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Quantitative and Qualitative Determination of Gliotoxin and Acetaldehyde Toxins in Yeasts Isolated from some Respiratory Patients and Study of Yeast Resistance to some Antibiotics

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Abstract

The concentrations (64, 128, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125) mg/ml were used to calculate the lowest inhibitory concentration of antibiotics for Candida yeast by microdilution method. The antibiotics fluconazole and itraconazole were used to complete this experiment, which is based on the results of testing the API Candida system by demonstrating the resistance of yeasts using several mechanisms, which serve to phenotype each organism selected in the framework. In this test, it was found that there is resistance to the antibiotics itraconazole and fluconazole in the types of yeasts under experiment. The toxin-producing yeasts extracted from yeasts showed that the toxin-producing yeast species were only 27 out of 30 samples of yeast, despite the growth efficiency of all samples. While 30 samples of yeast isolated from patients' sputum, it was found that all samples under the current study contain different amounts of acetaldehyde toxins with different concentrations.

Keyword: Gliotoxin; acetaldehyde; candida; itraconazol; fluconazole.

Introduction

During the past two decades, the incidence of diseases has become high and The species of pathogenic fungi increased significantly. The species of Candida yeast became the cause of The infection that is frequently encountered, especially at the present time, and which can cause the spread of a group of diseases that cause injuries in humans and animals, especially in the mucous membranes of the respiratory tract.[1] Candida albicans yeast is considered one of the main causes of death, especially in immunocompromised persons, while other species belonging to the genus Candida such as C. glabrata and C. krusei are also considered pathogens, but to a lesser extent than Candida albicans.[2] The ones that cause infections of yeasts, especially Candida, are the possession of multiple virulence factors that enable them to attack the host and to attach more, such as changes in physiology, hydrolysis, and adhesion.[3]

Aims of study:

1-Detection of Gliotoxin and acetaldehyde from yeasts isolated from the respiratory tract.

2-Determination of the minimum concentration of fluconazole and itraconazole inhibitors for Candida yeasts isolated from the respiratory tract.

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Material and Method

Antibiotic Agents

The antagonists used in this study are 150 mg/ l for fluconazole and 100 mg/l for Itraconazole .

Preparation of solutions

RPMI-1640 Medium It is a growth medium used in cell culture, with acidity up to, this medium was used for the purpose of testing the sensitivity of *Candida* yeasts.[4]

Sensitivity test to determine minimum inhibitory concentration (MIC)

The standard method described here aims to describe the method of performing a sensitivity test used for *Candida* yeasts and measuring their PURPOSE To determine the ability of yeasts to resist antifungals or susceptibility to antifungals used in the experiment in order to obtain data matching known laboratory standards (NCCLs) in fungal control. This test is also consistent with the results of the US National Committees for Clinical Medicine, and is among the most important internationally approved standards M27-A2.[5] BROTH MEDIUM RPMI 1640 medium supplemented with 2 mM Glutamine, 4.5 g glucose with pH meter[6] was used.

Fungicides

Antifungal drugs, including itraconazole and fluconazole, were prepared from the drug's manufacturing bottle, which was provided with the generic name of the drug, biological activity expressed in international units, micrograms per milligram of antifungal powder, expiration date as well as storage conditions. Prepared antifungals can be stored in the refrigerator as recommended by the manufacturers.[7.]

Prepare stock solutions I prepared solutions for antifungal drugs by equation

$$\text{Weight (mg)} = \frac{\text{volume (mL)} \times \text{desired concentration (mg/mL)}}{\text{antifungal potency (mg/mg)}}$$

Detection of mycotoxins Acetaldehyde and Gliotoxin

Mycotoxins were detected using HPLC technology for Gliotoxin and absorption spectroscopy for acetaldehyde toxins as follows

High-performance liquid chromatography(HPLC) for Gliotoxin

Gliotoxin separation was performed in a column made of C18 (250X4.6) particles of 5 µm size (Knuaer, Germany) according to[8], 20 µl of each biological sample was analyzed using reverse phase HPLC technology with UV detection (RP-HPLC). -UV) by HPLC system from Knauer (Germany) as shown in the table below.

