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Antimicrobial and Antibiofilm Activity of D-amino acids Combined with Nanoparticles against *Candida albicans*

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ABSTRACT
For the examined acids of D-amino, our findings indicated that D-aspartic (D-asp) acid was of high antifungal potentential, and then D-glycine. Also, the current study aimed to determine the effects of D-amino acids and nanoparticles against *Candida albicans* adhered cells and biofilms, results illustrated the D-asp concentration of 50 µg /ml caused inhibition as the highest, whereas the least was of D-gly 100 µg /ml of against mature bio-film cell adhesion and. The results proved that the combination of amino acid (D-glycine and D-aspartic acid) and nanoparticles (silver and lithium) at sub MIC concentrations ($p \leq 0.05$) inhibited cell adhesion and mature biofilm. Scanning electron microscope (SEM) results in observing the structure consisted of biofilm isolate *C.albicans* revealed, whereas therapy of D-asp had an effect on bio-film layers of more losing loss in the layers merged of bio-film and destroyed it. On the other hand, merged lithium along the D-asp effect destroyed cells and caused the bio-film single layer survival.

Keyword: D-aminoacids, antifungal, antimicrobial, nanoparticles, *C.albicans*.

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INTRODUCTION

Candida considered as natural flora in the body of human that colonizes various anatomical locations such as the digestive tract, skin, vagina and oral cavity (Seneviratne et al.,2008). In cases where host weakness occurs, or where the local climate changes to encourage overgrowth of Candida, that cause candidiasis (as infection) (Gow et al., 2011). As *Candida* transited to disease from a harmless communal, it renders pathogen to depend on the host immune system and *Candida* virulence factors (Yang et al., 2003).

Species of Candida pathogenicity is due to specific factors of virulence, for example adherence, the host defenses capability for evading, and the bio-films formation (on medical devices and host tissue) (Silva et al.,2011b). Species of Candida' ability for developing bio-films drug-resistant is an significant factor contributing to disease in human. As in the vast majority of microbial biofilms (Rajendran et al., 2010), In *Candida* biofilms, sessile cells are of low susceptibility to antimicrobials compare to cells of planktonic (Kuhn et al.,2004). The drug resistance development in bio-films of *Candida* was correlated with a increase parallel in the maturation process (Sard et al., 2011). For new agents of antifungal, the increase in resistant stresses needs new targets (White et al.,1998).

In Recent years, acids of D-amino have been known to make a significant role in controlling the disassembly and formation bio-films of bacteria, and show strategy of general bio-film prevention (Hochbaum et al., 2011). The enantiomers for L-normal are the D- and are components of abundant forth peptidoglycans cell membrane of bacteria. Within the cell, D-amino acids are important to maintain a high internal pressure of osmotic (Caprros et al., 1992). It was proved that bacteria exclusively do not reject D-amino acids (exogenous) that able to integrate in peptidoglycan in a manner similar to D-amino acids (endogenous) (Aaron et al., 2007). In this work attempted to know if there is significant role of acids of D-amino on bio-films formation in *candida albicans* isolate.

MATERIAL & METHOD

Isolations and Identification of *Candida*

The *Candida* were isolated and identified from different sources. All sample was cultured on agar of Sabouraud Dextrose (SDA), and then was aerobically incubated for 24-48 h at 37 ºC (Rajuand Rajappa,2011) . Identification of *Candida* isolate was performed reliant on the merits of morphology on culture media, germ tube formation, Chlamydospore formation, CHRO Magar (Hamester et al.,2010) and using system of Vitek two compact (Hata et al.,2007). Biofilm formation by *Candida spp*. isolate was detected (Christensen et al.,1985) and the interpretation of the results by tissue culture plate method was detected (D'Antonio et al.,2002).

