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Abstract

Several studies reported possible antimicrobial activities of selected D-amino acids against *Candida albicans*. The present study was undertaken to investigate the antifungal susceptibility patterns and growth inhibitory effects of certain D-amino acids, including D-glycine(D-gly) and D-aspartic acid(D-asp). Our findings indicate that D- aspartic acid is the most potent antifungal, among the examined D-amino acids, followed by D-glycine.Also The aim of this study was to evaluate the of D-amino acids and nanoparticles against *Candida albicans* adhered cells and biofilms, The results showed that the value of MIC for D-glycine was 50 µg /ml by well diffusion methods while in TCP methods the value of the MIC 100 µg / ml, MIC for zinc were 50 µg / ml for both methods. The effect of amino acid (D-glycine and D-aspartic acid) and nanoparticles (silver and lithium) in preventing the adhesion of cells on the surface of the polystyrene and the inhibition of the mature biofilm was studied. The results showed that the highest inhibition was at a concentration 50 µg /ml of D-asp , while less inhibition was given for the concentration 100 µg /ml of D-gly against cell

adhesion and mature biofilm. 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. The results of scanning electron microscope (SEM) in observing the structure consisted of biofilm isolate *C.albicans* showed that treatment with D-gly caused the loss of biofilm layers, while D-asp therapy caused more loss of in the merged layers of biofilm and destroyed it, while lithium merged with the effect of D-asp caused destroyed cells and the survival of a single layer of biofilm.In summary amino acid and nanoparticles may have the potential to be an effective alternative to conventional antifungal agents for future therapies in *Candida*.

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

Introduction

Candida is a part of normal flora of the human body colonizing various anatomical site like oral cavity, digestive tract, vagina and skin[1]. In cases where there is host debilitation, or where there is a change in the local environment promoting *Candida* overgrowth, this led to *candida* infection (referred to as candidiasis) [2]. The *Candida* which is transition from a harmless commensal to disease causing pathogen depends on the immune system of the host and virulence factors of *Candida* [3].

The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and on medical devices) [4]**.** The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease. As in the vast majority of microbial biofilms [5], sessile cells within *C. albicans* biofilms are less susceptible to antimicrobial agents than are planktonic cells [6]. The progression of drug

resistance within *Candida* biofilms has been associated with a parallel increase in the maturation process [7]. The increase in resistant strains necessitates a search for new targets for new antifungal agents [8].

Amino acids are mainly found as the L-enantiomeric form in all kingdoms of life. However, significant amounts of D-amino acids are produced by bacteria; the major producer of D-amino acids in the ecosystem [9]. In cross-linking of peptidoglycan [10]. Recently, it was shown that D-amino acids are released by diverse bacterial species in the stationary phase of growth and act as agents controlling cell wall assembly and modification [11]. Additionally, D-amino acids are used as an energy source via the metabolism by the D-amino acid dehydrogensae enzyme; a flavoenzyme that oxidatively transformsD-amino acids into their corresponding α-keto acids [12-14]

Recently, D-amino acids have been identified to play very important roles in regulating the formation and disassembly of bacterial biofilms, and may present a general strategy for biofilm prevention [15-17]. D-Amino acids are enantiomers of natural L-amino acids and are abundant components in the peptidoglycans of the bacterial cell membrane .D-Amino acids are vital for the maintenance of a high internal osmotic pressure inside the cell[18,19]. It has been found that bacteria do not exhibit an exclusive rejection of exogenous D-amino acids, which can be incorporated into the peptidoglycan in a similar way to endogenous D-amino acids [20,21]. In this work attempted to know if there is an important role of D-amino acids on biofilms formation in *candida albicans* isolate.

Material & Method

The *Candida* isolate from different sources were isolated and identified . All sample was cultured on Sabouraud Dextrose agar (SDA), then was incubated aerobically at 37ºC for 24-48 hrs [22]. *Candida* isolated were identified depending on the morphological features on culture medium , germ tube formation, Chlamydospore formation, CHRO Magar [23] and with the use of Vitek 2 compact system[24] .Biofilm formation by *Candida spp*. isolates [25].

