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ORIGINAL PAPER

The conventional identification and effect of diclofenac and aspirin on the *Candida species*

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RUSU ELENA[1], ENACHE-SOARE SIMONA[2], NEGRUTA OANA[2], VASSU TATIANA[3].
[1] Titu Maiorescu University, Faculty of Dental Medicine , Street Gheorghe Petrascu, no. 67A, sector 3, Bucharest, *Romania E –mail:elenarusu98@yahoo.com*
[2] MICROGEN- Center for Research in Microbiology, Genetics and Biotechnology, University of Bucharest, Faculty of Biology, 1-3 Aleea Portocalilor, sector 5, Bucharest 060101, Romania E-mail: simona_soare1979@yahoo.com (Simona Enache -Soare)
[3] University of Bucharest, Faculty of Biology, Department of Genetics, 1-3 Aleea Portocalilor, sector 5, Bucharest 060101, Romania E –mail: tatiana@botanic.unibuc.ro (Tatiana Vassu-Dimov)
Tel/ fax: 0040.213118077
** Author to whom correspondence should be addressed; E-mail: simona_soare1979@yahoo.com*

Abstract

The study was dedicated to investigate the effect of diclofenac and aspirin on planktonic cells and capacity to form germ tube of different Candida strais. The species of Candida genus are opportunistic dimorphic fungus that inhabits various host mucosal sites and cause both superficial and serious systemic diseases. Prostaglandins are small lipid molecules and may play an important rol in fungal colonization. Non-steroidal anti-inflammatory drugs (aspirin and diclofenac) are inhibitors of the cyclooxygenase isoenzymes. These drugs specifically block the biosynthesis of mammalian prostaglandins by inhibiting one or both of COX isoenzymes. We observed a 90% inhibition of suspension cells viability of Candida albicans that of untreated control cells in the presence on sodium diclofenac. In conventional assays for germ tube formation this drug produced significant inhibition to 80%. In the presence of aspirin, cells viability was 60% and the germ tube formation was 75%-60%, depending on the tested strain. Our results suggest that cyclooxygenase-depending synthesis of fungal prostaglandins is important for morphogenesis and the survivor of Candida albicans cells and may act as a regulator in these processes. We think that diclofenac sodium and aspirin might improve the traditional antifungal therapies against Candida albicans. The anti-inflammatory and analgesic properties of sodium diclofenac and aspirin might represent an additional advantage for their use in the management of infection with Candida species.

Keywords: *Candida*, aspirin, diclofenac.

Introduction

The *Candida* species, mainly *Candida albicans*, are the commonest causative agents of human fungal infection. The species of *Candida* genus are opportunistic dimorphic fungus that inhabits various host mucosal sites. They can cause both superficial and serious systemic diseases and are now widely recognized as important agents of hospital-acquired infection. The increase in the rate of opportunist infections by *Candida* has resulted from the growing use of broad spectrum antibacterial agents, as well as from an increasing number of immuno-compromised patients due to the use of aggressive chemotherapy for cancer and of immunosuppressive drugs in organ transplantation. Antifungal drugs are used increasingly

