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Antimicrobial Activity of Partial Purified Gengirol on Some Molecular Detectable Pathogens Isolated From Vaginitis

A Dissertation

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

(أَلَمْ تَرَ أَنَّ اللَّهَ أَنْزَلَ مِنَ السَّمَاءِ مَاءً فَأَخْرَجْنَا بِهِ ثَمَرَاتٍ مُخْتَلِفًا أَلْوَانُـهَا وَمِنَ الْجِبَالِ جُدَدٌ بِيضٌ وَحُمْرٌ مُخْتَلِفٌ أَلْوَانُـهَا وَغَرَابِيبُ سُودٌ)

صدق اللَّهُ العلي العظيم

سورة فاطر/ الآية (27)

Dedication

To….

Our great master **Muhammad** peace is upon him...

For those who planted the seeds of patience and steadfastness in my heart, my teachers, Dr. Ibtihal Moez Al-Husseini and Dr.Ali hussein Almarzouki…..

To everything in my life, to my hope in life, Mom

To the one who gave me strength, my grandmother, may God have mercy on her

To my honorary father, may God have mercy on him.

To all my classmates (Iman kareem, mervat kadhum and noor abbas) who supported me

.

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Summary

Vaginitis is an inflammation of the vagina. It usually results from a bacterial or fungal infection. Possible symptoms include a discharge, itching, burning, and possibly pain.

The current study was conduct on vaginitis in the College of Science - Department of Life Sciences in the fungi laboratory.

In this study, 150 samples were collect from the vaginal area of married women who do not carry any disease such as diabetes and other non-pregnant women from private women's clinics.

Where it was cultivated on fungal and bacterial food media after obtaining it and bringing it to the laboratory, and after culturing isolated bacteria and fungi on a number of specialized diagnostic media, the results showed that the total percentage of isolated bacteria was (55.3%), while the percentage of fungi was (54.7%). And the most important types of bacteria that appeared were *E.coli* with a percentage of 24.3%, *Klebsiella pneumoniae* 20%, *S.aureus* 55.7%. Through the results, *S. aureus* bacteria appeared to be more frequent than the rest of the isolated bacteria.

While *C. albicans* 52.6%, *C. glabrata* 23.7% and *C. krusie* 23.7% were isolated in clinical cases. Biochemical tests were performed for each sample, whether bacterial or fungal, and included an examination (indole, citrate, urea, catalase, oxidase, Coagulase and H2S), as the bacterial isolates showed a positive response to most of these tests. As for the fungal isolates, the result was positive for the catalase test and the blood analysis test, and the result was negative for the urea and oxidase test, while the germ tube test was positive only for *C. albicans* isolates. Through the results, S. aureus bacteria appeared to be more present than other isolated bacteria, with a rate of 81.2%.

As for fungi in clinical cases, C. albicans is more common than other types of Candida with a rate of 52.6%. The proportions of isolated microorganisms were high in women between the ages of 18-28 years.

The current work included the detection and molecular investigation of virulence factors of bacterial isolates isolated from the vaginal area, as well as the diagnosis of fungal isolates. Where 14 genes responsible for virulence factors were examined using Real Time PCR and PCR technology, and the PCR data showed that the proportion of genes was (*Nuc* 48.1, *Lip* 68.01, *Sak* 79.1, *hysA* 26.3, *mecA* 22.2, *icaA* 66.7, *icaD* 40, *sspB* 38.8, *Tst* 25, *Eta* 79.1, *Etb* 62.4, *Sea* 84.7, *Seb* 53.3, 16s *rRNA* 99). In addition, a pair of ITS1 and ITS2 primers was use in the diagnosis of Candida, where 32 fungal isolates were tested.

As for the plant used in the study, which is the rhizomes of *Zingiber officinal,* the qualitative detection results showed the presence of many phytochemical compounds that were tested using chemical reagents, namely alkaloids, phenols, flavonoids, tannins, coumarins, glycosides, saponins, resin, carbohydrates, and terpenes.