Preparation of nanoparticles.

The nanoparticles were insoluble in deionized water, according to the producer factory guidelines, 5% acetic acid in deionized water was used for solvent. 16mg of each nanoparticle [silver(Ag)and Lithium Oxide(LiO_2)] was solved in 1 mL 5% acetic acid and the stock solutions were filter-sterilized by passage through membrane filter (0.45 µm) and the resulted solutions were diluted 5 folds by the culture medium (SDB or YPD) [26]. After preparing the primary concentration, the final concentrations (200- 100-50-25- 12.5) µg/ml were selected.

Determination of Minimum Inhibitory Concentration (MIC) of Amino acids:

Sensitivity of one *C. albicans* isolates to amino acid (D-glycine and D-aspartic acid) was studied and the minimum inhibitory concentration (MIC) was determined:-

1:- The effect of D- amino acids Determination of Minimum Inhibitory Concentrations (MICs) [25, 27].

D-glycine and D-aspartic acid were prepared to determine the MIC for planktonic cells. A stock solution of 1 M of each amino acid was

prepared in distilled water. The stock solutions were filter-sterilized by passage through 0.45 µm membranes (Billerica, MA. USA). These were prepared to achieve different molarities of each amino acid, starting with 100 µg/ml and serial dilution was done with the medium to the end point concentrations. MIC test were conducted in 96 flat bottom microtiter plates (TPP, switerland). Each test well was filled with 100 ml Mueller-Hinton broth. A 100 µl of the stock solution was added to the first test well and mixed, then a series of dilutions was prepared across the plate after that 10 µl of liquid of the microorganism (*Candida albicans isolate*), was used to inoculate each microtiter plate well to achieve a final inoculum size of 1.5×10^8 CFU/ml well with overnight culture. Mueller-Hinton broth and fungi inoculum but without amino acid treatment were assigned as positive growth controls, whereas negative controls were D-amino acid treated wells but without inoculum. All control wells were prepared and incubated under the same experimented conditions. Plates were incubated for 48 hr at 37Cº. The wells were examined for microbial growth by naked eye. The MIC value was described as the lowest D – amino acid concentration that inhibited about 80% of microbial growth, relative to the negative and positive controls, microbial growth in the test wells was detected as turbid. MIC determination was carried out in triplicate.

1- Agar well diffusion method [28,29].

A- Yeast suspension was prepared in concentration 1.5×10^8 cells / ml.

B- A sterile cotton swab moistened with the inoculums suspension was used to apply to a 90 mm diameter plate containing Mueller-Hinton agar supplemented with 2% glucose and add some drop from methylene blue .

C- The plates were allowed to dry for(5-15) mins and 6 mm in diameter wells were done in Mueller-Hinton agar by using (5mm) cork borer size.

D- 0.1 ml from each concentration of Nanoparticles was added to each well by Micropipette and nystatin was used as positive control , and the plates were incubated at 37˚C for 48 hrs . The activity of each concentration was determined by measuring the inhibition zone around wells by using a metric ruler in millimeters.

Biofilm formation by *Candida*. isolate [25,30]

To study the ability of isolation to produce biofilm , one isolate of *Candida spp*. were grown in sabouraud dextrose broth(SDB) containing 8% glucose in 96-well polystyrene tissue culture plates and incubated at 37ºC for 48 hrs under aerobic conditions. After incubation, the planktonic cells were washed ten times with deionized water, and the adhering fungi cells in each well were fixed with 200 µl of absolute methanol for 20 mins. The plates were emptied and left to dry overnight. The adhering cells were stained with 200 µl of 0.1% crystal violet for 15mins, and excess stain was rinsed off. The plates were washed with distilled water and air-dried overnight. The crystal violate dye bound to the adherent cells was dissolved with 1ml of 95% ethanol per well, and the plates were read at 490 nm using micro ELISA auto reader. The experiment was performed in triplicates, and the absorbance of wells containing sterile SDB was used as the negative control the result calculate as in table (1).