both as prophylactic and curative agents which have led to the widespread of resistant strains. This situation has prompted the search for alternative anti-*Candida* therapeutic agents. Furthermore, many currently used drugs have high costs and lead to serious side-effects. Prostaglandins are small lipid molecules that have diverse biological activities, including the modulation of host immune responses (8). They are known to be produced by pathogenic fungi as well as by mammalian cells. The pathogenic yeasts *Candida albicans* and *Cryptococcus neoformans* produce eicosanoids (both prostaglandins and leukotrienes) *de novo* or via conversion of exogenous arachidonic acid. Treatment of these yeasts with cyclooxygenase inhibitors has proven to be toxic in vitro, suggesting a requirement for an eicosanoid product or biosynthetic pathway in growth (10). In mammalian systems arachidonic acid, formed by cleavage of phospholipids, is converted to prostaglandin H_2 by the cyclooxygenase (COX) isoenzymes, COX-1 and COX-2. It is thought that COX-1 is expressed constitutively in most tissues of the body and COX-2 is mainly an inducible enzyme involved in regulation of inflammation (7). COX-1 plays important roles beyond thromboxane A_2 (TXA_2) production in platelets. Of particular importance is the production of the cytoprotective prostaglandins by gastric mucosa. Unlike platelets, gastric mucosal cells possess the biosynthetic machinery necessary to overcome COX-1 inhibition and, therefore, recover the ability to synthesize prostaglandins within a few hours after exposure to aspirin. COX-2, a second cyclooxygenase isoenzyme primarily responsible for synthesis of the platelet inhibitors PGI_2 by endothelial cells (5) is induced in response to inflammatory stimuli, and is less sensitive to the effects of aspirin. The inducing stimuli include pro-inflammatory cytokines and growth factors, implying COX-2 in both inflammation and control of cells growth (12). Aspirin is 170-fold less effective at inhibiting COX-2 than COX-1. Little is known about the role of prostaglandins in fungal biology. Considering their known effects on mammalian cell biology, prostaglandins secreted by pathogenic fungi could also promote colonization and chronic infection by these organisms. The aim of this study was to investigate the effect of aspirin and diclofenac on planktonic cells of different *Candida albicans* strains. Aspirin irreversibly inhibits acetylation of the amino acid serine at position 529 thereby preventing arachidonic acid access to the COX-1 catalytic site through steric hindrace (3). Aspirin and diclofenac are non-steroidal anti-inflammatory drugs (NSAID) which are the most widely used in therapeutics, primarily for the treatment of pain and inflammation, especially arthritis. These drugs specifically block the biosynthesis of mammalian prostaglandins by inhibiting one or both COX isoenzymes.

Materials and Methods

Yeast strains. Three strains of *Candida albicans* (C1, C4 and C6) isolated from the "Matei Bals" Hospital patients with oropharyngeal candidiasis were used. Clinical isolates were first characterized and taxonomically identified based on colony morphology and microscopic appearance. It was examined shape, colour, colonies type, cells aspect and ability to form buds. The identification studies had been completed by the urease and yeast API 20 Caux tests (bioMerieux, France). The species of *Candida* genus have not the capacity to degraded urea because they don't have urease enzyme. Yeasts were initially isolated on YPG agar, stored in glycerol, and frozen at -70ºC until analysis. We used also two reference strains: *Candida albicans* 10231 and *Candida krusei* CMGB 94.

Medium and culture conditions. Strains were grown overnight at 37ºC (log phase culture) in Yeast Peptone Glucose (1% yeast, 2% peptone and 2% glucose) medium. Cell suspensions were standardized to a cell density of 0.5 McFarland.

COX inhibitors. Stock solutions (100mM) of diclofenac sodium and aspirin (acetylsalicylic acid) were prepared in ethanol. For viability experiments, COX inhibitors were used at final concentrations of 1 mM and for germ tubes tests the final concentrations was 0.1mM.

Viability counts. CFU of C*andida* treated by the test drug and untreated (controls) were counted after plating serial dilutions of suspensions in YPG agar. Colonies were counted after 24 h incubation at 37ºC.

Germ tube tests. Cultures of *Candida albicans* strains grown overnight in YPG-medium. *Candida krusei* has not the ability to form germ tubes. That is why, the test for filamentation had released to *C. albicans* strains. For this experiment we used a HeLa cell line for determination the ability of *Candida* cell to form germ tubes. The cells were resuspended in the potassium phosphate buffer (pH=7.4) and placed on the HeLa cell line. COX inhibitors were added to a final concentration of 100 μM. HeLa cells with *Candida albicans* suspensions were incubated at 37ºC for 1.5 h. For visualization we used Giemsa solution. For examination the percentage of germ tubes present we used a light microscope; 100 cells were counted each time.

