University of Thi-Qar Journal of Science (UTJsci)
E-ISSN: 2709-0256, P-ISSN: 1991-8690, Vol. 11, No. 2, Dec. 2024

Effective Antifungal Compounds of *Lactococcus lactic* Against Vulvovaginal Candidiasis for Women in Thi-Qar Governorate

Haneen A. Alshadood[a*] and Iman Alfayyadh[b]

Department of Pathological Analysis, College of Science, University of Thi- Qar, Thi Qar , Iraq
[a]E-mail:Haneen.mohammed@utq.edu.iq
[b*]Corresponding author: Iman_pa@sci.utq.edu.iq

Received: 2024-02-22, Revised: 2024-03-18, Accepted: 2024-05-06, Published: 2024-12-05

***Abstract*—One hundred and fifty-four samples were collected from Bint Al-Huda Teaching Hospital in Thi-Qar Governorate divided to 104 women infected urinary tract infection (UTI) and 50 healthy women as control group. Vulvovaginal Candidiasis (VVC) is the most opportunistic yeast infection produced by species of *Candida* in both pregnant and non-pregnant women were examined in this study.** *Lactococcus lactic* **(ATCC 11454) extracted and chemical compounds identified by Gas Chromatography, the extract of *L. lactic* inhibited all *Candida* isolates in agar well diffusion assay and the inhibition zone, it were (12, 11, 11, 11, 10 mm) for *C. albicans, C. dublniesis, C. glabrata C. krusei* and *C. parapsilosis* respectively, while minimum inhibiter concentration were (64, 128, 128, 128, 128µg/ml) for *C. albicans, C. dublniesis, C. glabrata C. krusei* and *C. parapsilosis* respectively, the chemical compounds of *L. lactic* unable inhibit efflux pump of all clinical *Candida* isolates after treated with (40, 20, 10, 5 mg/ml) of ethidium bromide.**

Keywords: **Antifungal, *Candida* species, Efflux-pumps, GC-mass, *Lactococcus lactis***

I. Introduction

Vulvovaginal candidiasis is the most common opportunistic yeast infection produced by species of *Candida*, mostly caused by *Candida albicans*. It is a part of the typical microbiota of the vaginal canal. Among fertile women [1,2].

Vaginal infections are common, that are brought on by a disease that causes the natural microflora to multiply or a sexually transmitted infection, including infections caused by *C. albicans* [3].

This is something that happens to around 75% of fertile women at some time in their lives and 20% of women experience a medical condition annually [4].

Forty to fifty percent of cases may result in more than one infection episode and twenty to fifty percent do not show any clinical symptoms. The symptoms include burning, itching, vulval redness along with foul-smelling and cottage cheese-like vaginal discharge. The severity of the symptoms is largely determined by how long the candidiasis remains untreated [5].

L. lactis is a gram-positive, spherical, homolactic, non-sporulating, and facultative anaerobic gut bacterium. The food and drug administration usually considers it harmless. It has been used for millennia in the fermentation of food, particularly cheese, yogurt, and sauerkraut. Additionally, *L. lactis* is being used as a model for genetic engineering and the generation of different recombinant proteins [6].

Antifungal medications are crucial for the treatment of fungal infections, and their elimination of often necessary to prevent recurrence [7,8].

This study was conducted to understand the inhibition ability of primary metabolites of *L. lactic* on *Candida* isolates

II. Materials and Methods

A. Samples collection

This study has included 104 patients with various urogenital diseases, which included pregnant women and non-pregnant women were suffered from urinary tract infections from Bint Al-Huda Teaching Hospital. Their ages were between 17 and 45 years. After the clinical initial diagnosis of the condition based on signs of fungal infections such as cream-colored vaginal discharge and severe itching. Vaginal swabs were collected under the aseptic condition by gel cotton swabs and transmitted immediately to the laboratory.

B. Identification of Candida species

1) Morphological Identification:

This type of diagnosis is based on the shape, size, color, texture and height of the colony after growth in Sabouraud dextrose agar medium [9].

