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Extraction of *Klebsiella pneumoniae* and *Candida albicans* Biofilm and Studying their Cytotoxic Effects on Human Lymphocytes

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Abstract

The microbial polymeric biofilm matrix belongs to a single microbial species or multiple microbial species that are directly or indirectly responsible for over 80% of all microbial infections. A six-month laboratory study was conducted to extract the biofilms from *Klebsiella pneumoniae* and *Candida albicans* isolates to study their properties and their toxic effects on blood lymph. The results showed that treating 20 samples of *K. pneumoniae* and 100 samples of *C. albicans* biofilms with gentamicin and fluconazole antibiotics led to the inhibition of biofilm production for all the studied isolates by 74.9% and 64.32%, respectively. The results of screening electron microscopy (SEM) showed that the diameters of some *K. pneumoniae* cells ranged from 0.18-0.47 µm, while the diameters of *C. albicans* cells ranged from 121.82-133.89 nm. Fourier-Transform Infrared Spectroscopy (FTIR) of biofilmrevealed the presence of compounds in the matrix of the biofilm of *K. pneumoniae*, such as mannans and carboxylate (C=O), indicating the presence of sugar acids, proteins, and hydrocarbons. The results of the analysis of the biofilm produced by *C. albicans* showed a high percentage of sugars, as well as carboxyl and 2' acetyl. The Biofilm produced by *K. pneumoniae* inhibited the lymphocytes by 34.8% from the 150 µL sample. The lymphocytes showed higher inhibitory activity against *C. albicans* alone and against the combined biofilm of *C. albicans* and *K. pneumoniae*, where the highest percentage of inhibition reached 74.82% and 69.5% for the two treatments, respectively, at a sample volume of 100 µL. This leads to the conclusion that the biofilms of *K. pneumoniae* and *C. albicans* are responsible for over 80% of microbial infections. The use of antibiotics such as gentamicin and fluconazole led to the inhibition of biofilm production by up to 74.9% and 64.32% respectively, indicating the effectiveness of antibiotics in controlling biofilm growth.

Keywords: Human lymphocyte, Extraction, Fungal-Bacterial biofilm, scanning electron microscope, FTIR, Co-infection, *Klebsiella, candida*

1. Introduction

The respiratory system is exposed to many different diseases resulting from bacterial, viral, and fungal infections which usually occur by inhaling air through the respiratory tract. It is followed by an exudative effusion containing red and white blood cells, large phagocytic cells, fibrin, and biologically active microbes that may reach the bloodstream in sufficient numbers, causing septicemia with the possibility of metastatic abscesses in distant organs such as the brain and kidney (Nelson, 2001;Ibrahim *et al.*, 2017).

Biofilms are complex surface-attached communities of microorganisms held together by self-produced polymer matrixes mainly composed of polysaccharides, secreted proteins, and extracellular DNAs. A biofilm can consist of a single microbial species or a combination of different species of bacteria, protozoa, archaea, algae, filamentous fungi, and yeast that could be strongly attached to each other or to biotic or abiotic surfaces (Muhammad et al, 2020). The microbial biofilms by which complex microbial cells are surrounded are found in diverse locations and ecosystems (Hall-Stoodley*et al*., 2012; Nouri *et al.*, 2015) with a very diverse potential as in biological wastewater treatment (Rassin*et al.*, 2015; Geladari *et al.*, 2019), biotechnological productions of chemicals or drugs, and bioremediation of soils and groundwater purification processes (Flemming*et al.*, 2016,Abdullah*et al*.,2019).

From a pathological perspective, they are behind tremendous problems in the infections and the diseases they cause to their hosts (Rather et al., 2021). The humanmicroflora, consisting of bacteria, fungi, and viruses, invariably show that they produce polymicrobial biofilms that are even more complex to manage than monomicrobial ones (Anju *et al.*, 2022, Costa-Orlandi *et al.*, 2017,Hashim *et al*.,2023).

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Polymicrobial biofilm-related infections complicate the diagnosis and treatment and cause a higher mortality rate compared to single-species biofilm infections. Particularly, infections caused by fungal-bacterial biofilms are serious public health concerns (Galdiero *et al*. 2021, Anju *et al* 2022).

A recent review summarizes an insight into biofilm structure, diffusion, and advection, i.e. mass transport. Due to these diverse impacts and implications, biofilms are the subject of interdisciplinary research, encompassing biology, chemistry, analytics, and engineering (Herrling, 2019). However, biofilms still need to be further studied and understood. In biological systems, biofilms that produce a matrix of highly hydrated extracellular polymeric substances (EPS) cause infections with high resistance to conventional drugs (Galdero *et al*. 2021), especially those -biofilms- caused by both fungi and bacteria. Recent estimates suggest that biofilm-associated infections are directly or indirectly responsible for over 80% of all microbial infections in humans (Nobile and Johnson, 2015).