Results and discussions

The examination of culture characters shows us the shape, colour and the colony type. Morphological analyses shows us that strains C1 and C4 had the same characters with the reference strain *Candida albicans* 10231. These isolates formed convex, round and cream-coloured colonies. Strain C6 had a different aspect, like *Candida krusei CMGB 94*. The colonies of these strains were white, convex, round, with toothed surface and greater than the other strains (fig. 1).

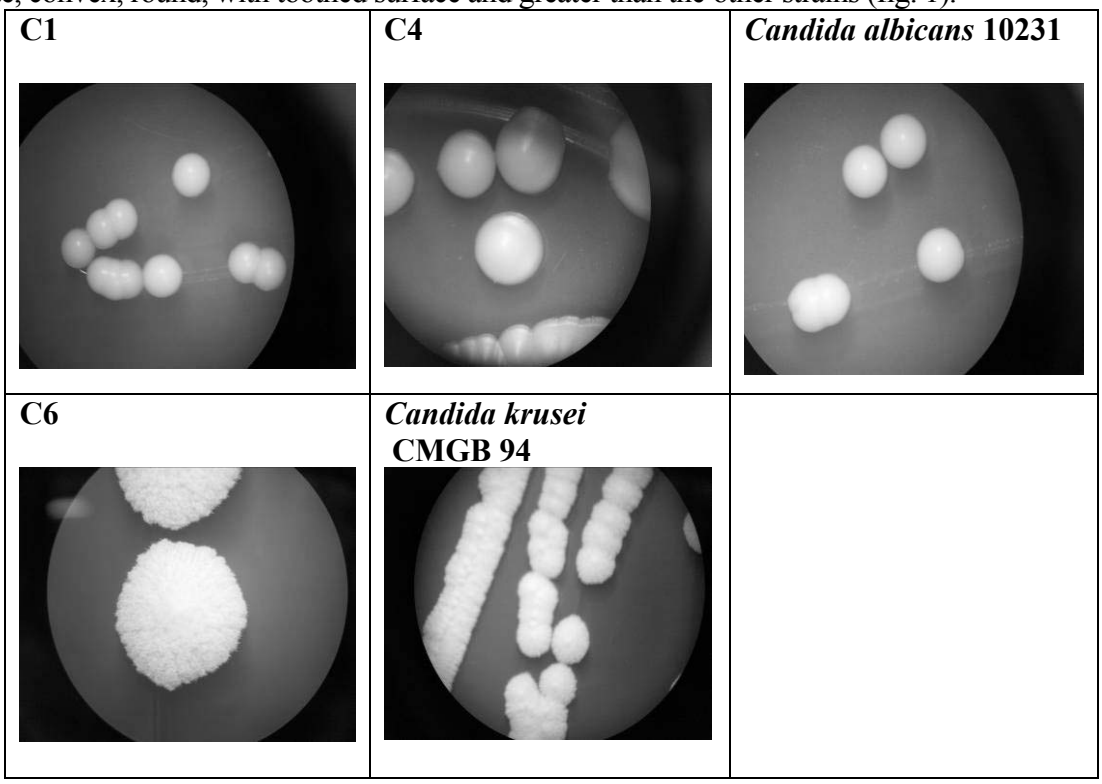

Figure 1. The macroscopic characters of tested strains

The cells shape and the mode of reproduction represent criterions of taxonomic identification. Cells of C1 and C4 strains have round and oval shapes with apical buds (fig. 2).

This type of cells is specific for the budding yeasts. The cells of C6 strain were elongated and had same aspect like *Candida krusei* MICROGEN cells.

