. albicans* strains ($P < 0.0001$). Likewise, cycloheximide (100 μM) significantly ($P < 0.0001$) inhibited **(Table 2)** the biofilm formation capacity of all *C. albicans* strains, and this effect was even three fold more pronounced than that of EDTA inhibition. Heparin at the concentration used (800U/ml, benzyl alcohol preservative) unanimously enhanced the biofilm formation in all tested *non-albicans spp.* ($P = 0.567$) and *C. albicans* ($P = 0.414$) strains, yet this enhancement, as compared to respective saline-control was not significant **Table 3** (see supplementary

material) & **Figure 3**. Whether this observed biofilm-enhancement is due to heparin as such and/or the associated benzyl alcohol preservative is not clear and warranted further investigation.

Additionally, **(Table 3)** summarizes the *in vitro* susceptibility testing (MIC_s) of 126 *C. albicans* and 31 *non-albicans spp.* strains against amphotericin B, 5-flucytosine and itraconazole by Etest method. The data were computed as the concentrations of antifungal agent necessary to inhibit 50% of the isolates (MIC₅₀) and concentration to inhibit 90% (MIC₉₀) of the isolates. *C. albicans* isolates taken together gave (MIC₉₀) value for amphotericin B, 5-flucytosine and itraconazole of 0.047, >32 and 2 $\mu\text{g/ml}$, respectively. Whereas, *non-albicans spp.* isolates taken together gave MIC₉₀ value for amphotericin B, 5-flucytosine and itraconazole of 0.064, 0.064 and >32 $\mu\text{g/ml}$, respectively **(Table 3)**. Amphotericin B MICs showed a very narrow range for the species tested. In contrast, 5-flucytosine and itraconazole MICs showed a broad range for all the species. As expected, most of *C. glabrata* strains were susceptible to amphotericin B and 5-flucytosine but resistant to itraconazole **(Table 3)**. Results also showed that all *Candida spp.* including *C. albicans* were susceptible to amphotericin B and also to 5-flucytosine with the exception 14% of the *C. albicans* strains. Mean while as much as 32 % of *non-albicans* isolates (mostly *C. glabrata*) were resistant to itraconazole, but only 14% of *C. albicans* isolates were resistant this drug.

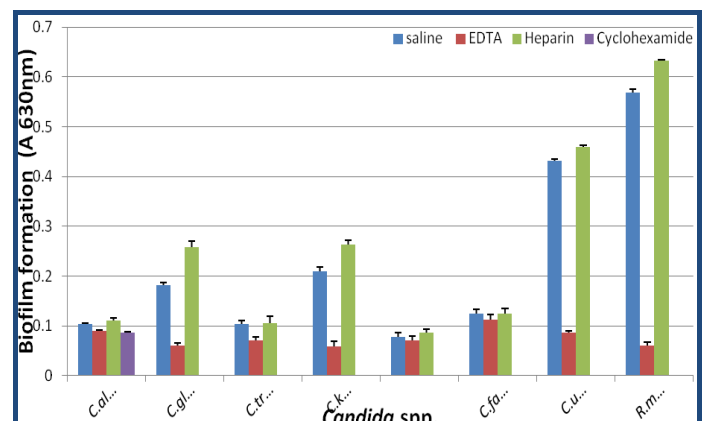


Figure 3: Effets of EDTA, cycloheximide and heparin on tested *Candida spp.* capability to form biofilms at 37°C for 48h.