Klebsiellapneumonia species, which are Gram-negative rods, remain one of the most significant causes of bloodstream infections in patients with immunity-related issues. One of the *K. pneumoniae* several intrinsic factors responsible for its ability to cause infection is its ability to produce biofilms on medical devices. It contains two types of antigens, namely the Somatic antigen (O), and the Capsular antigen (K), segregating the strains into 8 and 88 serological patterns respectively (Greenwood *et al*., 2007). The colonies of this bacteria are characterized by their large size, exhibiting pink color when grown on MacConkey agar due to their ability to ferment lactose, and by their mucoid consistency due to the presence of the capsule. These bacteria colonies also tend to be raised above the medium and have a regular spherical shape (Todar, 2007). The bacteria have been a major cause of death among enteric bacteria with drug-resistant cases. The biofilm forming ability allows it to colonize the host tissues and medical devices, survive in hostile environments, and resist antibiotics and host immune response (Tutelyan *et al*., 2022).

Samples of mixed-species infections often exhibit the presence of *C. albicans* and *K. pneumoniae*, especially from those found in the bloodstream and oral infections or respiratory diseases.

Fungal infections caused by molds are a leading cause of death among immunocompromised patients (Baddley*et al*. 2003) such as aspergillosis, which is caused by the *Aspergillussp.* Another notable fungus, Candida sp. causes systemic mycoses, especially in immunocompromised patients. Factors that contribute to their growth on mucosal surfaces include exposure to moisture, availability of glucose as a food source, and little competition from commensal bacteria, as well as dysfunction of epithelial and lymphoid cells (Brooks *et al*., 2001). *C. albicans* is a member of the human digestive and reproductive flora, with a prevalence of 40% to 60% in healthy adults (Erdogan and Rao, 2015). It is usually a commensal organism but, can become pathogenic in susceptible hosts. It is one of the few Candida species that causes human candidiasis resulting from fungal overgrowth (Martins *et al*., 2014; Erdogan and Rao, 2015).

Many *in vivo* and *in vitro* studies have shown that different bacteria and fungi have complex interactions in mixed-species biofilms in human health which is a phenomenon that shows how bacterial infections may play a modulatory role in fungal infections (Bandara*et al*., 2009). Maione *et al*. (2021) pointed out that although infections associated with mixed-species biofilms including *C. albicans* and *K. pneumoniae* have certain clinical significance, the description of interactions between *C. albicans* and *K. pneumoniae* in mixed-species biofilms is limited to a few observations.

Biofilms exhibit several characteristics that are very important to their survival strategy. Some general characteristics of biofilms include three-dimensional structure, presence of one or more microbial species, adherence to each other, adhesion to surfaces, and adherence to solid/liquid, or liquid/air interfaces. Although the extracellular matrix produced by the cells forming biofilms is a critically important factor for their structural integrity, the chemistry and physiology of biofilms can fluctuate depending on the resident microbes, and the surrounding environment (McCarty *et al*., 2014).

The nature of biofilm structure and physiological features also endow organisms with an inherent resistance to antimicrobial agents. Although the defense mechanisms of biofilms against antimicrobials are not yet clearly understood, some possible causes have been suggested by several scientists (Sánchez-Gómez et al., 2015).

The current study aims to extract and study the biofilms produced by *K. pneumoniae* and *C. albicans* and to determine their effects on lymphocytes. The objective of this study was evaluating the ex vivo biofilm formation potential of *Candida* and *Klebsiella*, and examining the produced biofilm's toxicity toward lymphocytes.

2. Materials and Methods

A laboratory study was conducted from September/2022 to February/2023 at the laboratories of the Department of Biology, College of Science, University of Baghdad, and the University of Al-Nahrain Center/ Al-Nahrain University. This study included 120 samples.

2.1. Collection of yeast samples:

100 yeast isolates which include *C. albicans*, *C. glabrata*, *C. kefyr*, *C.krusei*, *C. parapsilosis*, *C. rangosa*, and *C. tropicalis* were collected from Al-Tib Hospital and Yarmouk Hospital in Baghdad province, all isolates were collected from individuals with respiratory diseases. The isolates were identified using biochemical, and physiological tests, and the Vitek2 Compact system (Aubertine et al., 2006).

2.2. Collection of bacterial samples:

Klebsiella spp. isolates were obtained from educational laboratories in Al-Tib City/Baghdad from 20 patients admitted with infections in the respiratory system. The isolates were diagnosed using the Vitek2 Compact system (Aubertine et al., 2006).

2.3. Culture and identification of isolates:

Some steps were taken to check and confirm the microbial diagnosis as follows (Ellen *et al*. 1994):

Activating isolates on agricultural media: Isolates taken from the respiratory tract of the120 patients were cultured

and grown on solid Sabouraud Dextrose Agar (SDA). The cultured plates were incubated at 37 °C for 2-4 days. To reactivate them, three replicates were made for each isolate, and the characteristics of the surface colonies were observed included morphological characteristic shape, color, diameter, and height the growing colony on corn-meal agar, these isolates were transferred to a chromo-agar medium and incubated at 37 °C for 48–72 hours (Hospentha *et al*, 2006). microscopical characteristic studied when staining the yeast on glass slide, mixed with a drop of lactophenol cotton blue to observe the chlamydospores and yeast cells (Ellis *et al*., 2007). The ability of Candida spp. to develop biofilms:

The ability of *Candida* isolates to develop biofilms was tested using Congo Red Agar (CRA) medium, as described by Oliveira and Cunha (2010). inoculating "young colonies at the age of 24 hrs ,SDA medium" on Concho red medium using planning method, then the culture were incubated in aerobic conditions at a temperature of 37 C for 24 hrs. biofilm-forming isolates are black color.