Table 1: shows the methods for detecting Gliotoxin toxins using HPLC technology

No.	Component	Model or version
1	Binary high pressure gradient pump	P6.1L
2	Diode array detector	DAD 2.1L
3	Sample loop (20 µl) and injector	D1357
4	Analyses and system control software	Claritychrom , V 7.4.2.107

Micro-Detection of Acetaldehyde

A. solutions used in produce

1. Reagent solution 0.2% DNPH in 2m h HCL
2. 2 of chelating solution (also called plank) 2 molar hydrochloric acid.
3. 20% w/v Trichloro-acetic acid
4. 50% absolute ethanol
5. Ethyl acetate.
6. A solution of 6.0 M quinidine hydrochloric acid dissolved in a 0.5 M solution of potassium phosphate (5.2 PH).
7. Quartz cell.

B. Colorimetric method for the determination of Acetaldehyde

Colorimetric determination of acetaldehyde with a formula of dinitrophenylhydrazone in aqueous solution was carried out by quantitative drawing in carbon tetrachloride by following the working method that includedDivide the 20 mL volume between 5 and 35 µg of the acetaldehyde-containing sample, 5 mL of 2 4-dinitrophenylhydrazine reagent was added and left for 30 min at room temperature. The solution was shaken vigorously with 20 ml of carbon tetrachloride for one minute, after which the formed aqueous

layer was withdrawn again and placed with 5 ml of carbon tetrachloride, the sample was shaken again for 15 seconds, the formed tetrachloride extracts were transferred and titration was done to 50 ml, then 2 ml of ethanolic sodium hydroxide were added. Measure the optical density of the red solution formed within 10 minutes after adding the alkali.[9]

3.3.8. ELZA The ELISA device consists of two parts, one is the analysis base and the "reassembly base" with the absorbance of the material according to the specified wavelength.[10]

Result

Determination of the minimum inhibitory concentration of *Candida*spp(MIC)

The concentrations (64, 128, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125) mg/ml were used to calculate the lowest inhibitory concentration of antibiotics for *Candida* yeast by microdilution method as in Table (4.6) the antibiotics fluconazole and Itraconazole were used for completing this experiment, which is based on the results of the API *Candida* system test by showing the resistance of yeasts by using several mechanisms, which serve to determine the phenotype of each organism selected in the framework of this test, including In yeasts such as *Candida*, and when the test results interfere with the method of determining the minimum inhibitory concentration, they correspond to a database of no more than a phenotype and its distributions. These patterns correspond to the defense mechanisms of the organism. This process allows correcting the biological and therapeutic course by giving correct treatment reports about the possibility The use of antibiotics and changes in the permissible.

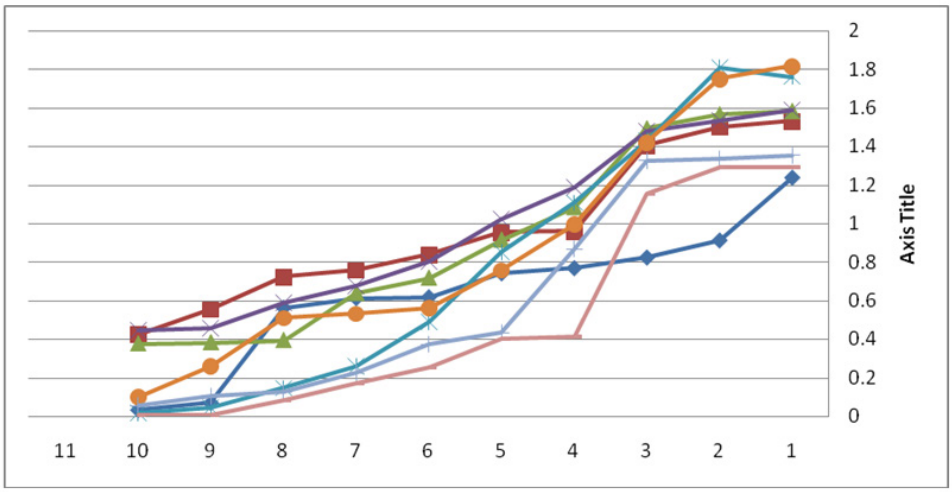


Figure 1: Shows the inhibition and resistance curves of Itraconazole by Candida yeasts