Minimum Inhibitory Concentration (MIC) Determination of D-amino acids

Isolates of multi-drug resistant sensitivity of single *C. albicans* isolate to amino acids (D-glycine and D-aspartic acid) was determined and the concentration of minimum inhibitory (MIC) was measured by method of Agar well diffusion (Nothan et al.,1978) and measuring MIC through the method of plate tissue culture (TCP) (Vazquez-Munoz et al.,2014).

Effects of acids of D-amino treatment on formation of Bio-film

Assays for the formation of biofilms were carried out by utilizing 96 plates of well microtiter, according to the documented protocols (Christensen et al., 1985; Silva et al., 2011a), with minor modifications. Briefly *C. albicans* isolate were cultured in SD Broth rapid where the culture was diluted as (1:100) (SDB + glucose 1% w/v). Two hundred µL

of suspension cells were replaced in a single well and then incubated at 37 ºC for 48 h, after formation of bio-film for 48 h. Each microtiter well of plate was filled with medium of 100 ml and 100 µl of D-glycine 100 µg/ml and D-aspartic acid 50 µg/ml, except a standard well of no any amino acid. A single concentration was triply assayed for a single measured amino acid. Incubation was then done to the plate for 24 h at 37 ºC. Planktonic fungi was eliminating by shaking the dish with sterile distilled water over a waste tray. Thereafter, crystal violet 0.1 % w/v solution was applied to single well where plate stayed stained for 10 min at room temperature. After that, solution of crystal violet was eliminated through plate submerging in a water tray. Plates were then inverted and overlaid on paper towels to absorb excessive fluid and put for air drying. Wells that were stained then treated at room temperature with 95% v/v ethanol for 10 min for dye solubilization. Fungi suspension for a single well was mixed well and at 490 nm its OD measured by microplate reader. Also the effect of mixing D-Glycine 100 µg/ml with Li02 25 µg/ml of, silver 50 µg/ml and D-aspartic acid 50 µg/ml with Li02 25 µg/ml, silver 50 µg/ml 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.

Scanning Electron Microscopy analysis (SEMa).

SEM was applied for detection of *Candida* isolate bio-film construction produced in the untreated plates of 96-well micro-titer and treated D-glycine, D-aspartic acid and in both D-aspartic acid with LiO2 as explained above. Bio-film for analyzing, was" fixed and then dehydrated in a variety of solutions of ethanol (in 70% and 95% for 10 min, and for 20 min in 100%)," and by using desiccator, the plates were held. wells" bottom sections (1× 1cm) with a scalpel blade were cut, mounted onto aluminium stubs, sputter coated with gold and observed" under scanning electron microscope a S-360 (Monteiro et al.,2012b and Monteiro et al.,2013).

Statistical analysis

The experiments were laid as factorial experiments (2×6), for a single treatment replication was done triply. SPSS program 2010 was applied for analyzing the data. Differences' between means was done according to Analysis of variance (ANOVA) and Duncan' Multiple test at ($p < 0.05$), Capital and small letters for rows and columns were used, respectively where similar letters are of no significant (Quinn and Keough 2002).

RESULT AND DISCUSSIONS

Minimum Inhibitory Concentrations (MIC_s) Determination of D-amino acids:

D-amino acids (glycine and aspartic) MIC_s were evaluted on isolate of *C. albicans* that able to yield some factors of virulence besides this isolate proved to have the resistant of multi-drug pattern.

Table (2) revealed the values of MIC Nystatin and nano-particles on isolate of *C. albicans*. D-aspartic acid MIC was 50 µg/ml for the 2 methods. D-glycine MICs was 50 µg/ml and 25 µg/mL, respectively in compairson to Nystatin MIC which was 2 µg/ml.

Table 1: Means of Nystatin and D-amino acids MIC of against isolate of *C. albicans*.