2.4. The ability of Klebsiella to develop biofilms:

The ability of Klebsiella spp. isolates to form biofilms were detected using the microtiter plate method according to Adriana *et al*. (2013), as follows: -

2.5. Preparation of Biofilm:

The Yeast and bacteria used in the study were stimulated by growing them on culture media. Nutrient broth with 2% sucrose and SD broth with 2% sucrose was prepared. The fungi were grown in Sabaroud broth with 2% sucrose and the bacteria were grown in Nutrient broth with 2% sucrose. Then, catheters were placed in the media to aid in the isolation of the biofilm. Incubation was carried out for 4 days at a temperature of 37 °C. The culture media was poured off and 2-3 mL of normal saline was added to dissolve the biofilm attached to the walls and bottom of the conical flask (Adriana *et al.* 2013).The biofilm suspension and normal saline were placed in closed test tubes and subjected to sonication to kill the bacteria and fungi (the bacterial suspension was sonicated for one and a half hours, while the fungal suspension was sonicated for two hours). After that, the emulsion was cultivated on the media in Step 2 to ensure that it was free of bacteria and yeasts and that there was no growth. Different volumes of biofilms (150,100,50,25) were obtained and the suspension was prepared to study their effects on lymphoid cells.

2.6. Collection of Blood Samples to isolate lymphocytes:

The source of blood was a healthy 30-year-old male donor. Ten milliliters of blood was collected into sterile 5 mL tubes containing EDTA anticoagulant, and this blood was used for isolating lymphocytes. Lymphocytes were isolated according to the method described by Rafael and Vaclav (2000). Two hundred microliters of the isolated lymphocytes was taken and added to a plate, then the bacterial and fungal biofilm was diluted with PBS and filtered using 0.22-micron filters. Different volumes of the filtered biofilm were added to the plate (150-100-50-25 microliters) and incubated at 37°C for 24 hours in an incubator containing 5% CO_2. Then, MTT dye was added to the plate and incubated for 4 hours, followed by measurement of the results using an ELISA reader.

2.7. FTIR Spectroscopy Analysis:

FTIR spectral analysis is a fast and non-destructive technique that relies on the principle that atoms in molecules that are not closely bound absorb energy when exposed to infrared radiation (between 300 and 4000 cm-1). The FTIR spectrum gave us information about the molecular structure of the sample and type of bond . This analysis was performed following the method described by Mostafa *et al.* (2012), where an appropriate amount of the biofilms was mixed with Potassium Bromide (KBr) and transferred to an FTIR spectrometer within the wavelength range of (400-4000) nanometers.

2.8. Scanning Electron Microscopy (SEM) studies:

The SEM samples were washed in a phosphate-buffered saline (PBS) solution and fixed with 1.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH=7.4, for 24 hours at 48°C on a rotary shaker. The samples were then dehydrated through a graded series of acetone solutions (70, 80, 90, 96, and 100% acetone) for 20 minutes at room temperature. The critical-point-dried samples were then oriented, mounted on a metal stud, and coated with gold using a Polaron 5000 Sputtering System (Watford, England) before imaging. The samples were examined under a Japanese-made JSM6400 scanning electron microscope (JEOL) with digital imaging capabilities. The images were collected at an accelerating voltage of 15 kV, a filament current of 555 µA, and a working distance of 10 mm². All images were numbered as high-resolution TIFF files and then converted to high-quality JPEG files using Adobe Photoshop 7.0 (Kania*et al*., 2010). The detection of biofilm formation was assessed in 40 samples employing Congo Red Agar medium following (Oliveira and Cunha, 2010,Dheeb*et al*.,2022).

3. Results and Discussion

3.1. Detection of biofilm production using Congo Red Agar:

Out of the 40 isolates tested, 40% were found to produce a biofilm on the medium. This result agrees with the study conducted by Saxena*et al*. (2014), who reported a biofilm-producing isolate rate of 41.8% on Congo Red Agar medium. However, the results do not agree with those of (Khalaf, 2016, Awad *et al*.,2020), where the biofilm-producing isolate rate was 51.6%.

These results were obtained by observing the colony morphology on Congo Red Agar medium (Figure 1) as described by (Oliveira and Cunha, 2010). Biofilm-producing isolates appear as dry, black, or shiny colonies, while non-producing isolates appear as light pink colonies. The color change in colonies that occurred in the later stages of the incubation period might be due to the presence of secondary metabolites. The use of 5% sucrose or glucose is described as a key factor in determining the production of exopolysaccharides using nutrient-rich media (Oliveira and Cunha, 2010). The color change might also be due to the direct binding of Congo Red dye to certain sugars that make up certain complexes (Hassan *et al*., 2011).

