Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

Studying the Effects of Active Compounds in Clove Plant and their Impact Against Certain Types of *Candida* Species

Sarab Fadhil [1]*, Sabah Abd Fleih [2], Zuhair Hameed Abboud [3]

[1] Department of Biology, College of Education for Pure Science, University of Kerbala, Karbala, Iraq

[2] Faculty of Agriculture, University of Kerbala, Karbala, Iraq

[3] College of Science, University of Kerbala, Karbala, Iraq

Emails: sarab.fadhi@uokerbala.edu.iq [1], sabah.alrubaay@uokerbala.edu.iq [2]

Abstract

The study included the detection of active compounds by using the high-performance liquid chromatography (HPLC) method . It revealed about 7 effective compounds, as the clove plant contains volatile oils and the most effective and concentrated compound was Eugenol acetate. The genus *Candida* spp. was identified based on cultural and microscopic characteristics was revealed in a way of smooth, shiny, convex colonies, white to milky in color, and distinguished by its distinctive odor. The shape of the cells appeared spherical to oval or longitudinal, single and budded. The virulence factors of *Candida* species were studied Its ability to produce Protease enzyme and the possibility of isolation from yeast *C. albicans* on the enzyme production, followed by *C. tropicalis* yeast while the other species do not have the ability to produce the protease enzyme. The ability of *Candida was* to produce the hemolysin enzyme especially the two species *C. albicans* and *C. glabrata* have hemolytic ability, while other isolates portability was non-hemolytic, and their ability to produce phospholipase enzyme was demonstrated by the isolate of *C.albicans* which recognized by the appearance of a white dense area around the growing colonies, which is the area of deposition on the medium of solid egg yolk, while the rest of the isolates did not produce the enzyme phospholipase. The alcoholic extract of Cloves plants was prepared for the purpose of identifying the antagonistic activity against *Candida* species, it was noted that the clove plant inhibited all species.

Keywords: HPLC, *Candida spp* ,volatile oils, Eugenol acetate

Introduction

Cloves belong to the Myrtaceae family; they are large evergreen trees, the leaves are simple, opposite, and thickly petiolate. The flower buds are bright red when picked (1). It is native to Malaysia and Southeast Asia. It is abundant in Sumatra and Zanzibar. Medical importance: Eugenol found in clove oil helps to stimulate regeneration of the mucous membranes of the stomach in people suffered from gastric ulcers. It also reduces acidity and activates digestive yeasts; its use is beneficial in cases of indigestion and gases resulting from gastritis (2). Eugenol is one of the substances widely used in treating caries teeth due to their anesthetic and antiseptic properties, and it has been found that the concentration of (1%) of clove oils has an antiseptic ability that is three to four times greater than that of phenol, as well as helps

to strengthen uterine contractions during childbirth, and also used as a raw material in Vanillin industry (2). The goal was to study the active compounds of cloves and their effect on five types of Candida.

Materials And Methods

Culture Media

Sabouraud Dextrose Agar (SDA)

It was prepared by dissolving 65 g of (SDA) in 1000 ml of distilled water and adding 250mg of chloramphenicol to the bacterial growth inhibition. (3).

Proteinase activity medium

The test medium contained of Bovine Serum Albumin solution and Water Agar. Prepare: Adjust the pH of BSA solution to 3.5 and sterilize by filtration, then mix with 140 ml of autoclaved water agar and poured in Petri dishes. (4).

Phospholipase activity medium

The test medium contained 13 g of SDA, 11.7 g of NaCl, 0.11 g of CaCl2, 10% of sterile egg yolk and 184 ml of D.W. The components were mixed (without egg yolk) and sterilized, then add 20 ml of supernatant of centrifuged egg yolk solution (500g\10 min at room temperature) to the sterilized medium, mixed well and poured in Petri dishes. (5).

Hemolysin activity Medium

It was prepared by dissolving 3% of glucose with 6.2 of SDA in 100 ml of D.W, then adding 7 ml of human fresh blood to the sterile medium(6).

Identification of Yeast

Microscopic Examination

The first examination was performed by a dissecting microscope to identify the morphological characteristics of the colonies. Then a slide of the yeast was prepared with a drop of Lacto phenol cotton blue stain and apart of yeast colony taken from SDA plates and covered with the cover and examined under an objective lens 10x, then 40x. After examination, the isolates were transferred to a slant culture medium in clean, sterile tubes and preserved at 4 ^{o}C until being identified and differentiating between yeast spp. depending on morphological, biochemical and molecular methods.