HPLC technology was use, which confirmed the presence of the active compound, gingerol, at a concentration of 39.58 ppm, purified in a molecular way. It is consider anti-inflammatory through its ability to inhibit the growth of bacteria and candida. The gingerol compound, using three concentrations (10, 20, and 30 mg/ml) showed a clear effect on bacteria and Candida isolates isolated from the vaginal area compared to antibiotics used on bacterial and fungal isolates. Fungi, the inhibition rate was 19 mm for *C. albicans.*

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List of Abbreviations

Abbreviated Form	Meaning
ANOVA	**A One Way Analysis Of Variance**
Sig.	**Significant**
bp	**Base pair**
CLSI	**Clinical Laboratory Standards Instituite**
MIC	**Minimal Inhibitory Concentration**
S. aureus	***Staphylococcus aureus***
PDA	**Potato Dextrose Agar**
SDA	**Sabouraud Dextrose Agar**
Mm	**Millimeter**
Mg	**Milligram**
H2O2	**Hydrogen peroxide**
PCR	**Polymerase Chain Reaction**
DNA	**Deoxyribonucleic acid**
EDTA	**Ethylene diamine tetra acetic acid**
Nuclease	**Nuc**
Lipase	**Lip**
Staphylokinase	**Sak**
Hyaluronidase	**hysA**
Intercellular adhesion D	***icaD***
16s rRNA	**16s rRNA**
Penicillin binding protein 2a	**mecA**
Intercellular adhesion A	**icaA**

Serine protease	***sspA***
Cysteine protease	***sspB***
Toxic shock syndrome toxin-1	***tst***
Exfoliative toxin A	***eta***
Exfoliative toxin B	***etb***
Enterotoxin A	***sea***
Enterotoxin B	***seb***
Enterotoxin C	***sec***
Candida	**C.**

Chapter One
Introduction

Introduction

Vaginitis is an inflammation of the vagina that can cause discharge, itching, and pain. This is due to the constant change in vaginal bacteria. A drop in estrogen levels also causes it after menstruation and certain skin types (Deka *et al.*,2021).

The age of the woman, how her hormones are managing, her sexual activity, and her exposure to hygiene agents used in vaginal irrigation can all have a significant impact on the micro biota makeup (Pironti *et al.*,2021).

Vaginitis is one of the most common diseases that affect women's health, over 50% of women experiencing at least one episode of vaginal infection in their lifetime. Vaginal disease can be infectious, non-infectious, and chronic in nature.

C.albicans a typical component of the human micro biota, frequently colonizes the vaginal lumen asymptomatically. However, exuberant mucosalinflammation that is predominantion brought on by fungal overgrowth in the vagina and follow by epithelial invasion and the synthesis of virulence effectors can result in symptomatic infection, Itching burning, soreness, and redness in the vagina are typical illness signs (Willems *et al.,* 2020). These frequently come with a vaginal discharge that contains immune cells, yeast, sloughed epithelium, and vaginal fluid (Menon *et al*.;2021). vulvovaginal candidiasis is the most common human *candida* infection, affecting 75% of women at some point in their lifetimes, according to estimates 4 Risk factors for VVC are the use of antibiotics, sexual activity, high-estrogen containing oral contraceptives, pregnancy, use of sodium glucose transporter

2(SGLT2)inhibitors, and uncontrolled diabetes mellitus(Irene *et al.*, 2023).

One of the most frequent vaginal disorders linked to abnormal alterations in the vaginal microbiome (VMB) is bacterial vaginosis (BV), also known as vaginal symbiosis (Gardella *et al*.,2022).

Staphylococcus aureus and *candida albicans* are ubiquitous opportunistic pathogens and important nosocomial strains that can cause mild to severe illnesses (Sarra, 2022). Microorganisms acquire antibiotic resistance by means of several underlying mechanisms, including the synthesis of enzymes that degrade the active part of antibiotics, drug efflux, modifying antibiotic binding sites, and biofilm formation (Singh *et al*.,2021). *S. aureus* and *C. albicans* have been founding to form persistent biofilms on inanimate surfaces or within a host. The interaction between these biofilms is a precursor to increased drug tolerance, immune evasion, and virulence, with the outcome of this being increased mortality (Muiruri, 2022). For the last few decades, scientific communities have been in search of new, cost-effective, and potent antibiotic agents to treat infections caused by multidrug-resistant strains(Liu *et al*.,2022).