2) Chromogenic Candida Agar (CCA test):

The Chromogenic Candida Agar (Hi-Media) was prepared by dissolving 45.9g in 1L of distilled water. They were heated to boiling point and then placed in Petri dishes. The samples were cultured on the medium and left in the incubator at the temperature 37^0C **t**he colors of the developing colonies on the medium were monitored and the colors of each colony were used to distinguish yeast species compared to the standard [10,11].

https://doi.org/10.32792/utq/utjsci/v11i2.1175

C. *Beneficial bacteria sample:*

L. lactic (ATCC 11454) was obtained from the Biotechnology Research Center/Al-Nahrain University as identified isolate [12].

D. *Extraction primary product for L. acidophilus:*

The active and broad-spectrum *L. acidophilus* was subjected to the submerged state fermentation method to produce crude extract. Fresh cultures of each antifungal compound-producing isolate were inoculated separately in a 250 mL Erlenmeyer flask containing 100 mL MRS broth in anaerobic conditions at 30°C for 24. After incubation each sample was centrifuged at 6000 rpm for 15 min at 4°C to obtain cell-free culture supernatant (CFS). The cell-free solution obtained concentrated to dryness under vacuum using a rotary evaporator at a temperature of 70°C.

E. *preparation of chemical compounds of extract of L. lactic by GC-mass*

The active constituent products of *L. lactis* were identified by GC- mass (Agelint tech) by injection 1µl from each product and using a scan range (25-1000 m/z). Helium gas as a carrier, under 11.93 psi pressure [13].

F. *Agar well diffusion assay*

The five isolates of *Candida* were activated on SDA by spreading them on the medium and kept in the incubator at 37°C for 15min. after that make wells on the SDA and adding 100µl of the primary products of *L. lactis* in these wells and kept in the incubator at 37^0C for 24 hours. To study the effect of ingredients produced by *L. lactis* on *Candida* growth [14].

G. *Minimum Inhibitory Concentration (MIC)*

This study has employed the minimum inhibitory concentration to identify the concentrations of primary products of *L. lactic* that can suppress *Candida* growth. The microdilution method was used to determine the MIC. Chosen five typical *Candida* isolates (*C. albicans, C. dublliensis, C. krusei, C.glabrata and C. parapsilosis*) from pregnant women were infected of VVC from Bint Al-Huda Teaching Hospital.

1. Each *Candida* isolate was reactivated in SDA and incubated at 37^0C for 24 hr.

2. Inoculum of a yeast cell was taken, transferred to 5 ml of normal saline, and the cell count was adjusted to 1 x 10^8 CFU/ml equal to 0.5 McFarland. 20 µl of PDB was taken and added to the wells.

3. The initial solution was prepared and diluted from 512µl of the base product and transferred to 5 ml of PDB

4. The concentration of the primary product was diluted using two-fold dilution method: a series of dilutions that reduces the initial concentration to half by subsequent addition and removal from one well to another and the final 2.5 ml was discarded and it was transferred about 160µl to each well

5. After the dilution of the primary product, the inoculum was transferred to each well except the wells of the negative control. Finally, each well contains 180µl.

6. The microtiter was at $37C^0$ for 18h.

7. After incubation 30ul of resazurin indicter was added to every single well and incubated in the incubator for 4hr. at $37C^0$ [15].

H. *Phenotypic detection of efflux pumps activity by ethidium bromide cartwheel (EtBr-CW) method*

Phenotypic detection of efflux pumps activity by ethidium bromide cartwheel (EtBr-CW) method, In this method the accumulation of a substrate molecule inside the yeast cell was measured to infer the level of an efflux pump such as ethidium bromide. The hypothesis is lower the level of efflux the higher the concentration of substrate accumulated within the yeast cell. The SDA plates were prepared fresh on the previous or same day of the experiment and kept protected from light. Yeast strains were grown in 5 ml Potato dextrose broth (PDB) at 37°C overnight and in the following day their concentration adjusted to 0.5 of a McFarland standard. Preparation of the SDA plates containing EtBr concentrations (40, 20, 10, 5 mg/L) according to as the (64, 128, 128, 128,128 mg/L) are the best MIC for *Candida* isolates.