Inoculum Preparation

Each isolate's inoculum was produced using the Densi check plus equipment to evaluate the turbidity of the suspension, which was about 1.80-2.20McF.

Extracellular Enzymes Production

To detect the ability of the yeast to secrete the Proteinase, Phospholipase, Hemolysin and Lipase was activated the yeast isolates by sub cultured on SDA at 37°C for 24-48 h.

Proteinase Production

Upon McFarland scale standard (approximately 108 yeast CFU), a suspension of the yeast was set up with clean typical saline relying. On Bovine Serum Albumin medium (BSA), 10 µl of the suspension was vaccinated. Plates were incubated for 7 days at 37oC. Through the measurements of the hallowed zone around the colony, proteinase activity was detected (4).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

Phospholipase Production

By inoculating 10 µl aliquots of the yeast suspension (approximately 108 yeast cells/ml) into the wells punched onto the surface of the egg-yolk medium, the activity of extracellular phospholipase was detected. After two days' incubation period at 37°C, the diameter of the precipitation zone surrounding the well was measured (5).

Hemolysin Production

The plate of which media was inoculated by 10µl of yeast suspension from each isolate that was prepared initially and incubated at 37oC in 5% CO2 for two days (7).

Testing the inhibitory effectiveness of plant extract on Candida growth

Attend the middle SDA It was poured into dishes and left until it solidified, then the method of spreading in the sheets was followed by drilling In testing the sensitivity of yeasts to the extract yeasts in the middle and make five holes of equal dimensions in the middle SDA With a diameter of 5 mm, it is mediated Cork Borrer Concentrations of the extract were added at a rate of 0.2 mg/ml to each hole, with different concentrations of Plant extract. The dishes were left in the incubator for 24 hours for the plant extracts to diffuse into the medium culture the diameter of the inhibition zone was measured using a ruler (8).

Results and discussion

Analysis of some active compounds in clove extract: Table (1) shows the presence of various types and concentrations of active compounds in clove flowers, it has been found that Eugenol acetate has the superior of the most concentrated compound With a retention time of (5.977), this compound is considered as one of the important volatile oils that is used in medicine as sterilizer, anesthesia, and resistance to fungi and bacteria, cloves are also used to preserve foods from spoilage because of their inhibitory effect on *Streptococcus* and *Staphylococcus* bacteria (9) and is used as a mouth wash , It is an antiseptic and toothache reliever, It is also used to treat foot pain, stomach ulcers, and to treat dermatic fungi (10), especially ringworm. It is a repellent for gases and worms, and is also used in the treatment of Myasthenia gravis and nerve stiffness (9). As in Figure (1)

Table (1) Compounds that were separated using chromatography technologyHPLC Clove plant

Number compound	retention time	Compound concentration	Compound name active
1	1.897	0.829	α-pinene
2	2.638	0.497	Vanillin
3	3.718	0.528	Methyl salicylateVolatile Oil
4	5.04	0.391	eugenol
55.977	9.274		Eugenol acetate
6	6.638	0.704	Caryophyllene

Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

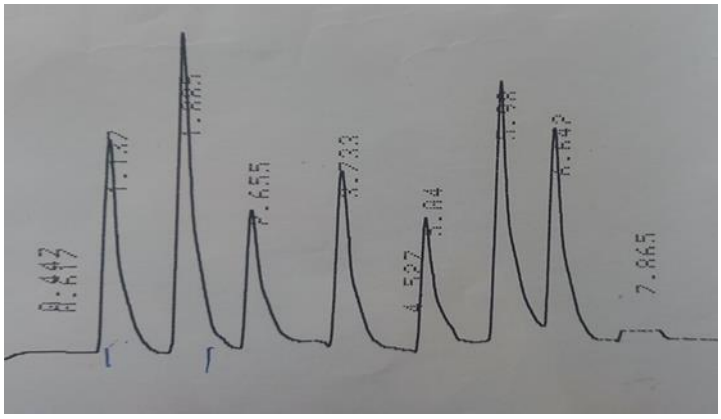

Figure (1) Compounds that were separated using chromatography technology HPLC Clove plant

Cultural Characteristics: The genus *Candida* spp. was diagnosed based on the cultural and microscopic characteristics and biochemical tests according to what was stated by (11). When these types of the studied species were cultured on SDA medium for (24-48) hours at (37°C), it appeared as smooth, shiny, convex, and white to milky in color colonies as illustrated in Figure (2) and is distinguished by its distinctive smell.