Plants have an important role in serving humans, as they are an important source for many medicines and medical preparations, and the importance of medicinal plants lies in the fact that they contain effective substances that can be used as a medicinal substance in the treatment of humans and animals after studying and purifying them. Therefore, we note that some medicinal plants can be used in the treatment of different pathological conditions. Such as diabetes, rheumatism, and diseases of

the body systems such as circulation, digestive, nervous, respiratory, and reproductive, as well as skin diseases.

The ginger plant (Ginger Zingiber officinale) is one of the medicinal plants commonly used since ancient times as a treatment for many diseases, in addition to its use as one of the common spices for food and drinks, due to its distinctive flavor. The ancient Chinese and Indians used it to treat cases of headache, nausea and rheumatism, as well as Colds and its widespread use at the present time due to its multiple therapeutic properties, where ginger extract was used as an anti-emetic, allergy and anti-inflammatory, and a reducer of blood sugar level(Arablou and Aryaeian, 2018).

Recent research that attempted to identify the chemical components of ginger plant tubers indicates that they contain the following active substances: zingiberene, gingerols, shogaols, zingerone, paradol, and 6-gingerol. The plant's effectiveness is attributed to the presence of these active substances(Shahrajabian *et al*.,2019).

The Aim of study

Evaluate the prevalence of bacteria and fungi associated with Vaginosis disease in women and controlling it with several antimicrobials and ginger plant extract.

Objective of study

1. Identification of Characterization microbial in women vagina

2. Isolation of morphologically associated fungi and bacteria using PCR technique, and their molecular diagnosis.

3. Detection of virulence factors.

4. Detection of the anti- and active substances of ginger plant using HPLC technique.

5. Use antimicrobials and ginger plant extract to controlling on microbial growth.

6. Determining the bioactivity of Ginger on microbial.

Chapter Two
Literature Review

2. Literature review

2.1. The Human Normal Vaginal Flora

The female genital tract is composed of a sequence of cavities. The external genital tract (vulva) leads into the vagina that connects in succession to the end cervix, the uterus and then to the Fallopian tubes. This passageway allows for the migration of the mature fetus and menstrual flow to the exterior, and for the movement of spermatozoa to the interior. The exposure of the female genital tract to the external environment carries with it the risk of potentially compromising reproductive functions. Among the defense mechanisms that are operational in preventing infections in this area, undoubtedly one of the most important is the composition of the microbial flora that colonizes the vagina (Liu *et al*., 2022).

Vaginal micro flora has been studied in a variety of populations using different sampling, isolation, and identification techniques. These differences between protocols have made the prevalence of a single bacterium difficult to define (Ison, 2020). The lining of the normal human genital tract is a mucosal layer made up of transitional, columnar, and squamous epithelial cells and the flora of female genital tract is greatly influenced by oestrogen level, which depends on the host′s age (Pekmezovic *et al*., 2019). In utero, the vagina of the fetus is microbiologically sterile. Organisms are first acquired from mother′s vagina at delivery, from the hands of care givers, and from the infant′s feces. For the first six weeks of life, the maternal oestrogens are present in the vaginal epithelium, providing it with the microbiology of the adult vagina (Worku, 2022). After estrogens have been metabolized, the flora contains skin organisms, e.g. coagulase-

negative staphylococci, and fecal organisms, e.g. *Escherichia coli* (Xie *et al*., 2020).