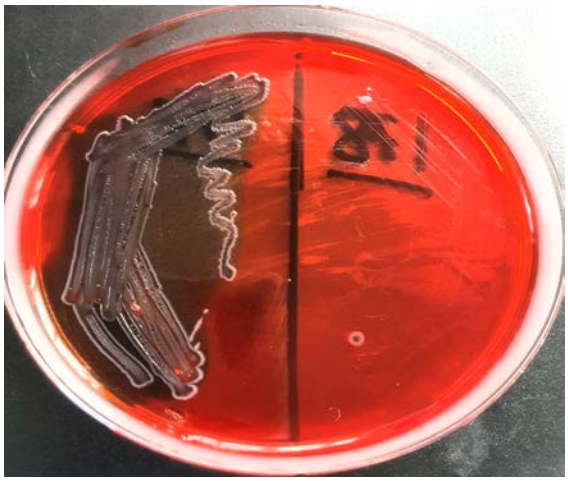

Figure 1.A biofilm-producing isolate of *C. albicans* is shown in bright black color on the surface of Congo Red Agar medium.

The effect of the antibiotic gentamicin on the biofilm production of *K. pneumoniae* isolates Table (1) shows the mean values of biofilm production from twelve K. pneumoniae strains and the inhibitory effect of gentamicin on biofilm production efficiency at 0%, 25%, 50%, and 75% concentrations. Isolate A12 had the highest significant (P≤0.05) biofilm production of 0.503 nm, while strain A6 had the lowest rate of 0.292 nm. A 75% concentration of gentamicin resulted in a 74.9% decrease in biofilm production to 0.187 nm compared to the control treatment, which recorded a biofilm production rate of 0.745 units. The interaction between *K. pneumoniae* strain production and the effect of gentamicin showed that strain A10 had the highest biofilm production of 0.949 nm. in control treatments, while isolate A11 had the lowest production of 0.157 nm. in the treatment of 75% concentration of gentamicin.

These results are consistent with those obtained by Cadavid and Echeverri (2019), who studied the gentamicin sensitivity of mature *K. pneumoniae* biofilms and found that adding gentamicin (1.0 µg/mL) to the mature biofilm formed in the presence of inhibitors (2-hydroxycinnamic acid and 3-methyl-2(5H)-furanone) led to a reduction of the remaining biofilm by 42.51% and 33.82%, respectively. The biofilms of *K. pneumoniae* isolates examined in this study showed a significant decrease in production efficiency due to exposure to several antibiotics, including gentamicin (Geladari*et al*., 2019). In an earlier study, *K. pneumoniae* biofilms in the laboratory revealed that the isolates were widely resistant to gentamicin; however, these isolates formed relatively low-mass biofilms (Naparstek*et al*. 2014). The differences in the abilities of the studied isolates to form biofilms were the cause of their sensitivity to antibiotics. Moreover, the thick matrix outside the cell impedes the spread of antibiotics, leading to decreased antibiotic activity (Flemming*et al*., 2016).

Table 1. Effects of concentration gentamicin on biofilm production in *K. pneumoniae* bacteria.

Biofilm Conc. (%) *K.pneumoniae* isolates	control	25	50	75	Biofilms average
A1	0.728 cde	0.626 def	0.217 j	0.202 j	0.443 ab
A2	0.690 c-f	0.430 gh	0.196 j	0.197 j	0.378 b-e
A3	0.768 bcd	0.321 hij	0.188 j	0.175 j	0.363 b-f
A4	0.737 cde	0.400 hi	0.194 j	0.163 j	0.374 b-f
A5	0.596 ef	0.242 ij	0.209 j	0.165 j	0.303 ef
A6	0.559 fg	0.207 j	0.226 j	0.177 j	0.292 f
A7	0.662 def	0.228 j	0.220 j	0.210 j	0.330 def
A8	0.718 c-f	0.273 hij	0.215 j	0.168 j	0.343 c-f
A9	0.776 bcd	0.293 hij	0.204 j	0.172 j	0.361 c-f
A10	0.949 a	0.320 hij	0.185 j	0.210 j	0.416 bc
A11	0.904 ab	0.313 hij	0.185 j	0.157 j	0.390 bcd
A12	0.848 abc	0.724 cde	0.195 j	0.245 ij	0.503 a
Concentration average	0.745 a	0.365 b	0.203 c		

*Means with the same letter are not significantly different according to the Duncan test (P ≤ 0.05).

3.2. Effect of the antifungal fluconazole on the biofilms produced by C. albicans

The data presented in Table 2 exhibits the average biofilm production of twelve strains of *C. albicans* and the effect of 0, 25, 50, and 75% concentrations of fluconazole on the efficiency of biofilm production. It is observed that isolate A12 recorded the highest significant (P≤0.05) biofilm production of 0.608 nm, while strain A6 recorded the lowest rate of 0.349 nm. Regarding the effects of fluconazole, a concentration rate of 75% led to a decrease in biofilm production to 0.223 nm, a decrease of 64.32% compared to the control treatment which recorded a biofilm production rate of 0.625 units. The interaction between *C. albicans* strains biofilm production and the effect of the antifungal fluconazole showed that isolate A12 had the highest biofilm production among the control treatments, reaching 1.117 nm, while the lowest production of 0.121 nm was recorded for isolate A6 in the 75% fluconazole concentration treatment.