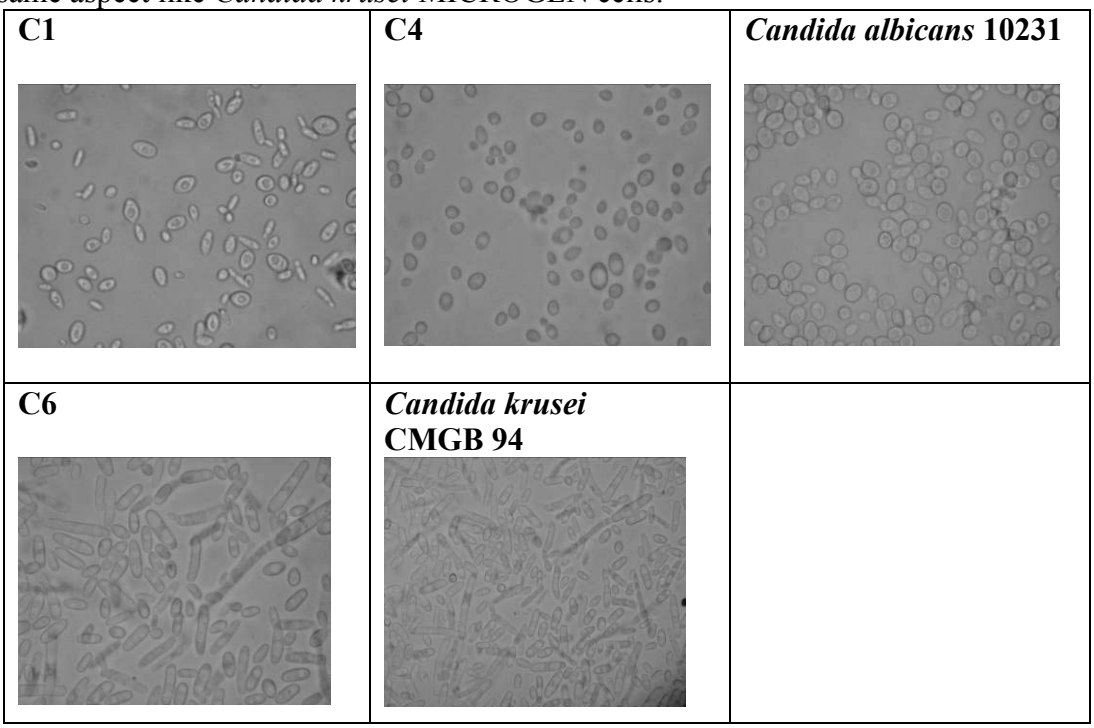

Figure 2. Microscopic aspects of tested strains

The results of the urea assay had been negative for all strains (fig. 3). The species of *Candida* genus have not the capacity to degraded urea. The colour of Christensen medium didn't changing the colour from yellow to pink. These results were confirmed by the yeast API 20 Caux tests.

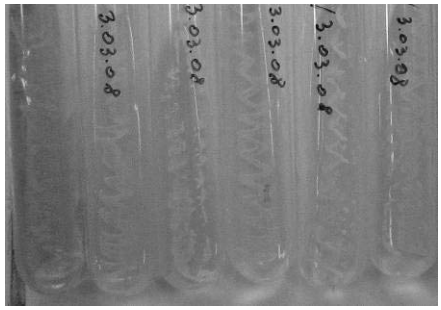

Figure 3. The results of the urea test

A number of registered non-antibiotic drugs possess antimicrobial effect that have generally been regarded as a side-effect, as in the case with anti-diuretic, anti-diabetic, β-blocker, and non-steroidal anti-inflammatory molecules (4). Prostaglandins production could be inhibited by aspirin, which also suppresses the growth of the yeast form and prevents the yeast to hypha transition of *Candida albicans*. Exogenous PGE_2 from either host or fungal sources enhances germ tube formation in *Candida albicans*, implicating fungal eicosanoids as a morphogenic factor (11). The yeast to hypha transition is often associated with progression of infection by *Candida albicans*, and therefore, fungal eicosanoid regulation of morphogenesis may be considered a virulence mechanism.

Viable counts of planktonic cells of five strains of *Candida* species were determined after growth for 24 h at 37ºC in the presence of 1 mM diclofenac or aspirin. Aspirin has been

reported to have antifungal activity of biofilm formation at concentrations lower than 1 mM (1). The results of viable cells number are presented in the table 1. Influence of aspirin and diclofenac is presented in fig. 4. Aspirin reduced the viability of planktonic cells between 78.3% and 40.15% (tab.2). Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of inhibitor.