SDA medium is used to isolate different types of yeast (12) and is considered an ideal medium for isolating *Candida* because the low PH of this medium promotes its growth and prevents the growth of many types of oral bacteria and adding the antibacterial increases its selectivity (13). As in Figure (2)

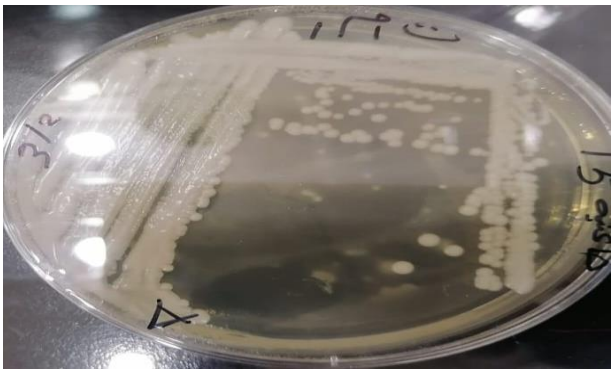

Figure (2) Yeast growth *C. albicans* on media SDA

Detection of virulence factors

The ability of Candida to produce the enzyme Protease

The results of the study illustrated in Table (2) showed the ability of *C. albicans* to produce the enzyme Protease, followed by the yeast *C. tropicalis*, while other species are not capable of producing the specific enzyme mentioned above as in Figure (3).

Table (2) Results of the protease enzyme production test

Candida species	result
C. albicans	+
C. galabrata	-
C. tropicalis	+

C. kefyr	-
C. Krusei	-

The results of the study also coincided with the results of (14) which showed that the susceptibility of yeasts *Candida* spp. to produce the same enzyme Protease. The reason for the inability of some yeast to produce the protease enzyme may be due to the difference between strains or due to the lack of activity of the SAP gene which is responsible for the production of the proteolytic enzyme that is considered as one of the basic components of the *Candida* genome (15).

Proteinase enzyme is one of the essential virulence factors Of Pathogenic fungi, which enable them to invade host tissues and have the ability to degrade albumin and proteins present in the skin, and inhibiting the immune response that causes inflammation (16).as in Figure (3).

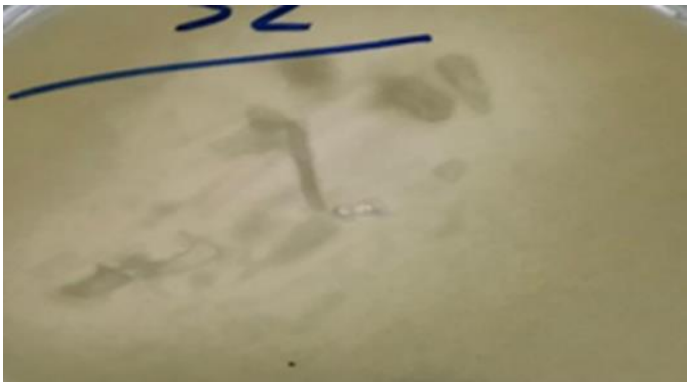

Figure (3) Protease enzyme production

The ability of Candida to produce the enzyme Hemolysin

The results shown in Table (3) showed that the isolate belonging to yeast C. *albicans* and *C. glabrata* was hemolytic, while the rest of the isolates were not hemolytic. These results agreed with the findings of (Rossoni 2013)(17), who showed that all isolates of yeast mentioned above had the ability to decompose blood, but they did not agree with them regarding yeast *C.tropicalis* their isolate was hemolytic.

Table (3): Hemolysin enzyme test results

Candida species	result
C. albicans	+
C. galabrata	+
C. tropicalis	-
C. kefyr	-
C. Krusei	-

The ability of yeasts to break down blood is an important factor of Virulence causes pathogenicity, and this is what many studies have stated, including the study (18). When the blood breaks down, iron will be released, which is a necessary factor for the growth of

Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

pathogenic organisms (19). Iron is an essential factor for the growth of fungi and yeasts and also stimulates various biochemical processes (20). (The process of decomposition of red blood cells is a strategic process for survival during Opportunistic infection, for example in the case of *C. albicans* facilitates blood breakdown and iron absorption from the invasion process during infection with candidiasis (21), as in the figure (4).

