Mitigation of the Biofilm Using Nanoparticles By Enhanced by D-alanine and D-proline

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Abstract

The present study was undertaken to investigate the susceptibility patterns as antifungal and growth of certain D-amino acids inhibitory effects, includes D-alanine(D-ala) and D-proline (D-pro). The obtained results indicated that D- proline acid is potent mostly as antifungal, among tested D-amino acids, then followed by D-ala. The study aim was evaluating the of D-amino acids and nanoparticles against *Candida albicans* adhered cells and bio-films. Results showed that D-ala MIC value was 25 µg/ml, while in methods of TCP, (D-pro) MIC 50 µg/ml value. Nanoparticles (lithium and silver) and amino acids (D-pro and D-ala) effect in preventing cells adhesion on polystyrene surface and mature bio-film inhibition was studied. Highest inhibition was obtained at D-pro concentration 50 µg /ml, while the lowest at concentration D-ala 25 µg /ml against mature bio-film and cell adhesion.

Keyword: *D-aminoacids, D-alanine, D-proline, bio-film, nanoparticles*

Introduction

Candida considered as part of human body normal flora that colonize different anatomical sites i.e. digestive tract, oral cavity, skin and vagina [1]. Candidacies are the cases where local change in the environment or debilitation of host take place and encourage overgrowth of *Candida* resulting in *candida* infection [2]. *Candida* species pathogenicity is reasoned to certain factors being virulence, i.e. adherence, evades host defenses ability, bio-film formation (on medical devices and on host tissue [3]**.** *Candida* species ability for forming bio-films drug-resistant is significant factor in their human disease contribution. Drug resistance progression within *Candida* bio-films correlated with a parallel elevation in process of maturation[4] .Resistant strains increase necessitates new targets search for new agent as antifungal [5]

In all life kingdoms, amino acids are basically found in L-enantiomeric form. Nevertheless, D-amino acids significant amounts are bacteria produced; as major D-amino acids producer at ecosystem [6]. In peptidoglycan cross-linking [7]. In recent years, D-amino acids are releasing via various species of bacteria in the growth stationary phase and behave as agents controlling modification and assembly of cell wall [8].

Nanoparticles for many years have been known for its importance as antimicrobial broad-spectrum activity against bacteria of G+ and G-, protozoa, certain viruses, and fungi [9], including antibiotic-resistant strains [10]. Ag, as agent being antimicrobial, is utilized in burn treatments, creams, wound dressings, and as coatings on various devices in medicine [11.]

Material & Method

The *Candida* isolate from different sources were isolated and identified. All samples were cultured on agar of Sabouraud Dextrose then were aerobically incubated for 24-48 h at 37 ºC [12]. Isolated of *Candida* were identified based on features of morphology on culture media, formation of germ tube, formation of Chlamydospore, CHRO Magar [13] and along Vitek 2 compact system utilizing [14] .Bio-film formation by *Candida spp*. isolates [15.]

Nanoparticles preparation

Nanoparticles in de-ionized water were insoluble based on producer [16].

Amino acids (D-ala and D-pro) Minimum

Inhibitory Concentrations Determination [15, 17].

Preparation of D-ala and D-pro was done for determining planktonic cells MIC. Solution of 1 M as stock for every amino acid was prepared in D.W. These solutions were filter-sterilized via membranes passage through of 0.45 μm (Billerica, MA. USA). Such were prepared to obtain various molarities for every amino acid, beginning with 100 μg/ml and following dilution series were preformed with the media till concentrations point ending. Tests of MIC were preformed in ninety six plate's flat bottom micro-titer (TPP, switerland). With hundred ml broth of Mueller-Hinton, every test well was filling. Addition of 100 μl of solution as stock to 1st test well and mixing, then dilutions series was applied across the plate after that ten μl of microorganism liquid of (*Candida albicans isolate*) was applied for inoculating every plate well of micro-titer to reach a final size of inoculums 1.5×10^8 CFU/ml well with culture overnight. Fungi inoculums along broth of Mueller-Hinton but with no a.a. treatment were considered as positive controls growth, while D-amino acid without inoculums considered as negative controls. Under same conditions of experiment, all wells as control were prepared and incubated where plates incubated at 37C° for 48 hr. Wells were tested via naked eye for growth of microbes. MIC values were described as the lowest concentration of D –amino acid inhibiting microbial growth of 80%, comparing to positive and negative controls, and growth of microbes in wells as turbid was detected. Determination of MIC in triplicate was done.