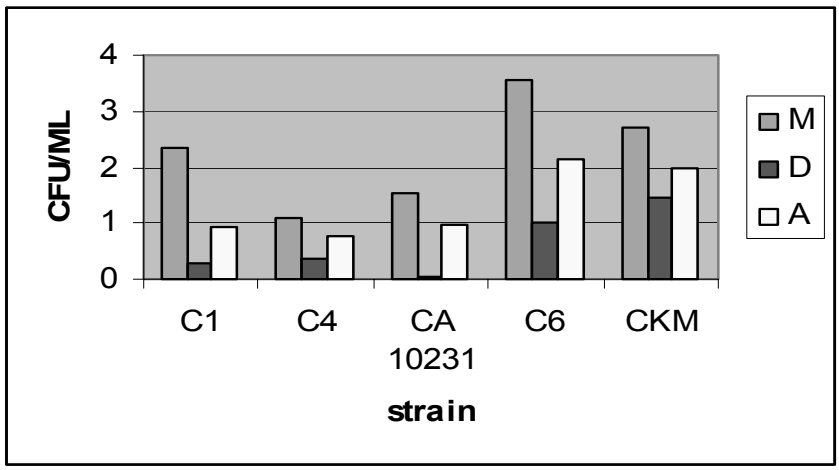

Figure 4. Influence of diclofenac (1mM) and aspirin (1mM) on planktonic cells on different strains of *Candida albicans*

Table 1.Viable cells number of *Candida* species after growth for 24 h in the presents of diclofenac and aspirin (1mM) or in absence of inhibitors.

Nr. Crt	Strain	CFU/ml untreated	CFU/ml treated with diclofenac	CFU/ml treated with aspirin
1	C1	2.342×10^9	0.29×10^9	0.94×10^9
2	C4	1.103×10^9	0.35×10^9	0.76×10^9
3	*C.albicans 10231*	1.52×10^9	0.049×10^9	0.96×10^9
4	C6	$3,57 \times 10^9$	$1,02 \times 10^9$	2.14×10^9
5	*C. krusei CMGB 94*	$2,72 \times 10^9$	$0,35 \times 10^9$	1.98×10^9

Diclofenac sodium had the greatest effects on the planktonic cells of *Candida albicans* (fig. 5). The viabilities cells had reduced to the 12.39- 3.22 % of that of untreated control cells and were depended on the tested strains (tab. 2).

Table 2. Viability of planktonic cells in the presence of different COX inhibitors

Strain	Viability (%) -Aspirin	Viability (%) - Diclofenac
C1	40.15	12.39
C4	78.3	31
C.albicans 10231	63.15	3.22
C6	59.94	28.57
C. krusei CMGB 94	72.79	12.86

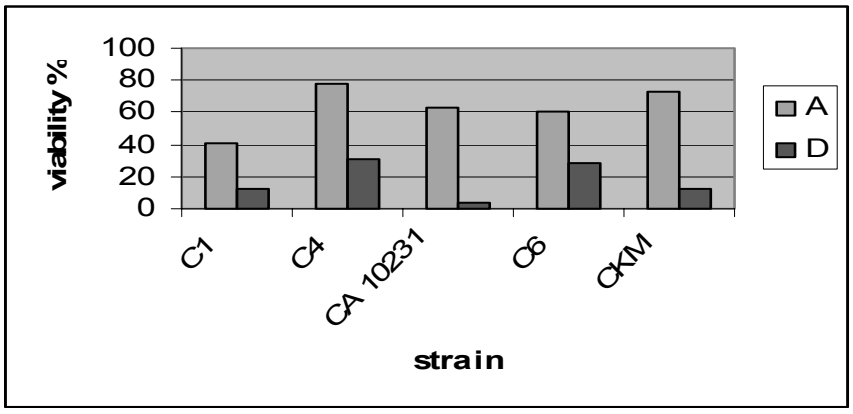

Figure 5. Effect of aspirin and diclofenac on different *Candida albicans* strains (viability %); (blue columns are aspirin; red columns are diclofenac).