Figure (4) Candida's ability to produce hemolysin

The ability of candida to produce the enzyme phospholipase

The results, shown in Table (4), showed that the isolate of C. *albicans* had the ability to produce phospholipase enzyme occurs through the appearance of a white dense area around the growing colonies, which is the sedimentation area on the culture media of solid egg yolk, a precipitation area appeared as a result of the formation of a calcium complex with fatty acids liberated from the phosphorylated fats present in egg yolk due to the effect of the phospholipase enzyme (Price), (22) as for the rest of the isolates did not produce the enzyme phospholipase.

Table (4) Phospholipase test results

Candida species	resul
C. albicans	+
C. galabrata	-
C. tropic	-
C. kefyr	-
C. Krusei	-

These results agreed with (23) that *C. albicans* produces the enzyme phospholipase.as in Figure (5)

Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

Figure (5) Candida's ability to produce phospholipases

The inhibitory effect of the alcoholic extract of clove plant

The results of the study showed that the alcoholic extract of clove plant has a very high inhibitory effect against the studied yeasts. The concentration of 100 mg/ml of the extract showed a very high inhibitory effect, as the average diameter of inhibition was 13.18 and 13.20 mm for each of the yeasts *C. tropicalis* and *C. kefyr* respectively, followed by the inhibition rate For yeast *C. glabrata* with an average of 15.19 mm, the highest inhibition rate was for each of the yeast *C. albicans* and *C. Krusei* with an average of 18.20, 18.17 mm, respectively. Table (5) and Figure (6) Our study was consistent with a previous study (24). They found that the aqueous and alcoholic extract of clove plant inhibited the growth of all the tested microorganisms, where it gave the highest inhibitory effect on the growth of bacteria and with all concentrations Compared to the extracts of the studied plants as in Table (5).

As for the concentration of 75 mg/ml, it was the highest inhibition rate of 16.19 mm for yeast *C. krusei* followed by the inhibition rate of 14.20 mm for yeast *C. albicans*, followed by yeast *C. kefyr* with an inhibition rate of 20.10 mm, and the lowest inhibition rate for yeasts *C. galabrat* and *C. tropicalis* with an inhibition diameter of 8.21, 8.22 mm, respectively. As in Table (5) And Figure (6). As for the concentration of 50 mg/ml, it was the highest inhibition rate of 10.20 mm for each of the yeast *C. krusei* and *C. albicans*, and the lowest inhibition rate was 8.20 mm for yeast *C. kefyr*, and the rest of the yeasts did not show any inhibition rate. As for the concentration of 25 mg/ml, only yeast *C. albicans* with an inhibition rate of 7.20 mm | As for the rest of the yeasts, they did not show any inhibition rate.

Table (5) Effect of different concentrations of clove plant extract on types ofCandida ssp.

Yeasts	Damping diameter rate of clove				(± standard error) Yeast rate
Concentrations are micrograms/ml					
	25.00	50.00	75.00	100.00	
C. albicans	7.20	10.20	14.20	18.20	1.25±12.45 a
C. Krusei	0.00	10.20	16.19	18.17	

Annals of R.S.C.B., ISSN:1583-6258, Vol. 28, Issue 1, 2024, Pages. 128 - 137

Received 10 January 2024; Accepted 24 January 2024

2.13±11.15 a				
C. kefyr	0.00	8.20	10.20	13.18 1.48±7.90 ab
C. galabrata	0.00	0.00	8.21	15.19 1.92±5.85 b
C. tropicalis	0.00	0.00	8.22	13.20 1.70±5.35 b
Concentration rate	0.77±1.44 D	1.26±5.72 C	0.87±11.40 B	0.60±15.60 A 0.83±8.54
LSD (p-value) Concentration:4.97 (0.001*)				
Yeasts: 2.57 (0.016*)				

* Significant differences at a probability level less than$P<0.05$

The different lowercase letters indicate significant differences between yeast types, while the uppercase letters indicate significant differences between concentrations

This study showed that the inhibitory effect of the alcoholic extracts is due to the chemical components Found in them such as alkaloids, flavonoids, phenols, saponins, tannins, resins, and terpenes, as These components interfere with the metabolic processes and with the growth of fungi and thus work to break down these fungi (25). In addition to its content of Eugenol compound, which is a type of phenolic compounds that have antimicrobial activity and that work to inhibit the mechanism of action of the cell membrane of microorganisms and thus Inhibiting the growth of the microorganism (26, 27) it is one of the phenolic compounds that have the ability to disrupt the adhesion Bacteria and enzymes and transfer proteins of the cell envelope, while the alkaloids can inhibit the growth of bacteria by affecting their DNA (28).