Discussion:

The phenomenon of *in vitro* biofilm formation by microbes on various abiotic surfaces has been extensively studied in bacteria and to a lesser extent in fungi, and there appears to be a direct relationship between the capability of organisms to form a biofilm and their pathogenicity. Many *Candida spp.* infections involve the biofilm formation on implanted devices such as intrauterine devices and urinary catheter. In this study, SDB medium supplemented with extra glucose (8%) was used to induce biofilm formation by examined *Candida* isolates. The ability of our yeast-isolates to form biofilm varied among different *Candida* species and even within the same species. The *non-albicans spp.* exhibited greater capacity in biofilm formation (68%) than that of *C. albicans* (29%). These findings are consistent with those previously reported by Shin *et al* [17], Silva *et al* [18], Parthak *et al* [19]. However, Kuhn *et al* [20]

reported, that isolates of *C. albicans* consistently form higher biofilm formation *in vitro* than non-*albicans* spp. EDTA disrupts microbial biofilm through its chelation of calcium, iron, and magnesium with established anticoagulant activity and some inhibitory activity against *Staphylococci* and *Candida* spp. [21] ..These findings were confirmed by ours, where EDTA significantly inhibited the capacity of biofilm formations by non-*albicans* spp. As well as *C. albicans* strains. According to previous studies, EDTA inhibits *C. albicans* biofilm formation through its inhibitory effect on filamentation. Whereas Percival *et al* [22] reported that EDTA is a more appropriate anticoagulant and antimicrobial catheter lock than heparin. EDTA has also been shown to inhibit biofilm formation in *S. aureus* and in *Pseudomonas aeruginosa* [23].

In the present study heparin at the concentration used (1000U/ml, benzyl alcohol preservative) unanimously enhanced the biofilm formation in all non-*albicans* spp. as well as *C. albicans*. In contrast one of the latest studies reported, that heparin with or without, methyl paraben, and/or propyl paraben inhibited *C. albicans* (only five strains) biofilm formation up to 90%. These controversial findings regarding the effect of heparin on *Candida* spp biofilm formation, has also been reported for its effect on biofilm formation by *Staphylococcus* spp. Thus in agreement with our findings on yeast isolates. Likewise, the use of systemic heparin has been identified as a risk factor for catheter-related sepsis in dialysis patients. In contrast, Sauer *et al* [24] reported that, heparin reduced biofilm formation in *Staphylococcus aureus*. Further, heparin markedly reduced biofilm formation by 87% of *Staphylococcus lugdunensis* strains [25]. These discrepancies may be attributed to differences in heparin associated preservatives and /or used medium cation-contents, particularly calcium and magnesium which have been reported to restore the *S. aureus* heparin inhibitory effect.

Our finding that heparin enhances biofilm formation by all tested *Candida* spp. including *albicans* is compatible with previous findings that the use of systemic heparin has been identified as a risk factor for catheter-related sepsis in dialysis. On the other hand cycloheximide is incorporated in fungal media to inhibit saprophytic fungi including yeasts. However, as a research tool in the elucidation of steps in biofilm formation, the effect of cycloheximide on *C. albicans* biofilms formation has not been previously explored. In the current study, cycloheximide strikingly inhibited biofilm formation by all *C. albicans* strains, presumably due its attenuation of *de novo* synthesis of proteins, enzymes [26, 27] and/or complex molecules necessary for the biofilm expression. In this context, several protein synthesis inhibitor antibiotics have been shown to reduce the formation of biofilm in several bacteria (McCool *et al.*, 2008; Flemming *et al.*, 2009). In contrast antibiotics which inhibit cell wall synthesis have been reported to stimulate biofilm formation in prokaryotic pathogens, apparently due to weakness of cell wall integrity which facilitate out diffusion of complex molecules for biofilm formation [28] (Bagge *et al.*, 2004).

In this study, the susceptibility testing profiles indicated that amphotericin B exhibited a very narrow range MIC₉₀ of (0.047 µg/ml and 0.064 µg/ml) for 126 *C. albicans* strains and 31 non-