It is clear from the above Figures that there is resistance to the antibiotics Itraconazole and fluconazole in the types of yeasts under experiment, and according to the concentrations used, it starts from the highest concentration of 128 mg in some types of yeasts such as fungi, which are characterized by their ability to resist azole antifungal compounds in most cases, while the lowest inhibitory concentration for most yeast species of Itraconazole antifungal was 16 mg/ml.The MIC values (g/ml) of Itraconazole in RPMI at concentration 16 mg/ml for *Candida* yeasts were as follows: 0.851, 1.454, 1.216, 1.46, 1.391, 1.365, 1.418, 1.338, 0.823, 1.404, 1.492, 1.478, 1.429, 1.421, 1.324, 1.152, respectively, for *Candida albicans*, while the MIC values (g/ml) for *Candidatropicallis* were as follows: 1.339, 1.409, and for *Candida dubliniensis* as follows: 1.4861.403,1.471,1.591,1.586. while the MIC values (g/ml) for *Candida glabrate* were 1.554. As for the MIC values (g/ml) of Fluconazole in the RPMI at the concentration of 32 mg/ml they were as follows: 0.484, 0.435, 0.46, 0.431, 0.489, 0.519, 0.302, 0.243, 0.575 ,0.55, 0.036, 0.266, 0.398, 0.362, 0.394, 0.257, for *Candida albican* while the MIC values (g/ml) for *Candida tropicallis* were as follows: 0.425, 0.424 and for *Candida dubliniensis* as follows: 0.447, 0.412, 0.569, 0.447, 0.507, while it was The MIC values (g/ml) for *Candida glabrate* are: 0.507.as shown in Table 2.

Table 2: Shows the minimum inhibitory concentration MICof *Candida*spp

N0	Isolate	MIC (g/ml) of Itraconazole	MIC(g/ml) of fluconazole
1	C. albicans	0.851	0.484
2	C. albicans	1.454	0.435
3	C. albicans	1.216	0.46
4	C. albicans	1.46	0.431
5	C. albicans	1.391	0.489
6	C. albicans	1.365	0.519
7	C. albicans	1.418	0.302
8	C. albicans	1.338	0.243
9	C. albicans	0.823	0.575
10	C. albicans	1.404	0.55
11	C. albicans	1.492	0.036
12	C. albicans	1.478	0.266
13	C. albicans	1.429	0.398
14	C. albicans	1.421	0.362
15	C. albicans	1.324	0.394
16	C. albicans	1.152	0.257
17	C. dubliniensis	1.339	0.461
18	C. dubliniensis	1.409	0.431
19	C. dubliniensis	1.486	0.512
20	C. dubliniensis	1.403	0.426
21	C. dubliniensis	1.471	0.577
22	C. trupicallis	1.591	0.469
23	C. trupicallis	1.586	0.548
24	C.glabrate	1.554	0.557