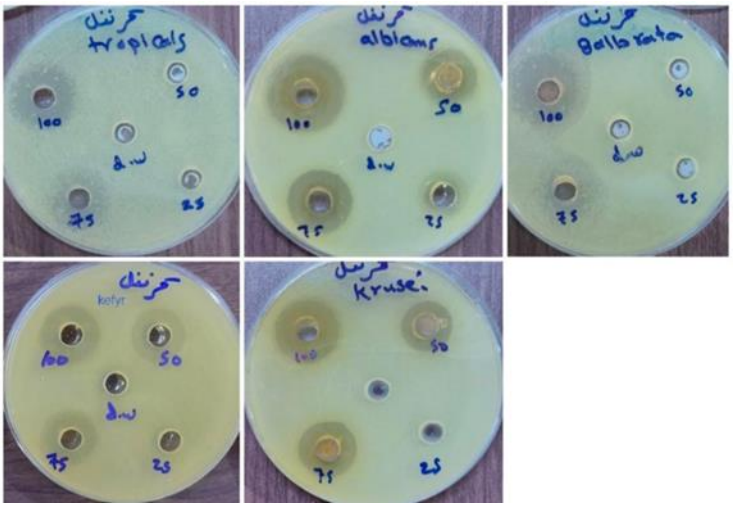

Figure (6) effect of clove plant extract on species Candida ssp.

Conclusion

The clove plant contains volatile oils, the ability of Candida to produce the protease enzyme, the production of the phospholipase enzyme, and its ability to produce the hemolysin

enzyme. The alcoholic extract of the clove plant also showed the ability to inhibit all Candida isolates

References

[1] Al-Mayah Abdel-Rida Akbar 2013. Medicinal plants and herbal medicine, Al-Basaer House and Library, Beirut-Lebanon.

[2] Aziz Rama Ahmed 2022. Medicinal and aromatic horticultural plants, Directorate of Books and Publications, Damascus University.

[3] Ellis, D.H. 1994. Clinical mycology. The human opportunistic mycoses. Pfizer, New York .166 P.

[4] Aoki, S., Ito-Kuwa, S., Nakamura, Y., and Masuhara, T. 1990. Comparative pathogenicity of a wild-type strain and respiratory mutants of Candida albicans in mice. Zentralblatt für Bakteriologie, 273(3), 332-343

[5] Samaranayake, L. P., Raeside, J. M., and MacFarlane, T. W. 1984. Factors affecting the phospholipase activity of Candida species in vitro. Sabouraudia: Journal of Medical and Veterinary Mycology, 22(3), 201- 207.

[6] Luo G ،Samaranayake LP ،Yau JY.2001. Candida species exhibit differential in vitro hemolytic activities. J Clin Microbiol. Aug;39(8):2971-4.

[7] Manns, J. M., Mosser, D. M., and Buckley, H. R. 1994. Production of a hemolytic factor by Candida albicans. Infection and immunity, 62(11), 5154-5156.

[8] Saxena, G., Farmer, S. W., Hancock, R. E. W., & Towers, G. H. N. 1996.Chlorochimaphilin: a new antibiotic from Monesesuniflora. Journal of natural products, 59(1), 62-65.

[9] Barbara Mc Ge ،M. G. 2003. encyclopedia of spices(cloves) exclusively foe the Epicenter.

[10] Prashar ،A.;Lock ،IC.; Evans ،CS.2006. Cytotoxicity of clove (suzygium) aromaticum oil and it major component to hu- -man skin cells ". Cell prolif. 39 : 241 – 248.

[11] Ellis ،D.; Stephan ،D.; Helen ،A.; Rosemary ،H. and Roben، B.2007. Description of Medical Fungi. 2nd ed. Ltd. Australia, PP: 147 –150.