albicans strains, respectively. In a similar study, Hanafy and Morsy [29], found that all the tested clinical isolates (39) were susceptible to amphotericin B. Whereas Kabli, [30] found that all the isolates (107) were also susceptible to the amphotericin B, tested by Etest strips. These findings confirm previous conclusion that amphotericin B was active against the majority of the tested yeast and filamentous fungi, including species known to cause rare and difficult to treat infections, and therefore this agent plays an important role in the management of invasive fungal infection [31, 32]. In contrast, in Taiwan [33] (Cheng *et al.*, 2004), reported that three isolates 2% (out of 170 isolates) of non-*albicans* spp. were resistant to amphotericin B. Whereas in Brazil Negri *et al.*, [34] reported, that two isolates, 2% (out of 100 isolates) of *Candida* spp., from Iran twenty isolates, 7% (out of 285 isolates) and from Iraq [35] 7 isolates, 47% (out of 15 isolates) of *C. albicans* strains were resistant to amphotericin B. This discrepancy confirms that *Candida* spp. susceptibility patterns varied from country to another, presumably due to frequent use of antifungal drugs *per se*.

Further our results indicated that with the exception of 14% of *C. albicans* (126 isolates) and all non-*albicans* *Candida* spp. were susceptible to 5-flucytosine. In comparison Mokaddas *et al.*, [36] reported, that 9% out of (107 clinical isolates) and 1% (out of 240 isolates) were resistant to 5-flucytosine, respectively. On the other hand itraconazole is among oral antifungal agents currently used in the management of sever fungal infections. However, in this study as much as 32% of non-*albicans* strains (mostly *C. glabrata*) were resistant to itraconazole, but only 14% of *C. albicans* strains where resistant the same drug. Whereas Hanafy and Morsy, found that 12 out of 18 *C. albicans* strains and 3 out of 21 non-*albicans* strains were resistant to itraconazole. A lower rate of resistance to this drug was found in Brazil (10%, 5/52); an even higher rate of resistance (40%, 17/43) to this drug was reported from Taiwan. Again as stated above this discrepancy may reflect extensive use and/or rational use of the drug *per se*. The resistance observed in this study against conventional antifungal agents should be viewed with concern and necessitates continuous monitoring through surveillance studies.

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The authors state no conflict of interest.

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Supplementary material:

Table 1: Distribution of biofilm-forming strains in relation to various *Candida spp.* isolates.

<i>Candida spp.</i>	Biofilm (+)	Biofilm (-)	Biofilm-forming (+) strains (%)	Total
<i>C. albicans</i>	36	90	29	36/126
<i>C. glabrata</i>	14	6	70	
<i>C. tropicalis</i>	2	2	50	
<i>C. kefyr</i>	2	0	100	21/31
<i>C. utilis</i>	1	0	100	(68%)
<i>C. famata</i>	1	0	100	
<i>S. cerevisiae</i>	0	2	0.0	
<i>R. mucilaginosa</i>	1	0	100	

Table 2: *In vitro* effects of EDTA, cycloheximide, and heparin on the abilities of *C. albicans* and non-*albicans* tested yeast-isolates to form biofilms.

Species number(n)	Saline % Biofilm positive	EDTA % Biofilm positive	Cycloheximide % Biofilm positive	Heparin % Biofilm positive
<i>C. albicans</i> (n=126)	36 (29)*	25 (20) ^a	8 (6) ^b	42(33) ^c
Non- <i>albicans</i> (n=31)	21(68)	0.0(0.0) ^d	NT	23(74) ^e

* As compared to saline-control: a, $P=0.015$; b, $P<0.0001$; c, $P=0.414$; d, $P<0.0001$; e, $P=0.576$, and NT; not determined.

Table 3: MIC₅₀ and MIC₉₀ values of E test-antifungal agents against tested *Candida spp.*

Species	E test (µg/ml)				
	Antifungal	Range	MIC ₅₀	MIC ₉₀	%Resistance
<i>C. albicans</i> (n= 126)	Itraconazole	0.002->32	0.125	2	14
	5-Flucytosine	0.002->32	0.064	>32	14
	Amphotericin B	0.002-0.38	0.023	0.047	0
Non- <i>albicans</i> (n= 31)	Itraconazole	0.002->32	0.75	>32	32
	5-Flucytosine	0.002-1.5	0.016	0.064	0
	Amphotericin B	0.002-0.19	0.023	0.064	0