The results of the present study indicate that continuous flow (60 ml per hour) of liquid through growing biofilms of *C. albicans* enhances matrix synthesis to a degree that significantly increases resistance to fluconazole. The biofilms of *C. albicans*grown under steady flow were

completely sensitive to the effects of fluconazole when exposed to high concentrations of the drugs. This is attributed to the sensitivity of the biofilm produced by *C. albicans* due to high drug penetration rates through these biofilms (Al-Fattani and Douglas, 2006; Dheeb*et al.*, 2015).

Table 2. Effects of concentration fluconazole on the production of biofilms by *C. albicans* isolates.

Biofilms Conc. (%) / *C. albicans* isolates	control	25	50	75	Biofilms average
A1	0.668 b	0.396 k-n	0.339 n-r	0.264 s-v	0.417 cd
A2	0.641 bc	0.370 l-p	0.272 r-v	0.169 way	0.363 f
A3	0.563 def	0.381 l-o	0.318 o-s	0.247 s-v	0.377 of
A4	0.485 g-j	0.444 i-l	0.341 n-r	0.211 vwx	0.370 f
A5	0.613 bcd	0.385 l-o	0.316 o-t	0.211 vwx	0.381 of
A6	0.502 f-i	0.417 j-m	0.358 m-p	0.121 y	0.349 f
A7	0.465 h-k	0.418 j-m	0.351 m-q	0.300 p-u	0.383 def
A8	0.667 b	0.527 e-h	0.370 l-p	0.242 tuv	0.451 b
A9	0.648 bc	0.524 e-h	0.226 u-x	0.227 u-x	0.406 cde
A10	0.539 e-h	0.475 hij	0.255 s-v	0.164 xy	0.358 f
A11	0.590 cde	0.482 g-j	0.375 l-o	0.240 uvw	0.4218 bc
A12	1.117 a	0.551 d-g	0.486 g-j	0.279 q-v	0.608 a
Concentration average	0.625 a	0.447 b	0.334 c	0.223 d	

*Means with the same letter are not significantly different according to the Duncan test ($P \leq 0.05$).

3.3. Biofilm examination using electron microscopy:

The present study employed a scanning electron microscope (SEM) by Japanese-made JSM6400 scanning electron microscope (JEOL), to examine and visualize the composition of biofilms on surfaces (Figure 2).

At low magnification, irregular formations packed on a flat surface were observed, and at high magnification (Figure 2- K1 and C1) these formations showed bacterial cells with disk-shaped and round shape outlines (Figure 2 K1).

In addition to the bacterial colonies, scattered *C. albicans* cells were observed, most of which had round cell-like structures (Figure 1C). Some surface areas showed scaffolds of bacterial cells and yeast-like single-celled fungal cells (Figure 2- K3 and C3). Bacterial cells were visualized on the external surface at a single-cell level (Figure 1- K4 and C4). Dense formations of microorganisms were observed in some areas, with disk-shaped structures of bacterial cells and single-celled yeast-like cells. Although differences in bacterial and yeast growth can be observed, overall, the biofilm of the pathogens showed abundant growth and dense colonization of the surface under SEM, as single-type cells of *C. albicans* and *K. pneumoniae* were well-cohesive and evenly distributed on the surface (Figure 2- K2 and C2).

Yeast buds were also seen clustered either in pairs or in groups with some recently formed yeast. Both the biofilm of *C. albicans* and *K. pneumoniae* showed increasing numbers of cellular layers with a recognizable extracellular matrix, and the biofilms of both pathogens were relatively thick and multilayered, although the extracellular matrix was barely visible (Figure 2- K4 and C4).

The diameters of some *K. pneumoniae* cells ranged from 0.18-0.47 nanometers (Figure 1- K2), while the diameters of *C. albicans* cells ranged from 121.82-133.89 nanometers (Figure 1- C5). However, in the visual examination using SEM, the biofilm showed few layers of cells, along with abundant cellular debris, alongside morphologically altered and degraded yeast cells (Figure 2- K2 and C2). Interestingly, most of the bacteria were observed adhering to the surface.