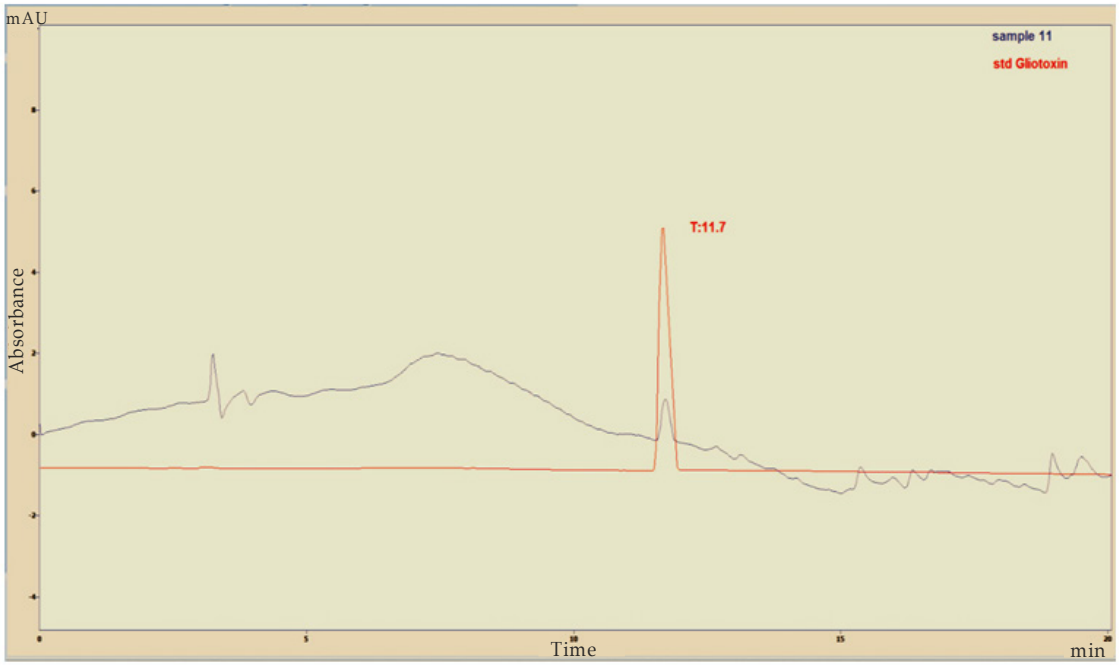
The above table shows that most of the *Candida* showed resistance to Itraconazole antibiotics at a concentration of 8 mg/ml, while *Candida* showed resistance to fluconazole at a concentration of 16 mg/ml, and this indicates that Candida yeasts show a high resistance to antibiotics, especially the azole group.

Detection of mycotoxin

Gliotoxins using HPLC

The specific liquid chromatographic (LC) parameters were as follows: mobile phase (A) water, TFA (99.95: 0.05 v/v); (b) Acetonitrile, either mobile phase flow rate 1 mL/min; As for the degree program, it was as follows : 10% b for 2 minutes, 10 to 90% B in 18 minutes, 90% B for 1 minute then 90 to 10% b in 1 minute Re-equilibrate to 10% B for 3 minutes. All analyzes were performed at 30°C. The detection wavelength (λ) was set at 254 nm. Glyotoxin was identified by its retention time and UV/VIS matching with standard substances. Then a quantitative measurement of 30 yeast samples isolated from sputum samples of patients with respiratory diseases in Thi-Qar Governorate was carried out by measuring the integrated peak area. The content was calculated using a calibration curve by drawing the peak area against the concentration of the peak area with the respective standard sample as it is shown in the following standard curve:

The HPLC gradient protocol is shown as in Figure 3, where the representative standard curve chromatogram of Gliotoxin of yeast samples cultured on RPMI medium shows the standard curve of Gliotoxin extracted from yeasts compared to the representative curve of Gliotoxin extracted from yeast species in the presence of the toxin in 27 samples Only from 30 samples of yeasts, despite the growth efficiency of all samples. Figure (4) shows the detection of Gliotoxin in small quantities and others ranged at higher rates. The cause may be due to the different genetic nature or environmental conditions specific to the production of Gliotoxin depending on the fungal type and yeast.



Figures 2: Show HPLC chromatograms for gliotoxin of some yeasts, where the retention time of glial toxins is determined in each chromatogram. HPLC chromatogram of Candida albicans isolate culture.