Standard method for quantitative biofilm detection test was used to detected the ability *Candida* isolated to produce biofilm from fresh agar plates were inoculated in 10 ml of Sabouraud dextrose broth(SDB)

with 8% glucose w/v. Broths were incubated at 37 ºC for 24 hrs. The culture were then diluted 1:100 with fresh medium and transferred to individual wells of sterile 96 well- polystyrene tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37 ºC for 48 hrs. After incubation, the content of each well were removed by gentle tapping. The wells were washed with sterile distilled water once. This removed free floating fungi. Biofilm formed by yeasts adherent to the wells were stained by (0.1%) w/v crystal violet. Excess stain was removed by using 1ml of 95% ethanol per well and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader at wavelength 490 nm, and the interpretation of the results was conducted as shown in table (1). The experiment was performed in triplicate and repeated three times .

Table (1): Classification of fungi adherence by tissue culture plate method [31-32]

OD values	Adherence	Biofilm formation
< OD c	Non	Non
OD < OD ≤ 2*Odc	Weakly	Weak
2*ODc < ODt≤ 2*ODc	Moderately	Moderate
4ODc<OD t	Strong	High

The effect of D-amino acids on Biofilm formation as treatment

Biofilm formation assays were performed using 96_ well microtiter plate, based on the protocol by [25,30,33], with minor modifications. Briefly *C. albicans* isolate were cultured in SD Broth overnight and the resulting culture was diluted to 1:100 (SDB + 1% w/v glucose) Cell suspensions (200 µL) were pipetted into each well and incubated for 48 h at 37ºC ,After biofilm formation for 48 h. Each well of microtiter plate was loaded with 100 ml of medium and 100 µl of 100 µg/ml D-glycine and 50 µg/ml D-aspartic acid , except a control well without any amino acid. Each concentration for every amino acid tested was assayed triplicate. The plate then incubated at 37Cº for 24 hrs. The planktonic fungi were removed by shaking the dish over a waste tray filled with sterile distilled water. Subsequently 0.1% w/v crystal violet solution was added to each well and the plate was left to stain for 10 min at room temperature. Next the crystal violet solution was removed by submerging the plate in a water tray. The plate was then inverted and topped on paper towels to remove excess liquid and left to air dry. The stained wells were then treated with 95% v/v ethanol for 10 min at room temperature to solubilize the dye. The fungi suspension in each well was mixed well and its optical density was measured in a microplate reader at 490 nm. Also the effect of mixing 100 µg/ml of D-Glycine with 25 µg/ml of Li02, 50 µg/ml silver and50 µg/ml D-aspartic acid with 25 µg/ml of Li02, 50 µg/ml silver were prepared by loaded 50µl 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). [34-35].

SEM was used to observe the structure of *Candida* isolate biofilm formed within the 96-well microtiter plates untreated and treated with D-glycine , D-aspartic acid and in combination D-aspartic acid with LiO2 as

described above .For this analysis , biofilm was fixed and dehydrated in a series of ethanol solutions (70% for 10 min, 95% for 10min and 100% for 20 min), and the microtiter plates were kept in a desiccator . Sections (1× 1cm) of the wells bottom were cut with a scalpel blade, mounted onto aluminium stubs, sputter coated with gold and observed under a S-360 scanning electron microscope .

Statistical analysis [36-37]

The experiments in this study was set up as factorial experiments (2×6),each treatment combination was replicated three times.

Analysis of variance (ANOVA) and Duncan Multiple test were used to explain the differences between means at ($p < 0.05$),Capital and small letters indicate to compare between rows and columns, respectively and the similar letters are non-significantly different. Using SPSS program 2010 .

Result and Discussions

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

The MIC_s of D-amino acids (D-glycine and D-aspartic acid) were determined on *C.albicans* isolate which had the ability to produce some virulence factors (biofilm and adherent cells) in addition to that this isolate revealed the multi-drug resistant pattern.