For the production of germ tubes, the diclofenac registered an important inhibition effect on *Candida albicans* cells (fig. 6 and 7). The yeast to hypha transition is often associated with progression of infection by *Candida albicans*. In the presence of diclofenac sodium (0.1 mM), the filamentation was reduced to the 10%-3 % (tab. 3). Germ tube formation is expressed as a percentage of that for control cells incubated in the absence of COX inhibitors (fig. 8). In the presence of aspirin, cells viability was 60% and the germ tube formation was 75%-60%, depending on the tested strain. In mammalian systems, all of the classic nonsteroidal anti-inflammatory drugs, such as aspirin, diclofenac and indomethacin inhibit both the COX-1 and COX-2 isoenzymes. The COX active site is created by a long hydrophobic channel that is also the site of drug binding. Aspirin is the only known non-steroidal anti-inflammatory drug that covalently binds to a serine residue and that inhibits COX-1 more than COX-2 (8). Both the beneficial and detrimental effects of aspirin are believed to be primarily due to inhibition of prostanoid biosynthesis, in particular that of TXA_2 and prostaglandins (PGE_2 and PGI_2). In this study, two COX inhibitors tested decreased viabilities cells by *Candida albicans* with aspirin and diclofenac, a preferential COX-2 inhibitor, producing the greatest effects. Diclofenac has a low to moderate preference to block the COX-2 isoensyme (approximately 10-fold) and have a somewhat lower incidence of gastrointestinal complaints than noted with aspirin. The relationship between non-steroidal anti-inflammatory drugs use and serious gastrointestinal complication has been examined in a number of studies. One of the most complete studies is a meta-analysis of reports between 1985 and 1994 (9) in which 11 NSAIDs were ordered for their association with serious complications. The order of the NSAIDs, from least to most damaging, was 1-ibuprofen, 2-diclofenac, 3-diflunisal, 4-fenoprofen, 5-aspirin, 6-sulidac, 7-naproxen, 8-indomethacin, 9-piroxicam, 10-ketoprofen and 11-tolmetin. This is consistent with idea thai NSAIDs produce serious gastrointestinal complications by significantly inhibiting the activity of COX. Further comparison of the COX-1 selectivities of these compounds demonstrates that compounds associated with the greatest gastrointestinal toxicity have the greatest COX-1 selectivity (13).

Table 3. Germ tube formation (%) in the presence of COX inhibitors (diclofenac and aspirin 0.1 mM)

Nr. Crt.	Strain	Control %	Test D %	Test A %
1	C1	93	5	75
2	C4	90	10	65
3	*Candida albicans 10231*	91	3	60