Prepubescent and postmenopausal women harbor primarily staphylococci and corynebacteria, whereas women of reproductive age harbor large numbers of facultative bacteria such as *Enterobacteriaceae*, streptococci, and staphylococci, as well as anaerobes such as lactobacilli, anaerobic non spore-forming bacilli and cocci, and clostridia (Vissamsetty *et al*.,2022). The increase in oestrogen at the onset of puberty causes a thickening of the vaginal epithelium with a concomitant deposition of glycogen. Lactobacilli are thought to metabolize glycogen and produce large amount of lactic acid. The resultant low pH would, therefore, select for acid-tolerant organisms and protect the vagina from colonization by pathogens (Kaur *et al*.,2020).

Despite the evidence that lactobacilli flora play an important protective role against pathogens within the genital ecological niche (Barrientos *et al*,2020), there is a significant proportion (7-33%) of healthy asymptomatic women, especially black and Hispanic women, lack appreciable numbers of *Lactobacillus* species in the vagina (Li and Ma,2020) which may be replaced by other lactic acid-producing bacteria, such as species from the genera *Atopobium*, *Leptotrichia*, *Leunostoc*, *Megasphaera*, *Pediococcus*, *Streptococcus,* and *Weissella* (Abdool Karim *et al*.,2019).

Therefore, all ethnic populations have vaginal microflora communities containing lactic acid producing bacteria (Greenbaum *et al*., 2019).

The vaginal flora changes during different phases of life and during menstrual cycle (Zhao *et al*., 2020). A difference can be seen in the flora as well as the vaginal pH in premenarcheal, premenopausal, postmenopausal,

and pregnant women (France *et al*., 2022) for example; in fertile women, the number of protective lactobacilli is reduced during menses (Saraf *et al*., 2021) possibly because of a changed physiological environment.

Furthermore, the microbiological flora have been associated with behavioral variables, such as use of contraceptive agents, frequency of sexual intercourse, use of showers or deodorant products, and utilization of antibiotics or other medications with immune-suppressive properties (Vega and González-Muñoz,2021).

2.2. History of bacterial vaginosis :

Doderlein in 1894 discover (BV) after along study in another studies of sepsis caused abortion or caesarean section. In 1955 (BV) was discovered as a vaginitis and specific pathogen like *Tricohmonas vaginilis* and *Candida albicans* was not specific to caused vaginitis .Gardener and Dukes in 1955 isolate *Haemophilus vaginalis* that nonspecific for vaginitis. After along studies *Corynebacterium vaginalis* change to *Gradnerella vaginalis* (Mahdey and Abdb, 2018)

2.3. Abnormal vaginal flora

Abnormal vaginal flora may occur as a result of sexually transmitted infections (STIs), e.g. trichomoniasis and gonorrhoeae (Sherrard *et al*., 2018) colonization by an organism that is not part of the normal vaginal community, e.g. *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Listeria monocytogenes* (Berkley,2021); or increased virulence of an organism that is a constituent part of normal vaginal flora, e.g. *E. coli* (Taseen, 2019).

The composition of the vaginal flora is not static but changes over time and in response to exogenous and endogenous factors (Liu *et al*., 2018). Alterations occurring in the vaginal environment may increase or decrease the selective advantages for specific microorganisms (Witkin *and* Linhares,2017). If a virulence factor of an organism is constitutive, the number of organisms present will determine the amount of the virulence factors available to promote infection (Vieira *et al*., 2020). An example, is bacterial vaginosis which is associated with dramatic changes in the types and relative proportions of a diverse community of bacteria, i.e. a shift in the vaginal flora from a homogeneous, *Lactobacillus*-dominated state to a heterogeneous state containing a complex population of anaerobic and microaerophilic organisms (Onywera *et al*., 2019).

In addition, a number of bacteria that may present in healthy vagina, such as Gram-negative coliforms (*E. coli* and *Klebsiella pneumoniae*); and Gram-positive cocci (group B streptococci and *Staphylococcus aureus*) have been implicated in a condition known as "aerobic vaginitis", which differs from bacterial vaginosis and defines as a disruption of the lactobacillary flora, accompanied by sings of inflammation, and the presence of a rather scare predominantly aerobic microflora composed of enteric commensals and pathogens (Sonthalia *et al*., 2020). These organisms have been found to be with dramatic changes in the types and relative proportions of a diverse community of bacteria, i.e. a shift in the vaginal flora from a homogeneous, *Lactobacillus*-dominated state to a heterogeneous state containing a complex population of anaerobic and microaerophilic organisms (Ruiz-Perez *et al*., 2021).