Table (2) showed the MIC values of nanoparticles and Nystatin on *C.albicans* isolate.MIC for D-aspartic acid was 50 µg/ml for both method . The MICs for D-glycine was 50 µg/ml and 25 µg/mL, respectively compared to MIC of Nystatin was 2 µg/ml.

Table(2). Mean of MIC of D-amino acids and Nystatin against *C. albicans* isolate.

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

In a local study accomplished by [38] reported the significant effect of D-glycine and D-aspartic acid were with concentration (50mM) on the *Staphlococus aureus* and *Escherichia coll* ,respectively. while 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 showed demonstrated moderate antifungal activities against*Candida albicans*. The MIC values for D-alanine and D-lysine were 39 and 18µg/mL^{-1} [39]. In other study , [40] recorded the antifungal activities of the D-isomers, the L-isomers did not show any noteworthy activity against whichever of the tested species (MIC > 200 µg/µL). Among the tested D-amino acids, D-lys revealed the highest activity against *Candida albicans* with a MIC value of 6 µg/µL. *Candida glabrata* was the least susceptible species to most D-amino acids treatment. *Candida krusie* growth was most extensively inhibited by D-lys followed by D-ala, whereas considerably higher concentration of D-ser was needed to inhibit the growth of all tested *Candida* species. Apparently, D-pro did not exhibit any significant anticandidal activity against whichever species tested.

Antimicrobial peptides (AMPs) or HDP are efficient and versatile immune molecules bioactive against all types of pathogens, including bacteria, viruses, fungi, parasites even cancerous cells [41-43] AMPs are short peptides between 12 and 50 residues, produced by all living

organisms, and they present not only antimicrobial activity but also immunomodulatory functions. Their mechanism of action can be diverse: (i) AMPs can bind and disrupt the membrane structural integrity, through pore formation or detergent like mechanisms [42,44]; (ii) AMPs disperse biofilms by reducing the adhesion to surfaces, killing of embedded bacteria or interfering with the metabolic pathways involved in biofilm formation [45-46]; (iii) AMPs influence inflammation and recruitment of dendritic cells, hence modulating the immune response [47-48]; (iv) some AMPs can induce apoptosis [49-50]. There are very limited studies that report at the ultrastructureal level the effect of D-amino acids with fungal cells. It was stated that exposure of *C.albicans* to D-amino acids produced significant changes to the membrane , the formation of "pits" on the cell surface , and finally the formation of pores and cell death[50]. These results in the study are shown by different type D-amino acids, although may change their effects in similar types. Unfortunately they do not access to more data for comparison.

Effect of D-amino acids on Biofilm formation as treatment

The combination was applied by using the D-amino acids (D-glycine and D-aspartic acid) with nanoparticles(Ag and LiO_2) in two different concentrations (subinhibitory concentration) to investigate the effect of combination in the inhibition or reduction of the adherent cells and mature biofilm of *C.albcans* isolate .

Results showed that the D-amino acids (D-glycine and D-aspartic acid)combination with nanoparticles(Ag and LiO_2) obtained significant inhibition in mature biofilm as compared with the adherent cells, Significant inhibition happened at most combinations of D-amino acids (D-glycine and D-aspartic acid) with nanoparticles(Ag and LiO_2) that