Substance	Minimum Inhibitory Concentration(MIC)(mM)	
	Agar well diffusion (µg/ml)	Tissue culture plate (TCP) (µg/ml)
Nystatin	2	2
D-glycine	50	100
D-aspartic acid	50	50

Significant effect was reported of D-glycine and D-aspartic acid were with concentration (50mM) on the *Staphlococus aureus* and *Escherichia coll*, respectively (Tawfeeq,2016). While L-tyrosin, L-isoleucine and L-serine have no activity to prevent plank tonic cells. The report observed that D-lysine and D-alanine had demonstrated strong antifungal efficacy against *Candida albicans*. D-alanine and D-lysine MIC values were 38 and 17µg/mL^{-1} (Bardaweel,2014). In other study Bardaweel et al (2014) recorded the antifungal activities of acids D-amino, D-lys showed MIC value of 6 µg/µL which was considered activity as highest a against *Candida albicans*. *Candida glabrata* was susceptible very low to treatment of acids of most D-amino. Growth inhibition of *candida krusie* was by D-lys mostly then by D-ala.

HDP or peptides of antimicrobial (AMPs) are bioactive and flexible molecules of immunity against pathogens of all types, i.e. viruses, bacteria, champignons, cancer cells, and parasites. Hong et al(2014) Their diverse mechanism of action may be: (i) AMPs binding and disturbing the integrity structural of membrane, by detergents or pores such as mechanisms Bahar, A. and Ren (2013) ; (ii) AMPs bio-films extending through adhesion surface reduction, embedded bacteria destroying or metabolic pathways interacting with the participation in the creation of biofilms (Segev-Zarko et al.,2015); (iii) AMPs affect inflammation and dendritic cell recruitment and thus modulate immune response (Lee et al.,2011); (iv) some apoptosis AMPs inducing (Mader et al., 2005). Various types D-amino acids are shown in the current study in spite of their effects for change in similar types. Unfortunately, no data available for comparison.

Effect of acids of D-amino on formation of Biofilm

The combination was applied by using the acids of D-amino (glycine and aspartic) with Ag and LiO_2 as nanoparticles in 2 various concentrations (sub-inhibitory concentration) to determine the combination effects in the reduction or inhibition of *C. albcans* isolate for the mature bio-film and adherent cells.

Inhibition of significance was noticed on D-amino acids (D-glycine and D-aspartic acid) combination with nanoparticles (Ag and LiO_2) in mature bio-film in comparison to adherent cells Table (3) and (Fig 1). Significant inhibition happened at most combinations of acids D-amino (glycine and aspartic) + Ag and LiO_2 nanoparticles, except, concentration Li25+A50 µg/ml where no inhibition of significance in bio-film was observed compared to adherent cells.

Table 2: Combination effect of amino acid and Ag and Lio_2 Nanoparticles on the adhered cells and mature bio-film

Amino Acid									
Concentrations µg/ml	Inhibition of adherent cell(%)					Inhibiton of Biofilm(%)			
	Mean		±	S.d.		Mean		±	S.d.
Con. A50	72.381	A a	±	1.443		60.048	A a	±	1.459
Con.G100	72.857	A a	±	5.188		56.558	B ab	±	8.875
Con. Li25+A50	73.810	A a	±	4.062		54.272	A ab	±	7.295
Con. Li25+G100	75.714	A a	±	2.474		51.143	B ab	±	3.960
Con. Ag50+A50	70.595	A ab	±	2.508		48.977	B ab	±	10.123
Con. Ag50+G100	58.929	A a	±	13.093		46.089	B b	±	14.260
LSD P ≤ 0.05	**12.730**								

* Capital and small letters indicate the comparison among rows and columns respectively where similar letters are of no significant different at ($p < 0.05$), according to Duncan Multiple test $p < 0.05$.

The highest mature bio-film and adherent cells were (72.381 and 60.048) % in D-aspartic acid 50 µg/ml with significant of high differences in comparison to other concentrations which were of no significant effect against mature bio-film and planktonic cells. In spite of the lower % value (46.089 and 58.929 %) of mature bio-film the and adherent cells respectively, combination of D-amino acids sub-MIC (Ag 50 +G 100) µg/ml and nanoparticles sub-MIC (Ag), respectively for *C. albcans* isolate inhibition was observed.