The center of the SDA was divided into up to five sectors by radial lines, forming a cartwheel pattern. The adjusted yeast cultures were then swabbed by dipping a swab in to each culture and streaking them on the EtBr-SDA plates starting from the center of the plate to the margin. The SDA plates were then incubated at 37ºC for 16-18 hours. After this period, the SDA plates were examined under a gel-imaging system (or a U.V. transilluminator); for fluorescence and the SDA plates photographed. [16]

I. *Statistical Analysis:*

One-way analysis of variance ANOVA (Duncan) was performed to test whether group significance was defined as $P \leqslant 0.05$ were expressed as mean ± standard deviation and statistical significances were carried out using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla, CA).

III. RESULTS

A. *Samples collection*

The total collected samples in this study were 104 samples of vaginal swabs included 60.58% & 39.42% pregnant and non-pregnant women respectively. The total number of yeast positive samples was 76.36% for pregnant women, while 23.64% for non-pregnant women.

B. *Identification of Candida spp*

1) Morphological Identification:

Isolated Samples were cultured on SDA medium at 37°C in the form of smooth white to cream colored colonies and the shape of the colonies is circular.

2) Chromogenic Candida Agar (CCA)

The results of culturing *Candida* isolates on CCA have been shown by colonies of the different isolates of *Candida* which have shown different colors. The light green color of the colonies is the indicator for *C. albicans*, after incubation for 24 hours. Dark green color observed for *C. dubliensis*, the yeast colony in pink a with a whitish border color had seen in isolates of *C. krusei* and Creamy color observed for *C. parapsilosis* and dark violet colonies.

The results also have shown that, the percentage of *Candida* isolates are (73%, 12%, 6%, 5%, 4%) for (*C. albicans*, *C. dubliensis*, *C. glabrata*, *C. krusei* and *C. parapsilosis*) respectively.as shown in figure (1)

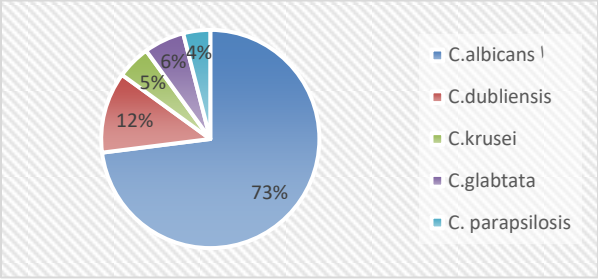

Fig. (1): Percentage of *Candida* isolated on CCA

C. *Agar well diffusion assay*

The antifungal results have releaved that the, extracted of *L. lactis* have antifungal properties and inhibit the growth of *Candida* and show inhibition zone ranged about (12, 11, 11, 11, 10 mm) for *C. albicans*, *C. dublniesis*, *C. glabrata C. krusei* and *C.tropicalis* respectively, as shown figure (2).

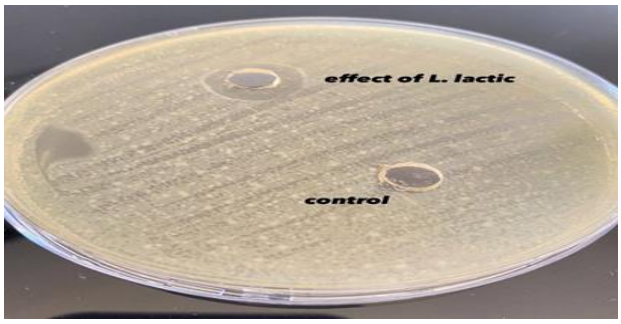

Fig. (2): inhibition zone of *L. lactic* of *C. albicans* on SDA

D. *Preparation of chemical compounds of extract of L. lactic*

The outcomes have suggested that, the active ingredient extracted from *L. lactis* may offer a natural and potentially effective alternative for combating Candida infections. The results have provided specific details about the active ingredient extracted from *L. lactis*, as in Figure (3)