Effect of combination of (D-ala and D-pro) and Nanoparticles(Ag and LiO_2) on the adherent cells and mature biofilm as treatment .

Bio-film assays formation were done utilizing 96_ micro-titer well plate, according to protocols of [15,18-19], with minor modifications. Briefly *C. albicans* isolate were cultured overnight in SD Broth and dilution preformed to the resulting culture as 1:100 (SDB + 1% w/v glucose). Suspensions of cells (two hundreds μL) were poured in every well and incubation at 37ºC for 48 h was preformed, after bio-film formation for 48 h. Every well of plate micro-titer was filled with hundred ml of media and hundred μl of D-pro fifty μg/ml and D-ala twenty five μg/ml, whereas well of control no amino acids were added. Every concentration for each tested amino acid was triplicate assayed. Incubation for plates was preformed for 24 h at 37 ºC. The fungi as planktonic were taken away via dish shaking over tray of waste loaded by sterile D.W. Solution (crystal violet) of 0.1% w/v subsequently was added to every well where plates were stand for staining at room temperature for 10 min. Solution (crystal violet) was taken away through plate submerging in tray of water. Inverting to plates was done and on paper towels was topped to take away liquid as excess and left for air drying. Ethanols of 95% v/v applied to wells stained at room temperature for 10 min for dye solubility. Suspension of fungi in every well was well mixed and its OD was read at 490 nm at micro-plate reader. Also the effect of mixing 25 μg/ml of D-ala with twenty five μg/ml of Lio2, fifty μg/ml silver and fifty μg/ml D-pro with twenty five μg/ml of Lio2, fifty μg/ml silver were prepared by loaded 50 μl of each amino acid and nanoparticle with 100 μl of medium after biofilm formation for 48 h and the other steps were the same.

Statistical Analysis

The experiments in the current study was designed as factorial experiments (2×6), every combination treatment was replicated 3 times. By SPSS 2010 program, Duncan Multiple test and ANOVA were applied to show differences among means at ($p < 0.05$).

Result and Discussions

D-ala and D-pro Minimum Inhibitory Concentrations (MIC_s) Determination

Table (1) expresses values of MIC for D-amino acids in respect to isolate of *C. albicans*. D-proline MIC was 50 μg/ml. The MICs for D-alanine was 50 μg/ml

Table (1): (D-ala and D-pro) MIC means against isolate of *C. albicans*.

Substance	Plate tissue culture (μg/ml) (MICs)
D-alanine	25
D-proline	50

values of MIC for D-lysine and D-alanine were 18 and 39 µg/mL^{-1} [20]. In other study, [21] recorded D-isomers antifungal activities, L-isomers with no detectable activity against species under test (MIC more than two hundreds µg/µL). D-lys as one among D-amino acids tested revealed activity as highest against *Candida albicans* with a 6 µg/µL value of MIC. Least species susceptibility was *Candida glabrata* of treatment to mostly D-amino acids. Growth of *Candida krusie* was extensively mostly inhibited via D-lys then by D-ala, while considerable D-ser higher concentration was required for growth inhibiting all species tested of *Candida*. It is obvious, D-pro exhibit no significant activity as anticandidal against tested species.