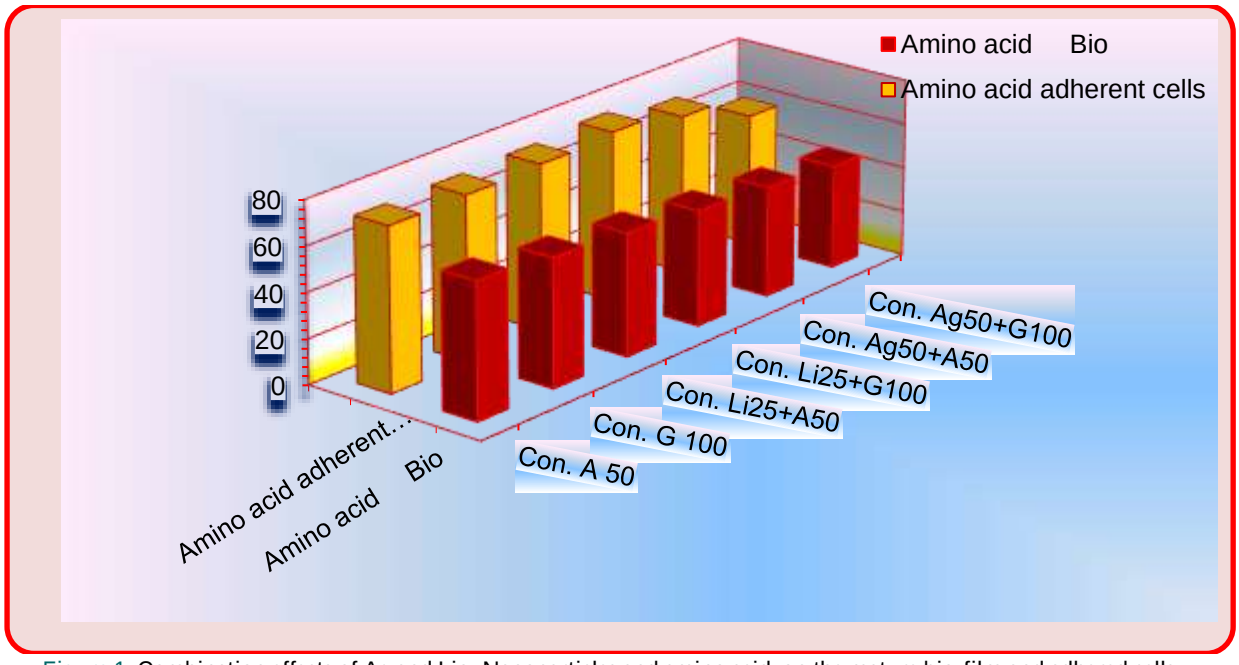

Figure 1: Combination effects of Ag and Lio_2 Nanoparticles and amino acid on the mature bio-film and adhered cells

D-amino acids utilizing is a recent struggle biofilm technique. Some studies have proposed that acids of D-amino may block the development of biofilms and spread established biofilms (Hang et al., 2015) .The report observed that showed in the assay of bio-film. All enantiomers of D- and L- of Asp, Glu, and Cys may substantially prevent the *S. mutans* bio-film development at their respective 40 mM concentrations, where the last amino acids did not. The anti-bio-film movement of Asp and Cys were more to that of Glu. At a 20 mM concentration of Glu did not significantly inhibit formation of bio-film, but Asp and Cys were active (Tong et al.,2014). The inhibition activity was mentioned in the article initially reporting the influence on growth of bio-film of D-amino acids (Cava et al., 2011). In another report, glycine revealed its effect as inhibitory on

the bio-film formation and was inhibition concentration-dependent (Tong et al.,2014).