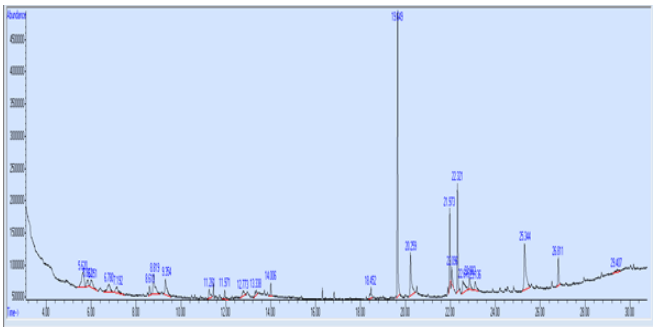

Fig. (3): GC-mass diagram of *L. lactis* extract

The results have provided information on the antifungal activity of various compounds against *Candida* spp, including the active compounds that were identified by GC-mass, as shown the table (1)

Table (1): The active compounds of L. lactic by GC-mass

NO	Compound	Cas number
1	Glyceraldehyde	2255 000056-82-6 10
2	Propanol	291 000071-23-8 14
3	Butanal, 3-hydroxy	4706 000623-50-7 10
4	Oxirane, (ethoxymethyl)-	4273 004016-11-9 38
5	Benzoic acid, methyl ester (3)	16311 000093-58-3 70
7	Benzaldehyde, 4-(1-methylethyl)-	22692 000122-03-2 38
8	l-Alanine, N-butoxycarbonyl-, hept adecyl ester	221351 1000313-36-8 53
9	1-Ethyl-3-[2-(octadecylthio)ethyl] thiourea	217873 296245-37-9 50
10	Hexadecanoic acid, methyl ester	119400 000112-39-0 98
11	Octadecanoic acid	131261 000057-11-4 76
12	7-Hexadecyn-1-ol	92513 000822-21-9 83
13	Methyl stearate	143126 000112-61-8 99
14	Oleic Acid	129337 000112-80-1 87
15	Vitamin E	222349 000059-02-9 98

E. *Minimum inhibitory concentration*

The broth microdilution method was used to determine the MIC of *Candida* in a 96-well microtiter plate. The results showed that the concentration of (64, 128, 128, 128, 128µg/ml) for the primary product of *L. lactic* was MIC of *C. albicans*, *C. dubliniensis*, *Candida glabrata*, *C. Kruse*, and *C. parapsilosis* respectively, which can inhibit growth, as shown in Table (2)

Table (2): The MIC and sub-MIC of the primary product of *L. lactic* against *Candida* isolates

Candida isolates	MIC	Sub MIC
C. albicans	64	32
C. dubleinsis	128	64
C. krusei	128	64
C. glabrata	128	64
C. parapsilosisl	128	64

F. *Phenotypic detection of efflux pump activity by ethidium bromide cartwheel (EtBr-CW) method*

The activity of the antifungal drugs for *L. lactis* studied haven not observed any changes in the susceptibility of the efflux pump for tested *Candida* isolates after being treated with (40,20,10 and 5 mg/ml) of ethidium bromide that have not inhibited the efflux pumps in all *candida* isolates.

IV. Discussion

SDA is a semi-selective plating medium which is used for the isolation and cultivation of yeasts and molds, and it allows for the isolation of *Candida* spp [17].

The significance of visible colonies on CCA that allows for the selective isolation and differentiation of *Candida* based on colony color and morphologies due to the production of enzymes that react with chromogenic substrates in the CCA medium. This reaction produces colonies of different colors, which can help identify certain *Candida* to the species level [18].

L. lactic has been shown to inhibit the growth and virulence of *Candida* species through the production of antimicrobial compounds and the regulation of biofilm formation [19].

The antifungal properties of *L. lactis* has been shown to inhibit the growth of *Candida* spp, specifically *C. albicans, C. dubliniensis, Candida glabrata, C. Kruse,* and *C. tropicalis.* The study reports inhibition zones ranging from 10 to 12 mm for each of these *Candida* spp [20].

GC-mass analysis demonstrated that the L. lactis extract inhibited the development of *Candida* growth by containing substances such as methyl ester of hexadecanoic acid that has antifungal properties according to [21] octadecanoic acid, 7-hexadecyn-1-ol, methyl stearate, oleic acid, and vitamin E these have antifungal effect this agreement with [22] referred to that cause the uncontrollable release of intracellular electrolytes and damage the fungal cell membrane . The inhibition of *Candida* growth observed in the extract suggests that the combination of these compounds may have synergistic effects, enhancing their antimicrobial properties. However, further studies would be necessary to understand the exact mechanisms of action and the potential for developing treatments or preventative measures against *Candida* infections according to [23]

The decreasing growth of *Candida* isolates decline in minimum inhibition concentration may be due to the presence of many compounds that affect bacterial growth such as Vitamin E which has been discovered to have antifungal activity via a variety of pathways. According to research, vitamin E can restore mitochondrial function and guard against lipid peroxidation in the mitochondrial membrane, which enables it to have antifungal effects [24].