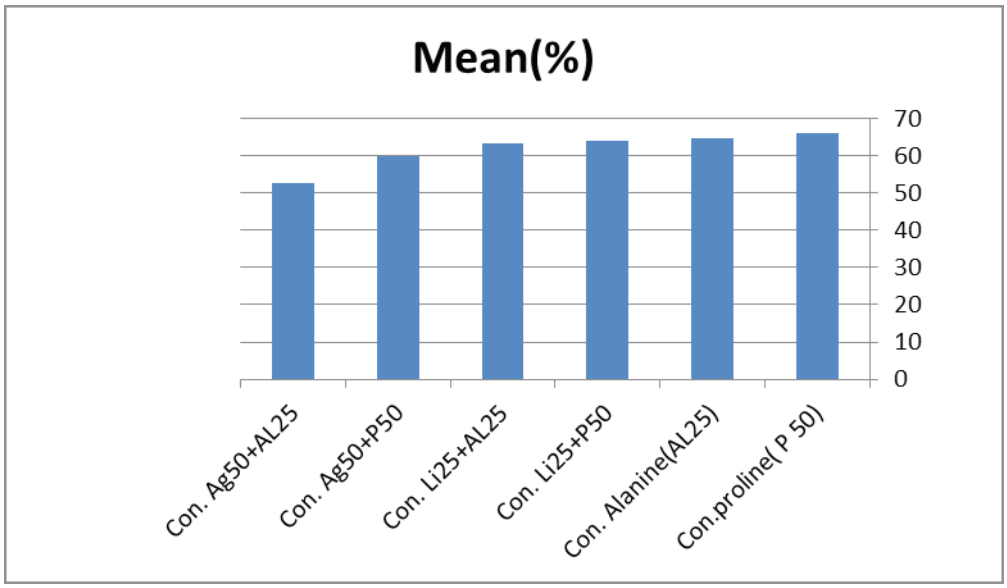
Antimicrobial peptides affect dendrites' cells recruitment and inflammation, thus immune response modulating [22-23]; some AMPs can induce apoptosis [24]. Limited studies were reported regarding ultra-structural level of D-amino acids effect with fungal cells. It was proposed that *C.albicans* exposure to D-amino acids yield membrane changes significant, cell surface pits formation and ultimately poress formation and death of cell[25].

Effect of combination of (D-ala and D-pro) and Nanoparticles(Ag and LiO_2) on the adherent cells and mature biofilm as treatment .

Highest mature bio-film and adherent cells were (70.714 and 52.848) % in D-Pro(conA) acid of 50 µg/ml with high differences of significant comparing to other concentrations which of no significant against mature bio-film and planktonic cells**.** Although the lower percentage value (52.509) of mature bio-film and adherent cells, inhibition was noticed for isolate of *C.albcans* at combination D-amino acids (Ag50+AL25) µg/ml of sub-MIC and sub-MIC of nanoparticles(Ag), respectively, Tabel(2),fig(1)

Table (2): Effect of combination of (D-ala and D-pro) and Nanoparticles(Ag and LiO_2) on the adherent cells and mature biofilm.

Factors	Treatments	Amino Acid			
		Mean (%)	±	S.d.	Sig.
A	Con.proline(P 50)	66.215	±	6.879	A
	Con. Alanine(AL25)	64.708	±	11.044	A
	Con. Li25+P50	64.041	±	11.933	A
	Con. Li25+AL25	63.429	±	13.778	A
	Con. Ag50+P50	59.786	±	13.554	Ab
	Con. Ag50+AL25	52.509	±	14.120	B
LSD P ≤ 0.05		9.003			
B	Amino acid. adherent cells	70.714	±	7.675	A
	Amino acid Bio	52.848	±	8.761	B
LSD P ≤ 0.05		5.198			
L.S.D.	Least Significant Difference				
	Significant Difference at p ≤ 0.05				
	Non Significant Difference				



Figure(1): Effect of combination of (D-ala and D-pro) and Nanoparticles(Ag and LiO_2) on the adherent cells and mature biofilm

Observed report showed that in assay of bio-film, both L- and D- Asp, Glu, and Cys enantiomers with 40 mM concentration inhibit significantly formation of *S. mutans* bio-film, where other A.A. not. Asp and Cys anti-bio-film activity were higher than of Glu. At 20 mM concentration, Glu significantly did not prevent formation of bio-film, [26.] The reported that D-aspartic acid inhibitid biofilm formation on tissue culture plates similar to [27],which observed that the high concentration above (10mM) inhibited the growth of *staphylococcus aeureus* planktonic cells. In recent years, D-amino acids proved to have significant roles in regulating disassembly and bacterial bio-films formation, and might express bio-film prevention general strategy [28-30]. Hassan *et al.*(2013) stated that results obtained revealed the Ag-NPs MIC50, Grisofulvin and Itraconazole on *T. mentagrophytes* and *C. albicans* which were (8±0.18) µg/ml, (4±0.25) µg/ml and (2±0.10) µg/ml respectively, on *C. albicans*. Such result confirms requiring investigation agents as alternative or drugs combinations, i.e. SN use with drugs as antifungal. Combinations use along lower concentrations drug able to elevate efficacy of drug and minimize drugs adverse effects [31]. Based on SN effect yeast cells via membranes attacking, hence membrane disruption potential. Such authors noticed, via microscopy as transmission electron, pits formation on the *C. albicans* membrane surfaces and ultimately pores formation and subsequent death of cell.[32]