[12] Liguori ،G. ; Di Onofrio ،V. ; Gallẻ ،F. ،Lucariello ،A. ; Albano ،L.; Catania ،M. R. and Guida ،M. 2010. Candida albicans identification : comparison among nine phenotypic systems and a multiplex PCR. J. Prev. Med. Hyg., 51: 121-124.

[13] Raju ،S. B. and Rajappa ،S.2011. Isolation and identification of Candida from the oral cavity. Int. Scholarly Res. Network, 2011: 1-7.

[14] Al-Zubaidi, S., Al-Ayafi, A. and Abdelkader, H., 2019. Biosynthesis, characterization and antifungal activity of silver nanoparticles by Aspergillus niger isolate. Journal of Nanotechnology Research, 1(1), pp.23-36.

[15] Julian ،R. Naglik ،S. ; Challacombe ،J.and Bernhard ،H. 2003. Candida albicans Secreted Aspartyl Proteinases in Virulence and Pathogenesis, 67(3): 400–428.

[16] Sardi JCO ،Scorzoni L ،Bernardi T ،Fusco-Almeida AM ،Mendes Giannini MJS.2013. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 62(Pt 1):10-24.

[17] Rossoni ،R. D. ،Barbosa ،J. O. ،Vilela ،S. F. G. ،Jorge ،A. O. C. ،and Junqueira ،J. C. 2013. Comparison of the hemolytic activity between C. albicans and non-albicans Candida species. Brazilian oral research, 27(6), 484-489.

[18] Nayak A.M.D.S. ،Ranganath N. Nayak M.D.S. ،Kishore Bhat M.D. 2012. Antifungal activity of a toothpaste containing Ganoderma lucidum against Candida albicans - an in vitro studyJ. Int Oral Health 2010 All right reserved JIOH, August 2010, Volume 2 (Issue 2).

[19] Bullen ،J. J. 1981. The significance of iron in infection. Rev. Infect. Dis. 3:1127–1138.

[20] Calera JA ،Haas H. Cations (Zn ،Fe). 2009. In: LatgeJP , Steinbach WJ , eds Aspergillus fumigatus and Aspergillosis. Washington, DC: ASM Press ;: 107 – 129.

[21] Odds ،F.C. 1979. Candida and Candidosis. Leicester University press. London. PP.381.

[22] Price, M. F., Wilkinson, I. D., and Gentry, L. O. 1982. Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia: Journal of Medical and Veterinary Mycology, 20(1), 7- 14.

[23] Ying, S. and Chunyang, L.2012. Correlation between phospholipase of Candida albicans and resistance to fluconazole. Mycoses.55:50-55.

[24] Al-Thuwaini, Amna Nimah Sinar, Salman Nasifu, Sabah Mahdi Hadi 2009. The effect of some aqueous and alcoholic extracts of cloves, black seeds, and bazoons on the growth of some pathogenic bacteria. Kufa University Journal. Volume 2, Issue 1

[25] Fazza, Souad Abd 2011. The effect of aqueous extract of clove flowers on some Bacterial isolates causing gingivitis, College of Science, Al-Qadisiyah University.

[26] Ibraheim, H. K., Fayez, R. A., Jasim, A. S., & Gharban, H. A. (2023). Role of nuc gene in Staphylococcus aureus to phagocytic activity in different cattle infections. *Open Veterinary Journal*, *13*(8), 1021-1026.

[27] Abd- Kreem H. Romi , Sabah Abd Fleih and Fatima Ali Hasan. 2023. Studying the Effect of Some MedicinalPlants Extracts and Antibiotics on some Pathogenic Isolates Bacterial, IOP Conference Series: Earth and Environmental Science, 1262 (2023) 052052.

[28] Kaur K. , Sandeep B. and Sumeet G. 2017. Phytochemical Screening And Antimicrobial Activity Of Eugenia Caryophyllata(Clove) And Plumbago Zeylanica (Chitrak) Medicinal Plants.International Journal of Scince Technology and Management.6(3):2394-1529.

[29] Anteithar Jabar Mohammad Al-Edani and Sarab Fathil Husseinand Ban A.H.Al-khafaji.2020.Evaluation of the activity of alcoholic extract of Gujarat plant(Hibiscussabdariffa L.) against some dermatophytes,IOP Conf. Series: Materials Science and Engineering 928 (2020) 062009.