Klebsiella pneumoniae is a gram-negative, lactose-fermenting, non-motile, aerobic rod-shaped bacterium. It has been a known human pathogen since it was first isolated in the late nineteenth century by Edwin Klebs (Choby *et al*., 2020).

Klebsiella pneumoniae resides as a normal member of human mucosal flora and is common in the gut. However, *K. pneumoniae* also causes severe opportunistic infection in some carriers and has emerged as an important bacterial pathogen causing hospital-acquired infections such as septicaemia, pneumonia and urinary tract infections (UTIs) that are resistant to multiple commonly used antibiotics ,*K. pneumoniae* can also cause community-acquired infections, such as pyogenic liver abscesses, sometimes complicated by meningitis or endophthalmitis, and soft tissue abscesses. Community infections are often caused by virulent clones (Rakotondrasoa *et al*.,2020).

K. pneumoniae can be transmitted from mother to infant and poses a high risk to colonized neonates. An estimated 20% of neonatal sepsis-related deaths due to treatment failure in the developing world are attributed to *K. pneumonia* (Peretz *et al*., 2017).

Escherichia coli general characteristics are enteric Gram-negative bacilli found most frequently in the genital tract of women. These microorganisms possess several virulence factors that allow them to cause vaginal and/or end cervical colonization and have been implicated in different infections in pregnant women, as well as in intra-amniotic, puerperal and neonatal infections both early and late neonatal sepsis, presenting sometimes with meningitis or urinary tract infections.(Behzadi *et al*.,2021)

The transmission of maternal *E. coli* colonizing the newborn can occur after colonization or infection of amniotic fluid, after membrane rupture or on passage of the neonate through the vaginal canal during delivery, and may cause early neonatal infection (Liu *et al*., 2019).

Data on the features and virulence factors of infection-causing *E. coli* strains in mothers and babies, and colonization of genital tracts of pregnant women by this microorganism are scarce. Neonatal sepsis by *E. coli* is related to a limited number of phylogenetic groups B2 and D, both considered as virulent (Guiral Vilalta , 2018).

Staphylococcus aureus is a Gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a usual member of the micro biota of the body, frequently found in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen (Nachiar *et al*., 2021).

S. aureus usually acts as a commensal of the human micro biota it can also become an opportunistic pathogen, being a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies (Nachiar *et al*., 2021).

The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Despite much research and development, no vaccine for *S. aureus* has been approved (Álvarez *et al*., 2019).

An estimated 20% to 30% of the human population are long-term *carriers S. aureus* which can be found as part of the normal skin flora, in the nostrils and as a normal inhabitant of the lower reproductive tract of women (Nachiar *et al.*,2021).

Staph normally lives in our noses and on our bodies, and it is kept in check by other microbes and general defenses. Problems can arise when there is broken skin or our defenses are down. Staph bacteria create biofilms, which adhere to your vaginal surfaces and make it harder to get rid of, compared to planktonic – free-floating – bacteria. MRSA is sexually transmitted directly into the vagina (and other areas), but because it is transmitted by skin-to-skin contact, any contact with anyone, including a sexual partner, can spread it. Condoms cannot prevent infection (Cheung *et al.*,2021).

S. aureus has numerous virulence factors that determine the pathogenesis of the attendant infections. The most important are: capsular polysaccharide, peptidoglycan and catalase, teichoic acids, clumping factor, fibrinolysis, plasma coagulase, hyaluronidase, lipases, phosphatidylinositol-specific phospholipase C, nuclease or phosphor diesterase, β-lactamase enzymes, (α, β, δ and γ) hemolysin, exfloliatins or epidermolytic toxin, exotoxins a through E, Hand I, a-toxin, leukocidin, exfoliatins, toxic shock syndrome toxin-1 (TSST-1) and enterotoxins. Some of these toxins act as super antigen, which recruit host defense cells that liberate cytokines and, therefore, produce systemic effects (Mahmood, 2021). :

S. aureus resists phago cyte kill at a number of different levels. Effective opsonization of the bacterium is inhibited by the polysaccharide

capsule, the surface expressed clumping factor and protein A. *S. aureus* (Cheung *et al*., 2021).