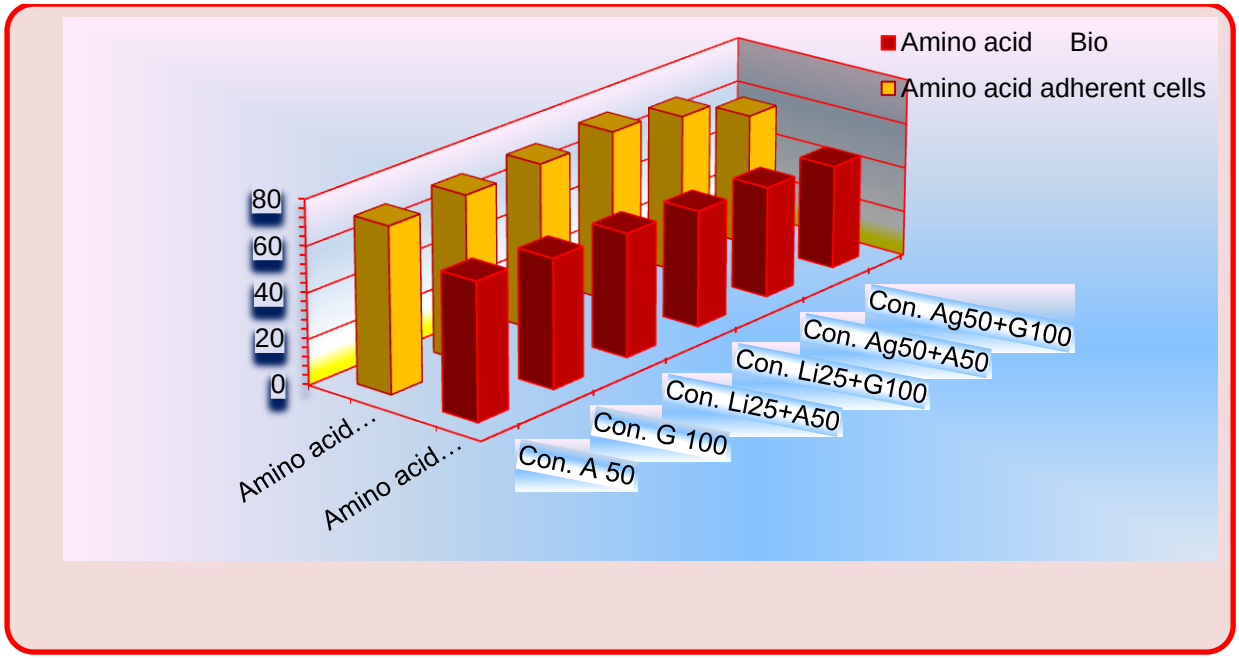
used, except, (Li25+A50 µg/ml) concentration which gave no significant inhibition in biofilm as compared to the adherent cells, Table (3), (figuer 1).

Table (3): Effect of combination of Amino acid and Nanoparticles(Ag and Lio2) on the adhered cells and mature biofilm as treatment.

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 to comparison between rows and columns respectively, and the similar letters are non- significantly different at (p < 0.05), according to Duncan Multiple test p < 0.05.**

The highest adherent cells and mature biofilm were (72.381 and 60.048) % in 50 µg/ml of D-aspartic acid with high significant differences as compared with other concentrations were not significant against planktonic cells and mature biofilm .Although the lower percentage value (58.929 and 46.089%) of the adherent cells and mature biofilm respectively, inhibition was obtained at combination of sub-MIC of nanoparticles(Ag) and sub-MIC of D-amino acids(Ag50+G100) µg/ml respectively for *C.albcans* isolate.



Figure(1): **Effect of combination of amino acid and Nanoparticles(Ag and Lio2) on the adhered cells and mature biofilm as treatment.**

The use of D-amino acids is a recent strategy for combating biofilms. Some studies have indicated that D-amino acids can inhibit biofilm formation and disperse existing biofilms [51] . The report observed that showed In the biofilm assay, both D- and L-enantiomers of Cys, Asp, and Glu at their respective concentration of 40 mM could significantly inhibit S. mutans biofilm formation, but the other amino acids did not. The antibiofilm activity of Cys and Asp were superior to that of Glu. At a concentration of 20 mM, Glu did not significantly prevent biofilm formation, but Cys and Asp were effective. At 10 mM, Cys, Asp, and Glu, with the exception of D-Asp, hardly prevented biofilm formation [52]. the inhibithion activity was mentioned in the article initially reporting the effect of D-amino acids on biofilm growth[53], in other report observed that glycine showd an inhibitory effect on biofilm formation and the extent of inhibition was concentration-dependent[54].