The results of the qualitative and quantitative detection of gliotoxin toxins using HPLC technology showed that 27 out of 30 samples contained gliotoxin toxins with rates ranging from 0.23-2.06 µg/ml as shown in Table 3:

Table 3: The amount of gliotoxin in yeasts

Candida spp	peak area	µg/ml (working solution)	µg/ml media
C. albicans	110.45	11.30	1.13
C. albicans	48.35	4.95	0.49
C. albicans	86.52	8.85	0.89
C. albicans	39.13	4.00	0.40
C. albicans	0.00	0.00	0.00
C. albicans	27.22	2.78	0.28
C. albicans	65.00	6.65	0.66
C. albicans	28.91	2.96	0.30
C. albicans	41.84	4.28	0.43
C. albicans	0.00	0.00	0.00

Contd... Table 3: The amount of gliotoxin in yeasts			
C. albicans	55.84	5.71	0.57
C. albicans	22.88	2.34	0.23
C. albicans	39.43	4.03	0.40
C. albicans	31.93	3.27	0.33
C. albicans	35.19	3.60	0.36
C. albicans	80.46	8.23	0.82
C. albicans	27.39	2.80	0.28
C. albicans	101.05	10.34	1.03
C. albicans	127.25	13.02	1.30
C. albicans	108.68	11.12	1.11
C. albicans	94.56	9.67	0.97
C. albicans	99.35	10.16	1.02
C. albicans	60.20	6.16	0.62
C. glabrate	58.23	5.96	0.60
C. dubliniensis	59.61	6.10	0.61
C. dubliniensis	201.70	20.63	2.06
C. dubliniensis	24.92	2.55	0.25
C. dubliniensis	142.73	14.60	1.46
C. dubliniensis	0.00	0.00	0.00
C. dubliniensis	67.68	6.92	0.69

Acetaldehyde

Using the spectrophotometer method for measuring the percentage of acetaldehyde in the 30 samples of yeast isolated from the sputum of patients it was found that all samples under the current study contain different amounts of acetaldehyde toxins with different concentrations.

When applying the recommended method for standard acetaldehyde solutions purified by ammonia derivatives and under the conditions required for detecting acetaldehyde, which start from the method of culturing samples on saline phosphite medium, it was found that the concentration of acetaldehyde ranged from 1.68 - 8.66mg /100 ml and As shown in Table 4.

Table 4: Show the concentration of Acetaldehyde toxin in yeast

No.	Yeasts species	Concentration of Acetaldehyde mg/100 ml
1	C. albicans	4.556575
2	C. albicans	5.382263
3	C. albicans	1.926606
4	C. albicans	2.232416
5	C. albicans	4.847095
6	C. albicans	2.293578
7	C. albicans	3.807339

Contd... Table 4: Show the concentration of Acetaldehyde toxin in yeast		
8	C. albicans	3.929664
9	C.dubliniensis	3.180428
10	C.dubliniensis	1.681957
11	C.dubliniensis	5.290520
12	C.dubliniensis	4.633028
13	C.dubliniensis	5.856269
14	C. kruzie	4.908257
15	C. kruzie	3.960245
16	C. kruzie	5.733945
17	C. kruzie	4.816514
18	Naganishiadiffluens	4.113150
19	Naganishiadiffluens	3.134557
20	Naganishiadiffluens	3.486239
21	Naganishiadiffluens	3.256881
22	C. tropicallis	4.418960
23	C. tropicallis	4.204893
24	Candida lusitaniae	4.571865
25	C.lusitaniae	4.954128
26	Magnusiomycescapitatus	4.40367
27	Magnusiomycescapitatus	8.669725
28	Magnusiomycescapitatus	4.051988
29	Magnusiomycescapitatus	4.678899
30	Magnusiomycescapitatus	3.501529

Discussion

MIC identification:

Antifungal susceptibility testing plays an important role in testing antimicrobial drugs such as fungi, as an aid in drug development and as a means for the purpose of tracking antifungal resistance in most epidemiological studies[11,] clinical and laboratory studies. studies. The subcommittee establishes criteria for the purpose of testing antifungals in a standardized manner, including the minimum growth inhibitor concentration test, which provides clinical information that can provide important information about antifungal resistance.[12] The curves of Candida antigens are shown according to the concentrations used in this experiment and the minimum inhibitory concentration (MIC) for yeasts, and according to the antigens used which included itraconazole and fluconazole, the MIC values were based on a point recommended by CLSI.[11]

Detection of mycotoxin:

Gliotoxin using HPLC:

The HPLC method confirmed that Twenty-seven samples containing gliotoxin toxins were obtained from a sample of only 30, according to what was mentioned in the results. isolated from clinical samples cultured on RPMI medium, and also consistent with what was found by [(12),] who was able to isolate gliotoxin from 30 yeast samples, mostly containing Candida yeasts. Gliotoxin is considered a carcinogenic and deadly poison when exposed to it, as it was set within the recommendations of the World Health Organization that the lethal

dose of gliotoxin is 50: 67 mg/kg, While the lethal intraperitoneal dose reached: LD50: 32 mg/kg depending on the recommendations of the World Health Organization, and these concentrations are considered lethal. By causing body dysfunction or causing cancer, especially in the lung, exposure to gliotoxin is also a major cause of cancer and highly immunosuppressive that exacerbates infectious diseases or causes immunosuppression and the development of malignant lymphomas such as breast tumors, i.e increase by 10 to 100 times compared to normal cases of the disease, or may increase lung tumors and lung weakness. In the case of low concentrations of the toxin.[14] These results are consistent with[15] stated, when they found that out of 100 strains of *Candida*, there are approximately 60% of the strains capable of producing gliotoxin toxins, especially the clinically important strains that were detected by HPLC technology.

Acetaldehyde

From the results obtained and as shown in Table (4), these results are consistent with what the researcher[16] mentioned and found. That all C. albicans isolates produced acetaldehyde at a concentration (>100 µM), which was isolated as a carcinogen in most smokers compared to non-smokers within the scope of the study and the differences were significant in most of the studied isolates. The results are also consistent with that reported,[17] who found that all Candida and other Candida isolates produce Acetaldehyde toxins and that all *Candida tropical* isolates produce acetaldehyde at higher concentrations than other species, with rates as high as *C. krusei* (54.6 ± 2.9). NS). *C. tropica* and *C. parapsilosis* also produce by yeast high amounts of Acetaldehyde toxins with high moral variations. Acetaldehyde is one of the carcinogenic toxins which is the main cause of cancers such as lung cancer, oral cancer and respiratory system, which have spread recently and in very high rates in most smokers and when yeasts or fungi are present in their respiratory system. The reason may be due to the fact that smoking helps metabolize Acetaldehyde toxins produced by most of the Candida species that spread in the upper cavity of the respiratory system, which are considered microorganisms that mainly inhabit the moist parts of the body.[18]