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References

1. Balazs, D.J; Triandafillu ,K.; Wood, P.;Chevolot, Y.;van Delden, C.; Harms, H.;Hollenstein, C .and Mathieu, H.J. Inhibition of bacterial adhesion on PVC endotracheal tubes by RF-oxygen glow discharge, sodium hydroxide and silver nitrate treatments. Bio. 2004.25:2139–2151.
2. Bardaweel,S.K. D-amino Acids: Prospects for New Therapeutic Agents. J. Medical and Bioengineering 2014,Vol. 3, (3).
3. Bardaweel.S.K; Darwish,R.M.; Alzweiri,M.H.and Al-Hiari,Y.M. Synergism and Efficacy of Some Naturally Occurring D-amino Acids Against Clinically Relevant Bacterial and Fungal Species. Jordan Journal of Pharmaceutical Sciences. 2014.7: (3).199-212.
4. Bjarnsholt, T.; Kirketerp-Møller, K.; Kristiansen, S.; Phipps, R.; Nielsen, AK.;Jensen, PØ.;Høiby, N. and Givskov, M Silver against *Pseudomonas aeruginosa* biofilms. APMIS.2007. 115:921–928.
5. Cava,F.;Lam,H.;de pedro, M.A.and Waldor,M.K.

Emerging knowledge of regulatoryroles of D-amino acids in bacteria ,Cell, Mol, life Scie2011 68:817-831.