In regard to this asignificant decrease in biofilm growth was observed at 4% concentration in *Escherichia coli*[55].The reported that D-aspartic acid inhibitid biofilm formation on tissue culture plates similar to Hang.Y.M et al(2015),which observed that the high concentration above (10mM) inhibited the growth of *staphylococcus aeureus* planktonic cells. The decrease cellular metabolism activity might be the reason for producing less protein and DNA in the matrix of the biofilm formed in the presence of aspartic acid.however varied inhibithion efficacies of aspartic acid were observed for biofilmformed by clinical *Staphylococcus aereus* isolates. There might be mechanisms other than decreasing the metabolic activity.e.g the biofilm phenotypes affecting the biofilm formation in the presence of asparti acid[27]. It is believe that the mode of action 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 [27]. Recently, D-amino acids have been identified to play very important roles in regulating the formation and disassembly of bacterial biofilms, and may present a general strategy for biofilm prevention [15-17].

D- Amino acids are enantiomers of natural L-amino acids and are abundant components in the peptidoglycans of the bacterial cell membrane .D- Amino acids are vital for the maintenance of a high internal osmotic pressure inside the cell[18-19]. It has been found that bacteria do not exhibit an exclusive rejection of exogenous D-amino acids, which can be incorporated into the peptidoglycan in a similar way to endogenous D-amino acids [20-21].

The study by [35] who showed the antifungal activity of silver nanoparticles in combination with nystatin and chlorhexidine digluconate

against *C. albicans* and *C. glabrata* biofilms. [56] reported that the obtained results revealed the MIC50 of Grisofulvin and Itraconazole and Ag-NPs on *C. albicans* and *T. mentagrophytes* which were (4±0.25) µg/ml, (8±0.18) µg/ml and (2±0.10) µg/ml respectively, on *C. albicans* .On the other hand, the mechanism of action of silver is multifactorial. Nanoparticles can bind to sulphur-containing proteins resulting in defects in the cell membrane, interact with phosphorus-containing compounds like DNA (preventing cell reproduction), and attack the respiratory chain causing the cell death[57]. In addition, the high surface to volume ratio of silver provides better interaction between particles and cells[58] Thereby, it is possible that the synergism found is due to both different and similar targets of the drugs on the biofilm cells[35].

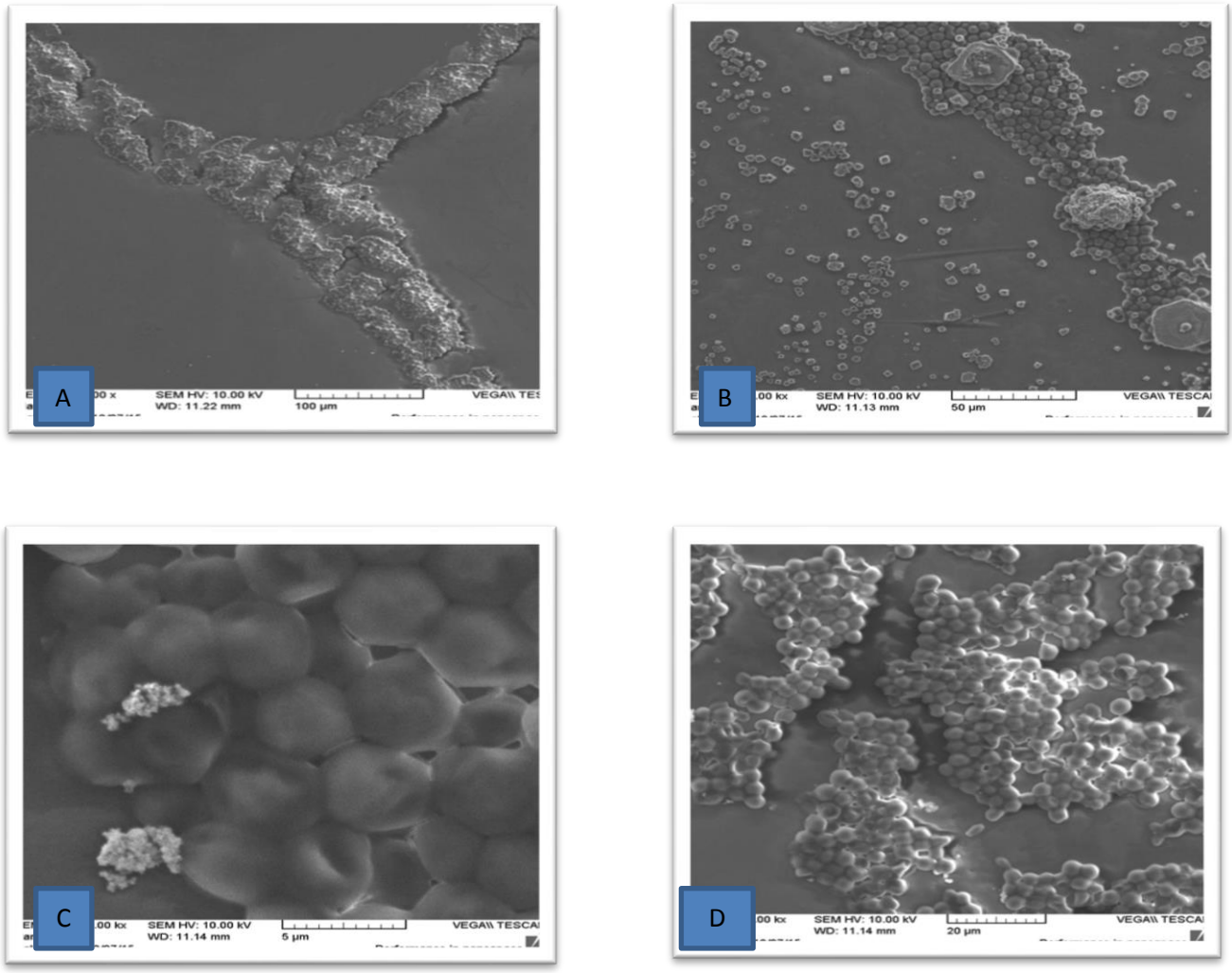
Scanning Electron Microscopy analysis (SEMa).