The activity of antifungal drugs for *L. lactic* its impact on the efflux pump susceptibility of *Candida* after treatment with ethidium bromide at varying concentrations (40, 20, 10, and 5 mg/ml) does not response to this assay, However, the results have provided relevant information about the antifungal activity of *L. lactis* and other LAB against *Candida* this agreement with [25] who found EtBr test may not be suitable for detecting efflux pump activity in *Candida*, and other methods such as the Rhodamine 6G accumulation assay may be more effective., while it wasn't in agreement with the results by [26], that referred to drug discovery targeting fungal efflux pump can lead to the development azole-enhancing combination therapy. This approach aims to inhibit efflux pumps.

V. Conclusion

Exploration of the effective anti-candida compounds produced by *L. lactic*. Study potential of *L. lactic* compounds on *Candida* isolates growth. Chemical compounds of *L. lactic* unable to inhibit the efflux pump of all *Candida* isolates.

Conflict of interest

Authors declare that they have no conflict of interest.

References

[1] M. Q. Waheeb, "An Effect entomopathogenic fungi in control Culex pipiens".*University of Thi-Qar Journal of Science*, vol10(1),2023

[2] J. Senthilganesh, S.Kuppusamy, R. Durairajan, S. B. Subramaniyan, A.Veerappan, & N. Paramasivam,. "Phytolectin nanoconjugates in combination with standard antifungals curb multi-species biofilms and virulence of vulvovaginal candidiasis (VVC) causing Candida albicans and non-albicans Candida". *Medical Mycology*, vo l60(2),2022.

[3] I. H. Al-Fayyadh & M. H. Al-Yasiri, "Detection of yeast species associated with respiratory tract infections using internal transcript spacer". *Journal of Cardiovascular Disease Research*, vol 12(5), pp 962-966, 2021.

[4] S. D. Hellier& A. F. Wrynn, "Beyond fluconazole: A review of vulvovaginal candidiasis diagnosis and treatment". *The Nurse Practitioner*, vol48(9), pp33-39,2023.

[5] E. H. A. Waheeb, M. H. AL-Yasiri & A. A. Maktoof, "The role of Candida species in the occurrence of dental caries in Thi Qar Governorate" *University of Thi-Qar Journal of Science*, vol10(1),2023.

[6] E. B. Kim, J. S. Son, Q. K. Zhang, N. K. Lee, S. H. Kim, J. H. Choi & Y. J. Choi, "Generation and characterization of thymidine/D-alanine auxotrophic recombinant Lactococcus lactis subsp. lactis IL1403 expressing BmpB". *Current microbiology*, vol61, pp29-36,2011.

[7] S. M. Saadaldin, "A Novel Approach to Overcome Adherence Property of Candida albicans. *Al-Nahrain Journal of Science*, vol14(3), pp 115-118,2011.

[8] B. Y. Bulgasem, M. N. Lani, Z. Hassan, W. M. W. Yusoff, S. G. Fnaish, "Antifungal activity of lactic acid bacteria strains isolated from natural honey against pathogenic Candida species". *Mycobiology*, vol44(4), pp302-309,2016.

[9] L. L. Horvath, D. R. Hospenthal, C. K. Murray, & D. P. Dooley, "Direct isolation of Candida spp. from blood cultures on the chromogenic medium CHROMagar Candida". *Journal of clinical microbiology*, vol41(6), pp2629-2632,2003.

[10] F. C. Odds, & R. I. A.Bernaerts, " CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important Candida species". *Journal of clinical microbiology*, vol32(8), pp1923-1929,1994.

[11] R. A. Hajjeh, A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, & D. W. Warnock, " Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program". *Journal of clinical microbiology*, vol 42(4), pp1519-1527,2004.