Conflict of Interest: Nil

Source of Funding: Self

Ethical Clearance: Not required

Reference

1. Sullivan DJ, Moran GP, Pinjon E, Al-Mosaid A, Stokes C, Vaughan C & Coleman DC Comparison of the epidemiology, drug resistance mechanisms, and virulence of Candida dubliniensis and Candida albicans. FEMS Yeast Res (2004);4:369–376.
2. Iman H. AL.Fayyadh. Isolation and Diagnosis of Fungi from Samples of Spices in the City of Nasiriya. University of Thi-Qar Journal Of Science (UTsci) The 4th Scientific Conference of Science College/ University of Thi_Qar.2019. Volume (7), No.2.
3. Richardson MD (2005) Changing patterns and trends in systemic fungal infections. J AntimicrobChemother 56: i5–i11. Calderone RA &Fonzi WA Virulence factors of Candida albicans. Trends Microbiol .(2001);9:327–335.
4. Claudio Kupfahl1, Thomas Ruppert2, Anneb¨ arbel Dietz1, Gernot Geginat1 & Herbert Hof. Candida species fail to produce the immunosuppressive secondarymetabolitegliotoxininvitro. _C Federation of European Microbiological Societies FEMS Yeast Res 7 (2007) 986–992 Published by Blackwell Publishing Ltd. All rights reserved.
5. Atlas RM, Snyder JW. Handbook of Media for Clinical Microbiology (2nd ed.). Boca Raton, Florida: CRC Press. (2006);p.427.
6. NationaL Committee for CLinicaL Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, 2nd edn. Approved Standard M27-A2. Wayne, PA: NCCLS, 2002.
7. EromtLing RA, GaLgiani JN, PfaLLer MA et al. MuLticenter evaLuation of a broth macrodiLution antifungaL susceptibiLity test for yeasts. Antimicrob Agents Chemother 1993;37:39–45.
8. J. L. Rodrı´guez-Tudela1 (Chairman), F. Barchiesi2, J. Bille, Johnson, G. R. A., Scholes, G., and Weiss, J., J . Chem. SOL, 1953, 3091 Sambrook, J.; Fritsch, E.F. and Maniatis, T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.(1989).
9. Bulgari, D., Fiorini, L., Gianoncelli, A., Bertuzzi, M., &Gobbi, E. Enlightening Gliotoxin Biological System in Agriculturally Relevant Trichoderma spp. Frontiers in microbiology, 11, 200. Characteristics of flucytosine determined by time-kill methods.Diagn. Microbiol. Infect. Dis. (2020). 36:101-105. 2.. 3.DOI:10.1111 /j.1567-1364.2007.00256.x18 May 2021.
10. Johnson, G. R. A., Scholes, G., and Weiss, J., J . Chem. SOL, Sambrook, J.; Fritsch, E.F. and Maniatis, T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (1989). 1953- 3091.
11. Bassett, Caroline "The computational therapeutic: exploring Weizenbaum's ELIZA as a history

of the present". AI & Society. (2019);34(4):803–812. doi:10.1007/s00146-018-0825-9.

12. Gopinath, S. M., T. B. Suneetha, et al. "Chemical prophylaxis and antibacterial activity of methanolic and aqueous extracts of some medicinal plants against bovine mastitis." International Journal of Advanced Biological Research IJABR.(2011); 1(1):93-95.
13. Akortha, E. E., V. O. Nwaugo, et al. "Antifungal resistance among Candida species from patients with genitourinary tract infection isolated in Benin City, Edo state, Nigeria." African Journal of Microbiology Research.(2009);3(11):694-699.
14. Gardiner DM, CozijnsenAJ, Wilson LM, Pedras MS &Howlett. BJ. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus Leptosphaeriamaculans. Mol. Microbiol. (2004);53:1307–1318.
15. Material Safety Data Sheet. NFPA. sc-201299). World Health Organization Akortha, E. E., V. O. Nwaugo, et al. (2009).
16. Shah, Darshana T.; Larsen, Bryan. "Clinical isolates of yeast produce a gliotoxin-like substance". Mycopathologia. (1991). 116 (3): 203–8. doi:10.1007/BF00436836. PMID 1724551. S2CID 12919491.
17. M. L. Gainza-Cirauqui, M. T. Nieminen, L. Novak Frazer, J. M. Aguirre-Urizar, M. D. Moragues, R. Rautemaa. Production of carcinogenic acetaldehyde by Candida albicans from patients with potentially malignant oral mucosal disorders . 22 August. 2012. JOU.ORAL PATHOLOGY &MEDICINE.https://doi.org/10.1111/j.1600-0714.2012.01203.x.
18. McClenny, N. B., H. Fei, E. J. Baron, A. C. Gales, A. Houston, R. J. Hollis, and M. A. Pfaller. Change in colony morphology of Candida lusitaniae in association with development of amphotericin B resistance. Antimicrob. Agents Chemother.2002;46:1325–1328.
19. Pelucchi C, Gallus S, Garavello W, Bosetti C, La Vecchia C. Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. Eur J Cancer Prev. 2008;17:340–4.