In significant respect, lessening in growth of bio-film was detected at concentration of 4% in *Escherichia coli* (Leungpailin and Dolye, 2000).The reported that D-aspartic acid inhibited bio-film development on tissue culture plates like to Hang.Y. M *et al* (2015),which observed that the high concentration above (10 mM) inhibited the growth of *staphylococcus aeureus* planktonic cells. It is believe that the mode of feat of D-amino acid in biofilm formation is by prevented initial attachment which is the primary steps of biofilm formation in bacteria by reducing extracellular polysaccharides and protein production in early growth stage (Vazquez-Munoz et al.,2014). Newly, acids of D-amino have been known to havea a significant role in blocking the disassembly and development of bio-films of bacteria, and may have general strategy for prevention of bio-film [9-10].

Ag-NPs showed antifungal action in combination with Chlorhexidine digluconate and Nys contra *C. albicans* bio-films (Monteiro et al.,2013). Hassan et al (2013) reported a revealed results that the Grisofulvin and Itraconazole MIC50 and Ag-NPs on *T. mentagrophytes* and *C. albicans* that were (4±0.25) µg/ml, (8±0.18) µg/ml and (2±0.10) µg/ml respectively, on *C. albicans*. In comparison, the process for silver operation is multi-factorial. Nanoparticles may connect to proteins having sulphate which trigger defects in the cell membrane, Interact with compounds containing phosphorous, such as DNA (prevention of reproduction of cell), Or strike the respiratory chain that induces death in cells (Rai et al.,2009). Also, the large volume-to-surface ratio of silver means stronger contact with particles and cells (Hassan et al.,2013) So, the similarity observed could be attributed to both specific and identical drug targets on the biofilm cells (Monteiro et al.,2013).

Analysis of Scanning Electron Microscopy (SEMa)

SEM supervised the bio-film structure. In the control, Figure (2, A),*C. albicans* bio-film seemed as multi-layer comprised ecompletely of yeasts or hyphae nestled in a dense matrix of extracellular. Amazingly, after handling by 100 µg/ml of D-gly alone Figure (2, B), the bio-film offered a lower yeast close layer than that in the standard group. Though, after a handling by 50 µg/ml of D-asp alone Figure (2, C), the bio-films illustrated a lower more tight structure. Moreover, some amino acid particles were attached to the biofilm matrixes (amino acid aggregation).

Nevertheless, when combination treatment of D-asp with 50 and 25 LiO_2 µg/ml, respectively Figure (2, D), the bio-films arising from this is aggregated *Candida* cells and lower amount of extracellular matrix.

Conversely, SEM findings presented here showed a style of D-gly, D-asp and combination D-asp with LiO2 aggregation. Rai et al., (2009) reported that the unprocessed biofilm of *Candida albicans*324A/94 revealed a complex oval network and fungal cells extension in a large extracellular matrix; ,if cured by 13.5 µg/ml silver no expressive morphological change was detected. Conversely, when cured by silver 22 µg/ml and nystatin 215 µg/ml only, the resulting bio-films displayed a somewhat less compressed structure and were more visible in the NYT-treated community, proposing a lower matrix amount extra-cellulaire. Amazingly, in the bio-films cured with the integrate. The cells of fungi synthesized significant quantities of extracellular polymer content and then covered by the matrix. These findings are in agreement with Monteiro et al (2012). Furthermore, such findings assist the assumption that the initial size of particle in bio-films could be a bad view of the actual nanoparticle size (Monteiro et al.,2012b). Fernandes et al (2016) Displayed the SEM photos verified these findings, showing reductions in the cells number by *C. albicans* in all formations of bio-film.