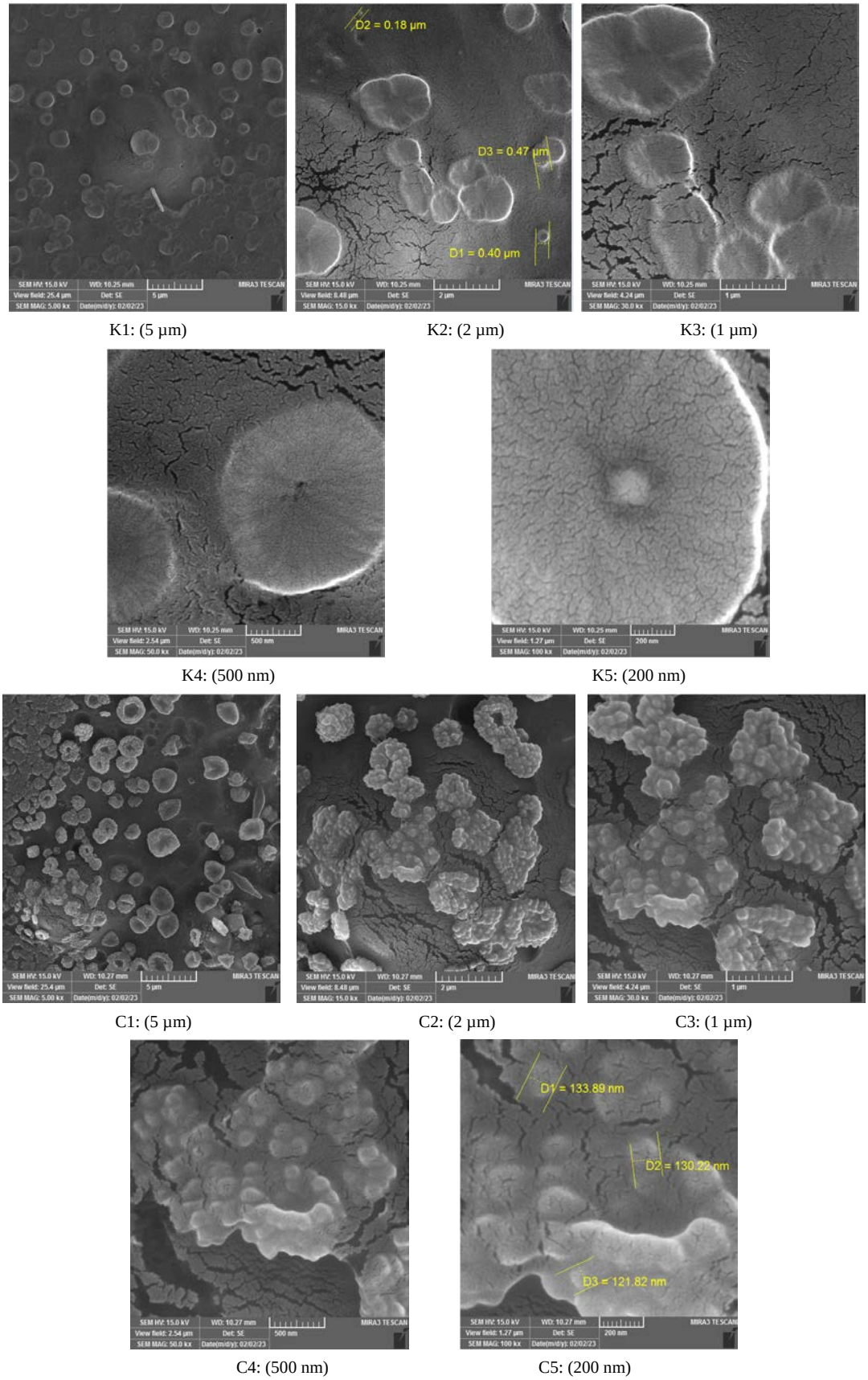

Figure 2. SEM results of the biofilms of *K. pneumoniae* bacteria and *C. albicans.*

Quantitatively, lower numbers of *K. pneumoniae* clusters were observed compared to *C. albicans*, and a thin and sparse biofilm was formed by fewer *K. pneumoniae* cells after initial colonization. In addition, there were variable-shaped exploding vacuoles of *C. albicans*, and the biofilm showed a greater thickness compared to *K. pneumoniae*. Both *C. albicans* and *K. pneumoniae* biofilms showed an organized structure to an acceptable degree,

where the yeast was evenly distributed with minimal extracellular material, dead cells, and cellular debris. The mature biofilm showed distinct and thick layered structures (Figure 2- K2 and C2). The presence of dark areas within the biofilm can also be explained by the water channels present, as well as the heterogeneous production of matrix and different external sugars within the biofilm.

3.4. Biofilms analysis using Fourier transform infrared spectroscopy (FTIR)

FTIR spectral analysis was recently used in microbial biofilm tests (Tugarova*et al*., 2017, Abed *et al.*, 2022) owing to its advantages of use. The study used the strains that showed the highest activities in producing and manufacturing biofilm to analyze the components of the biofilm produced by *C. albicans* and *K. pneumoniae.*

Figure (3) shows the results of FTIR analysis of the biofilms produced by *K. pneumoniae* bacteria. The results indicate a distinctive absorption band at the frequency of 3425 cm^{-1} (O-H stretching), as well as an intense band at 1647 cm^{-1} (C=O stretching of carboxylate). Medium bands were observed at the frequency of 526, 543, and 570 cm^{-1} (β1-6 of Mannans), 1060, 1074, and 1120 cm^{-1} (β1-3 of Glucose), 1409 and 1461 cm^{-1} (Bending of -δCH2, δCH3 from proteins-amide III), and 2981 cm^{-1} (C-H stretching). The remaining bands were weak.

The FTIR spectroscopic appearance obtained in the mid and far infrared regions revealed the absorption of many compounds present in the biofilm matrix of *K. pneumoniae*. The obtained spectral ranges showed associations with Mannans at low wavelengths (Singh *et al*., 2019). The band of the C=O carboxyl which was observed indicates the presence of sugar acids and a broad range of proteins (amide II) (Lal *et al*., 2010;Hussain *et al.*, 2017). The absorption of the carbonyl amide bond is attributed to the presence of γ-lactone and ketone carbonyls (Wu *et al*., 2011; Hussein *et al.*, 2019). These results confirm the presence of the lactone ring in biofilm-producing strains. The obtained spectra were very broad and, in some cases, overlapped with other ranges due to the presence of other compounds (El-Hilali *et al.*, 2016; Rosa *et al*., 2016).