6. Christensen, G.D.; Simpson, W.A.; Bisno, A.L. and Beachey, E.H. .Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. J.Infect Immun. 1985.37: 318-26 .
7. Gow, N.A.;van de Veerodonk,F.L.;Brown A.J. and Netea M.G.*Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat. Rev. Microbiol. 2011,10:112-122.
8. Hamester, M.; Wanderley, L.; Brustolin, J. and Fuentefria, A.M. .Incidence of *Candida spp*.in oral cavity of elderly with removable, total orpartial, dental prosthesis in the City of Coronel Freitas, State of Santa Catarina, Brazil . Revista Panamericana de Infectología 2010. 12(1):17-22.
9. Hang, Y., Wang, M., Yu, J., Wei, H. .Aspartate inhibits Staphylococcus aureus biofilm formation. Microbial Letters FEMS 2015.362, p p: 7-25.
10. Hata, D.J.; Hall, L.; Fothergill, A.W.; Larone, D.H. and Wengenack, N.L.A.. Multicenter evaluation of the New VITEK2 Advanced colorimetric yeast Identification card. J. Clin. Microbio. 2007. 45(4):1087-1092.
11. Hochbaum,A.I.;Kolodkly-Gal,l.; Foulston ,l.;Kolter, R.;Aizenberg ,J.and Losick,R.Inhibitory effects of D-amino acids on *staphylococcus aureus* biofilm development. J.Bactreiol 2011.193 .5616-5622.
12. Holtje, J. V. "Growth of the stress-bearing and shape-maintaining murein sacculus of escherichia coli," Microbiol Mol Biol R, 1998. vol. 62, pp. 181-203,
13. Kim, K.J.;Sung ,W.S.;Suh ,B.K.;Moon, S.K.;Choi ,J.S. and Kim, J.G .Antifungal activity and mode of action of silver nano-particleson *Candida albicans*. Biometals. 2009. 22:235-242.
14. Kim, S., Kim, I.-W., Kwon, Y.-N., Yun, E.-Y., and Hwang, J.-S. Synthetic Coprisin analog peptide, D-CopA3 has antimicrobial activity and pro apoptotic effects in human leukemia cells. J. Microbiol. Biotechnol. 2012. 22, 264–269.
15. Kolodkin-Gal,I.; Romreo ,D.; Cao,J.;Calrdy, R.;Kolter,R and Losick,R.D-aminoacids trigger biofilm disassembly, Science. 2010. 328: 627-629,
16. Kuhn, D. M. and Ghannoum, M. A. .*Candida* biofilms: antifungal resistance and emerging therapeutic options.Curr .Opin. Investig Drugs 2004.5: 186–197.
17. Lam ,H. ; Oh, D. C. ; . Cava, F.; Takacs ,C. N; Clardy, J. de Pedro, M. A. and Waldor, M. K. "D-amino acids govern Stationary phase cell wall remodeling in bacteria," Science , 2009. vol. 325 pp. 1552-1555.
18. Lee, E., Kim, J.-K., Shin, S., Jeong, K.-W., Lee, J., Lee, D. G., et al. . Enantiomeric 9-mer peptide analogs of protaetiamycine with bacterial cell selectivities and anti-inflammatory activities. J. Pept 2011. Sci. 17, 675–682.
19. Li, X. .The impact of D-amino acids on formation and integrity of biofilm-Effect of growth condition and bacteria type. M.Sc. Thesis. Biology Department. College of Science. 2013. Rice University.
20. Mader, J. S., Salsman, J., Conrad, D. M., and Hoskin, D. W. Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Mol. Cancer Ther. 2005. 4, 612–624. doi: 10. -77.
21. Raju S.B; and Rajappa S. Isolation and Identification of *Candida* from the Oral Cavity. ISRN. Dentistry 2011 ; vol: 7.
22. Sardi, J. C.; Almeida, A. M. and Mendes Giannini, M. J. New antimicrobial therapies used against fungi present in subgingival sites – a brief review. Arch Oral 2011.Bio.l56: 951–959 .
23. Seneviratne, C. J.;Jin, L. and Samaranayake, L. P. Biofilm lifestyle of Candida: a mini review. Oral Dis .2008..14: 582–590.
24. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W. and Azeredo, J. *Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS. Microbiol. 2011. Rev.36: 288–305.
25. Silva, S.;Negri, M.;Henriques, M.;Oliveira, R.;Williams, D. W. and Azeredo, J. . Adherence and biofilm formation of non *Candida albicans Candida* species.Trends2011 .Microbiol.19:241–247 .
26. Stobie, N.;Duffy, B.;McCormack, D.E.; Colreavy, J.;Hidalgo, M.;McHale, P.and Hinder, S.J. Prevention of *Staphylococcus epidermidis* biofilm formation using a low-temperature processed silver-doped phenyltriethoxysilane sol–gel coating. 2008. Bio. 29:963–969.

27. Tani, K., Murphy, W. J., Chertov, O., Salcedo, R., Koh, C. Y., Utsunomiya, I., et al. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. 2002. Int. Immunol. 12, 691–700. doi: 10.1093/intimm/12.5.691.

28. Tong,Z.; Zhang,L.; Ling, J.; Jian,Y.; Huang,L. and Deng, D. AnIn Vitro Study on the Effect of Free Amino Acids Alone or in Combination with Nisin on Biofilms as well as on Planktonic Bacteria of *Streptococcus mutans* 2014. PLoS ONE 9(6): e99513 .doi:10.1371/journal.

29. Vazquez-Munoz, R.; Avalos-Borja,M. and Castro-Longoria, E. Ultrastructural Analysis of *Candida albicans* when exposed to silver nanoparticles. PLOS. 2014.9(10): e108876.

30. Wei, G.X.;Xu, X. and Wu, C.D In vitro synergism between berberine and miconazole against planktonic and biofilm *Candida* cultures.Arch. .2011. Oral .Biol. 56: 565–72.

31. White, T. C.;Marr, K. A. and Bowden, R. A. .Clinical, cellular, and molecular factors that contribute to antifungal drug resistance.Clin. Microbiol. 1998.Rev.11:382–402.

32. Yigit, N.;Aktas, E.; Dagistan, S. and Ayyildiz, A.. Investing biofilm production, coagulase and hemolytic activity in *Candida* species isolated from denture stomatitis patients. The Eurasian J.2011. Med. 43:27-32.