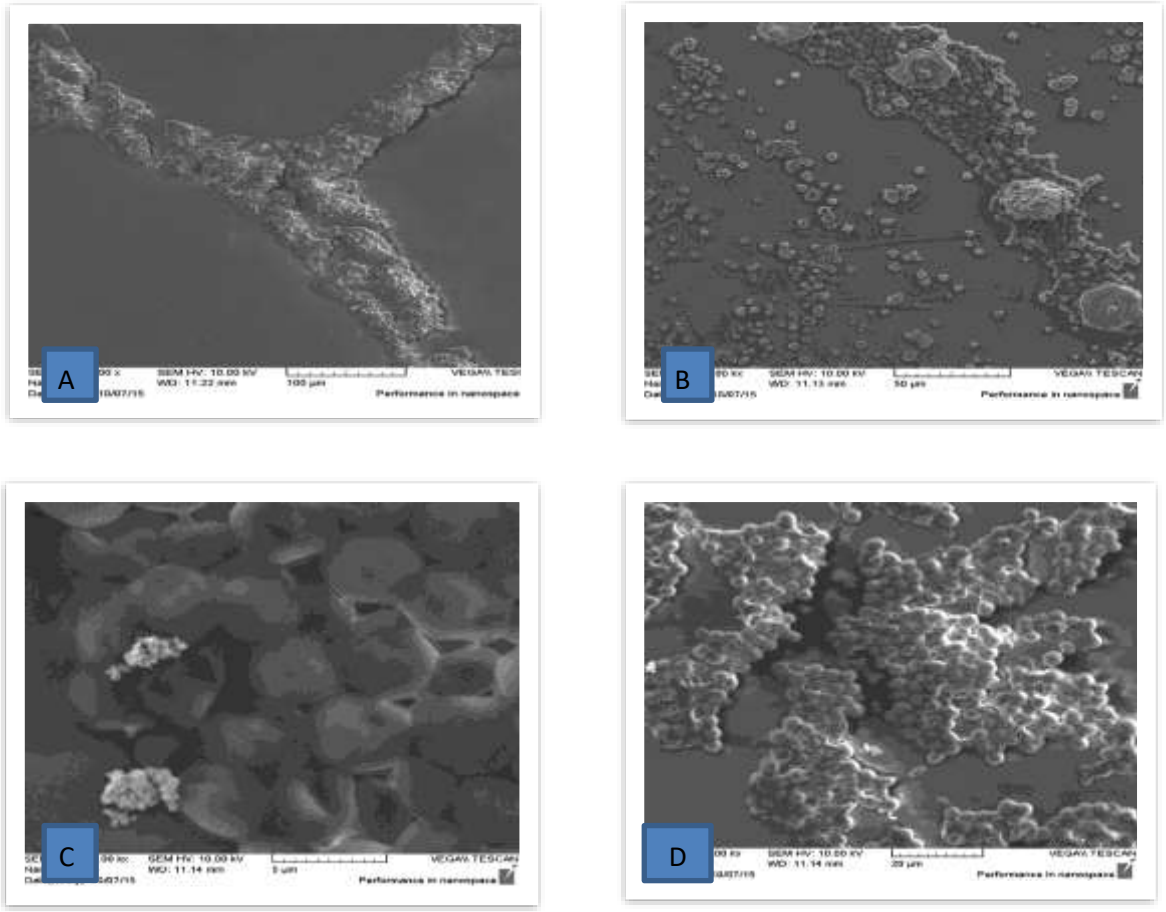

Figure 2: (SEM) supervation of the *Candida albicans* structure bio-films after dealing by various concentrations(µg/ml) of D-gly,D-asp and combination (A) unprocessed biofilm (control), (B-D) biofilms cured by 100D-gly, 50D-asp and 50 D-asp/25 LiO2 (combination).

CONCLUSION

In short, amino acid and nanoparticles considered as important replacement to traditional agents of antifungal for potential Candida treatments.

DECLARATION OF CONFLICT OF INTEREST

None.

ETHICAL APPROVAL

"Not required"

RANDOMIZED CONTROLLED TRIAL

"Not applicable"

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