The presence of carboxyl groups may provide some adaptive advantages regarding the isolation of divalent cations' needs. The carboxyl group may also act as functional parts to generate new or modified polymers, which may enhance bacterial infection or virulence or both (Dheeb *et al.*, 2014; Baum *et al*., 2009). The C-H stretching also indicates the movement or vibration of the carbon-hydrogen bond in the bacterialbiofilms. With this vibration, the bond between the C and H atoms expands and contracts several times per second regularly, and this vibration can reveal certain molecular properties such as its structure and chemical composition (Singh *et al*., 2019; Dheeb *et al*., 2019,Hashim *et al*.,2023).

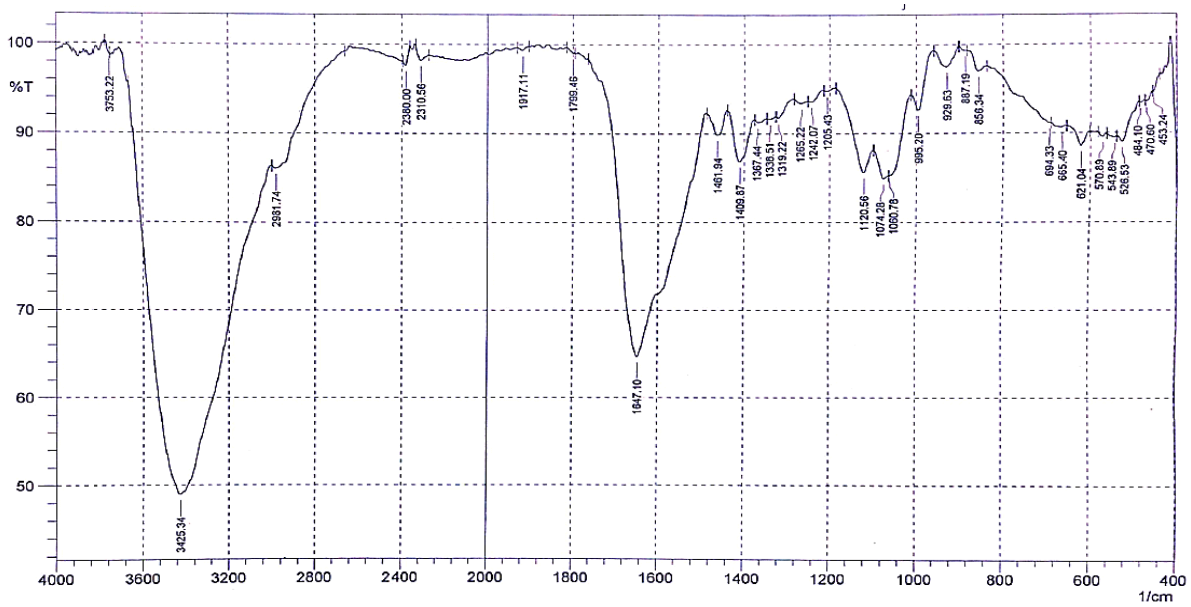

Figure 3. Analysis of the biofilm of *K. pneumoniae* bacteria using FTIR infrared spectroscopy, at spectral bands 400 - 4000 cm^{-1}.

FTIR spectroscopy allows for the comparison of the spectra of biofilms produced by *C. albicans* yeast. The FTIR spectra of the biofilm (Figure 4) exhibit distinctive absorption bands at frequencies of 3411 and 3429 cm^{-1} (O–H stretching), as well as intense bands at a frequency of 1641 cm^{-1} (C=O stretching of carboxylate) and a frequency of 1112 cm-1 (Glucose-Acetyl group compounds). The plotted data also revealed moderate bands at frequencies of 1053 and 1076 cm^{-1} (Glucose β1-3) and 1384 cm^{-1} (C-C ring stretching), while the remaining bands had weak intensities.

The high absorption peaks indicate the chemical compounds (carbohydrates) present in the biofilm produced by *C. albicans*. The asymmetrical stretching of the carboxyl group indicates the presence of uronic acids, while the presence of acetyl group peaks indicates the presence of 20 acetyl groups, which ultimately reveal the presence of acetylated uronic acids (Lal *et al*., 2010; Dahham *et al.*, 2019). The presence of carboxyl groups might provide some adaptive advantages in terms of isolating divalent cations for their needs. Moreover, the carboxyl group may also act as functional parts for generating new or modified polymers, which may enhance the pathogenesis of bacterial infections or fungi, or both (Baum *et al*., 2009; Singh *et al*., 2019,Salih *et al*.,2023).