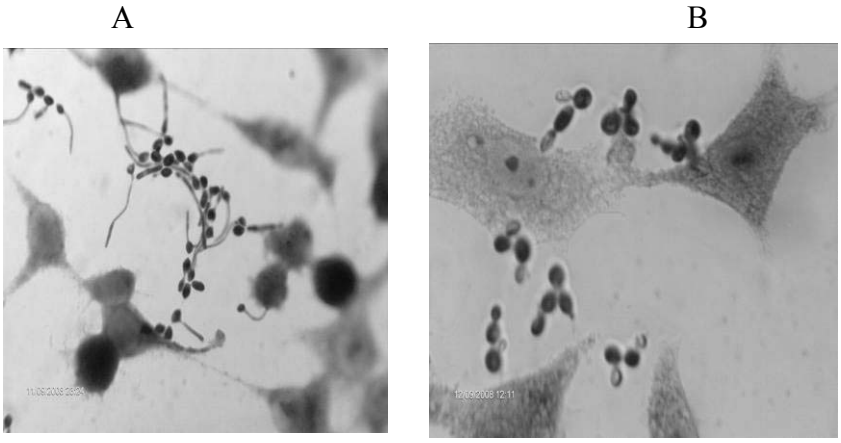

Figure 6. Strain C1
A.Control
B.Test- Diclofenac

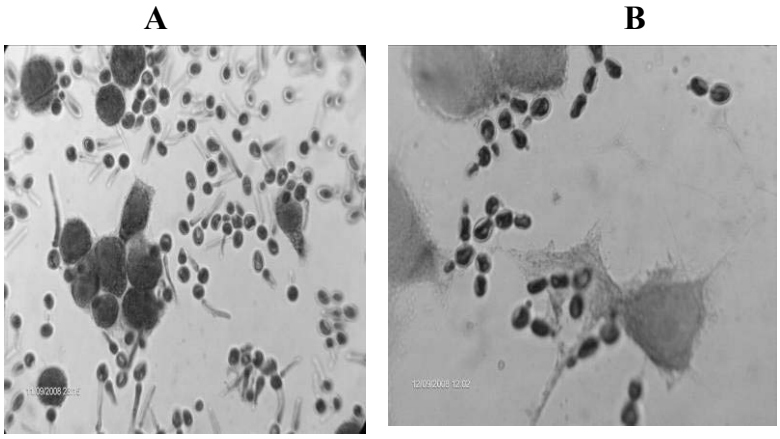

Figure 7. Strain C4
A.Control
B.Test –Diclofenac

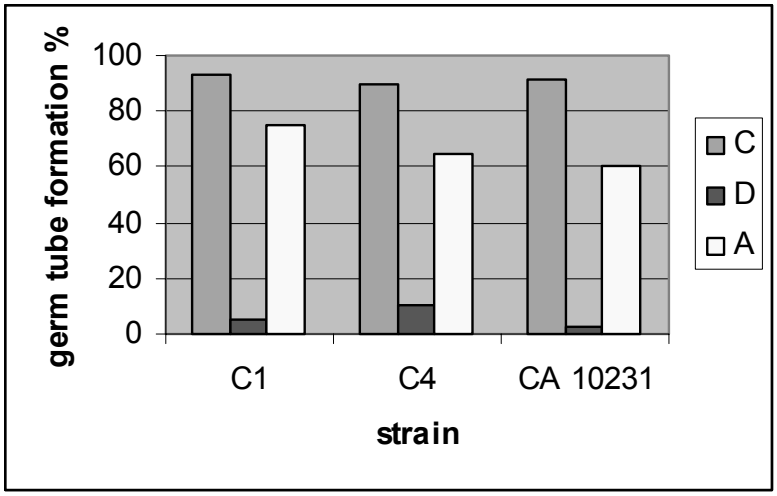

Figure 8. Effects of sodium diclofenac and aspirin (final concentration 0.1 mM) on germ tube formation by different *C. albicans* strains

There is some evidence that diclofenac inhibits the lipooxygenase pathways, thus reducing formation of the leukotrienes. Also, diclofenac may inhibit phospholipase A_2 as part of its mechanism of action. Phospholipase A_2 and phospholipase B have been identified in a large number of eukaryotic microbes, including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Phospholipases A and B cleave the fatty acid side chains of phospholipinds have been implicated in virulence in a number of parasitic and antifungal species, presumably via destruction of host cell membranes and subsequent lysis (11). These additional actions may explain the high potency of diclofenac.

Our findings suggest that cyclooxygenase-depending synthesis of fungal prostaglandins is important for morphogenesis and the survivor of *Candida albicans* cells and may act as a regulator in these processes. COX inhibitors of eicosanoids production (diclofenac sodium and aspirin) are effective in inhibiting morphogenesis and biofilm formation as well as exhibiting antifungal activity on biofilms (2).

Conclusion

Inhibitors of cyclooxygenase isoenzymes (aspirin and diclofenac) are effective in decreasing planktonic cells and germ tube formation of *Candida albicans.* Non-steroidal anti-inflammatory drugs specifically block the biosynthesis of fungal prostaglandins may be one strategy to combat fungal colonization and infection. The anti-inflammatory and analgesic properties of diclofenac and aspirin might represent an additional advantage for its use in the antifungal therapy of infections with *Candida* species.

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