[12] J. E. Kim, S. Y. Kim, K. W. Lee & H. J. Lee, " Arginine deiminase originating from Lactococcus lactis ssp. lactis American Type Culture Collection (ATCC) 7962 induces G1-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells". *British journal of nutrition*, vol102(10), pp1469-1476,2004.

[13] K. A. Azizan, S. N. Baharum, &N. Mohd Noor, " Metabolic profiling of Lactococcus lactis under different culture conditions". *Molecules*, vol17(7), pp 8022-8036,2012.

[14] M. S. Carmo, F. M. Noronha, M. O. Arruda, E. P. D. S. Costa, M. R. Bomfim, A. S. Monteiro, & V. Monteiro-Neto, " Lactobacillus fermentum ATCC 23271 displays in vitro inhibitory activities against Candida spp. *Frontiers in Microbiology*, vol7, 1722,2016.

[15] U. Binder, M. Aigner, B. Risslegger, C. Hörtnagl, C. Lass-Flörl & M. Lackner, " Minimal inhibitory concentration (MIC)-phenomena in Candida albicans and their impact on the diagnosis of antifungal resistance". *Journal of Fungi*, vol5(3), pp 832019,2019.

[16] H. A. Abbas, A. M. El-Ganiny, & H. A. Kamel, " Phenotypic and genotypic detection of antibiotic resistance of Pseudomonas aeruginosa isolated from urinary tract infections". *African health sciences*, vol18(1), pp11-21,2018.

[17] J. L. Richard, M. C. Debey, R. Chermette, A. C. Pier, A. Hasegawa, A., Lund & M. D. Connole, " Advances in veterinary mycology". *Journal of medical and veterinary mycology*, vol32(sup1), pp169-187,1994.

[19] C. Itapary dos Santos, Y. Ramos França, C. Duarte Lima Campos, M. R. Quaresma Bomfim, B. Oliveira Melo, R. Assunção Holanda, & V. Monteiro-Neto, "Antifungal and antivirulence activity of vaginal Lactobacillus spp. products against Candida vaginal isolates". *Pathogens*, vol8(3), pp150,2019.

[20] U. Roy, V. K. Batish, S. Grover, S. Neelakantan, " Production of antifungal substance by Lactococcus lactis subsp. lactis CHD-28.3". *International journal of food microbiology*, vol32(1-2), pp27-34,1996.

[21] M. N. Abubacker, & T. Deepalakshmi, " In vitro antifungal potential of bioactive compound methyl ester of hexadecanoic acid isolated from Annona muricata linn (annonaceae) leaves. Biosci". *Biotechnol. Res. Asia*, vol10(2), 879-884,2013.

[22] G. E. Dawwam, I. I. Saber, M. H. Yassin& H. F. Ibrahim, (2022). "Analysis of different bioactive compounds conferring antimicrobial activity from Lactobacillus plantarum and Lactobacillus acidophilus with gas chromatography-mass spectrometry (GC-MS)" *Egyptian Academic Journal of Biological Sciences, G. Microbiology*, vol14(1), 1-10.,2022.

[23] Y. Li, P. Jiao, Y. Li, Y. Gong, X. Chen & S. Sun, " The synergistic antifungal effect and potential mechanism of D-penicillamine combined with fluconazole against Candida albicans". *Frontiers in Microbiology*, vol10, pp2853,2019.

[24] L. Sun, X. Ye, D. Ding& K. Liao, " Opposite effects of vitamin C and vitamin E on the antifungal activity of honokiol". *journal Microbilogy and Biotechenology*. vol10,2019.

[25] E. E. Whittle, S.W, Legood, I. Alav, P. Dulyayangkul, T. W. Overton, & J. M. Blair, "Flow cytometric analysis of efflux by dye accumulation". Frontiers in Microbiology, vol10, 2319.2019.

[26] A. R.Holmes, T.S. Cardno, J. J. Strouse, I. Ivnitski-Steele, M. V. Keniya, K. Lackovic, & R. D. Cannon, "Targeting efflux pumps to overcome antifungal drug resistance". *Future Medicinal Chemistry*, vol8(12), 1485-1501, 2016.