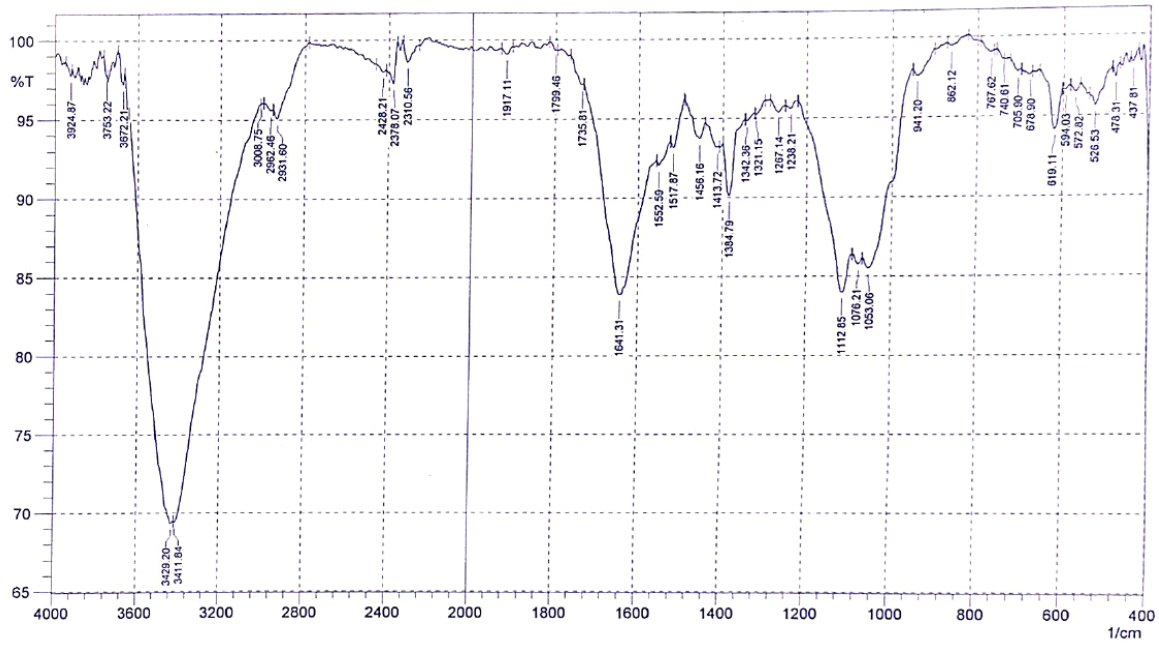

Figure 4. Analysis of the biofilm of *C. albicans* using FTIR infrared spectroscopy, at spectral bands 400 - 4000 cm^{-1}.

3.5. The effect of K. pneumoniae and C. albicans biofilms on lymphocytes:

The biofilms of pathogenic microorganisms might affect the ability of the immune system to recognize and fight them. For example, some pathogens like *C. albicans* can alter the structure of their biofilm to hide them from the immune system, making them less susceptible to targeting (Abdulbaqi *et al.*, 2018; Pontes *et al*., 2022). Clinically, infections related to multi-microbial biofilms lead to a complicated diagnosis and inappropriate treatment which might cause a much higher mortality rate compared to mono-species biofilm infections, especially those caused by mixed fungal and bacterial biofilms. The latter type of biofilm has become a major public health problem through its transmission via medical devices such as urinary catheters (Bander *et al.,* 2015; Rodrigues *et al*., 2019).

The percentage of cytotoxicity increased with increasing concentrations. The highest volume (100 µL/mL) of biofilm of *C. albicans* showed the highest inhibition of 74.82%, while the lowest concentrations showed 71.4%, 60.1%, and 55.2% of inhibition, respectively, compared with the negative control as shown in the Table (3).

As for *K. pneumonia*, the results in Table (3) show that the cytotoxicity percentage increased with increasing concentrations where the highest volume (150 µL/mL) of biofilm K. pneumonia showed the highest inhibition of 34.8%, while the lowest concentrations showed 31.9%, 30.03% and 26.08% of inhibition, respectively, compared with the negative control as shown in the Table (3).

According to the results in Table (3), the biofilm mixture of the bacteria and fungus used in the study showed the highest inhibition of 69.5% at a volume of (100 µL/mL).

Due to the high heterogeneity of human microbial flora (bacteria, fungi, and viruses), polymicrobial biofilms are often co-isolated from the body. In these microbial consortia, various types of interactions can occur between microorganisms, such as mutualism, antagonism, and coexistence, making polymicrobial biofilms more complex to manage than mono-microbial ones (Dheeb *et al.*, 2016,Costa-Orlandi*et al*., 2017; Al-Tekreeti *et al.*, 2017).

Table 3. Inhibitory effect of lymphocytes on the individual and combined biofilm volumes of *C. albicans* and *K. pneumoniae.*

Volumes of biofilm (µL)	*K. pneumoniae*		*C. albicans*		*K. pneumoniae +C.albicans*	
	Control	Growth inhibition (%)	Control	Growth inhibition (%)	Control	Growth inhibition (%)
150	2.024	34.8 a	1.549	71.40 b	0.751	65.51 b
100		31.9 b		74.82 a		69.5 a
50		30.03 c		60.10 c		26.63 c
25		26.08 d		55.20 d		5.85 d

*Means with the same letter are not significantly different according to the Duncan test ($P \leq 0.05$).

The results of cytotoxicity of *C. albicans* biofilm confirm that when using the volume o150 µL which led to the highest inhibition of lymphocytes, at the same time 100 µL for *C. albicans*, it gave the highest inhibition of lymphocytes, Whereas, when the combination biofilm of *C. albicans* and *K. pneumoniae* the inhibition rate of lymphocyte was higher in terms of volume 100 µL.

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