Biofilm structure was monitored by SEM . In the control fig (2,A),*C. albicans* biofilm appeared as multilayer composed entirely of yeasts and hyphae surrounded by a thick extracellular matrix. Interestingly, after the treatment with 100 µg/ml of D-gly alone fig (2,B),the biofilm presented a less compact layer of yeast than that in the control group. However, after a treatment with 50 µg/ml of D-asp alone fig (2,C),the biofilms showed a less more compact structure. Moreover, some amino acid particales were attached to the biofilm matrixes (amino acid aggregation) .

On the other hand, when treated with combination D-asp with LiO2 (50 and 25) µg/ml respectively Fig (2,D), the resulting biofilms were formed by aggregated *Candida* cells and lower amount of extracellular matrix.

On the other hand, SEM observations described here revealed a trend of D-gly, D-asp and combination D-asp with LiO2 aggregation.

[59] reported that the untreated *C. albicans* 324LA/94 biofilm displayed a dense network of oval and elongated fungal cells entangled in a thick extracellular matrix,when treated with 13.5 µg/ml silver no significant morphological alteration was observed .However, when treated with 27 µg/ml silver and 216 µg/ml nystatin only, the resulting biofilms showed a slightly less compact structure and it was more evident in the group treated with NYT, suggesting a less amount of extracellular matrix. Surprisingly, in the biofilms treated with the combination .The fungal cells synthesised great amounts of extracellular polymeric material and were hidden by the matrix, resulting in a very compact biofilm. These findings are in agreement with [60]. Additionally, these observations support the conclusions that the original particle size may be a poor exhibit of true nanoparticle size in biofilms [61]. [62] showed that SEM images confirmed these results, showing decreases in the number of cells in all Biofilm formation by *C. albicans*.



Figure(2) Scanning electron microscopy (SEM) observation of the structure of *C. albicans* biofilms after treatment with different concentrations(µg/ml) of D-gly,D-asp and combination (A) untreated biofilm (control), (B-D) biofilms treated with 100D-gly, 50D-asp and 50 D-asp/25 LiO2 (combination).

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