Produces a carotenoid pigment that imparts a golden color to its colonies. This pigment enhances the pathogenicity of the organism by inactivating the microbicide effect of superoxide's and reactive oxygen species within neutrophils,The eponymous golden carotenoid pigment protects *S. aureus* against neutrophil killing in vitro by scavenging oxygen free radicals (Ahanotu,2019).

2-4. Virulence Factors

Virulence factors are the genetic, biochemical, or structural features that enable an organism to produce disease (Rasheed and Hussein, 2021).

2-4-1. Intracellular Structure

2-4-1-1. Cell Wall

Gram-positive bacteria have a thick peptidoglycan layer envelops the cytoplasmic membrane of the cell. Lipoteichoic acid is another component of the cell wall. These components of the cell wall have received much attention as potential virulence factors (Ali and Maaroof, 2020).

2-4-1-2. Capsule

Capsule is a loose-fitting, polysaccharide layer (slime layer) is only occasionally found on staphylococci cultured in vitro, but it is believed to be more commonly present in vivo. More than 90% of clinical isolates of *S. aureus* produce capsular polysaccharides. Eleven capsular serotypes have been identified in *S. aureus*, with serotypes 5 and 8 associated with the

majority of infections, it encoded by *cap5* and *cap8* genes, respectively (Yeryomina and Kamyshny, 2019).

These capsules can also be divided into two distinct groups on the basis of colony morphology. Microcapsules include the remaining serotype 3 to 11 capsules, strains with these capsules have a thin capsular layer and form non-mucoid colonies on solid medium. Mucoid type capsules strains producing these capsules are heavily encapsulated and are mucoid on solid medium (Fischetti *et al*., 2000).

The capsule protects the bacteria from polymorph nuclear leukocytes as well as by inhibiting the proliferation of mononuclear cells after mitogen exposure. It also facilitate the adherence of bacteria to catheter and other synthetic material (e.g., grafts, prosthetic valves, shunts and joints). This property is particularly important for the survival of relatively a virulent coagulase-negative staphylococci. It has been shown that, *S. aureus* strains are defective in microcapsule production are more efficient at inducing experimental infective endocarditis (IE) than their parental strains suggesting that the microcapsule may obscure important surface-expressed cell wall proteins involved in IE pathogenesis (Tong *et al*., 2015).

2-4-1-3. Protein A

This protein is a component of the cell wall of coagulase positive strains. Protein A makes-up nearly 7% of the cell wall of *S. aureus*, and is present in over 95% of all pathogenic strain (Levinson and Jawetz, 2000).

Protein A has been proposed to be an important *S. aureus* virulence factor involved in allowing the bacterium to evade the host immune system because it binds to the FC protein of immunoglobulin G (IgG). Such binding

would result in the antibodies being oriented in the wrong direction for interaction with phagocytic cell and may actually provide a protective coating (Nair *et al*., 2000).

2-4-2. Extracellular Enzymes

2-4-2-1-Coagulase

Coagulase enzymes production is the principal criterion used in the clinical microbiology laboratory for the identification of *S. aureus* (Tong *et al*., 2015). *S. aureus* strains possess two forms of coagulase: bound (also called clumping factor) and free, coagulase bound to the staphylococcal cell wall can directly convert fibrinogen to insoluble fibrin and can cause the staphylococci to clump (Fischetti *et al*., 2000 ; Puah *et al*., 2016(. Although a few strains of *S. aureus* do not produce detectable amounts of coagulase, all strain seem to possess a coagulase gene (*coa*) (Foster *et al*., 2014).

2-4-2-2 Catalase

All Staphylococci produce catalase, which catalysis the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide (H2O2) can accumulate during bacterial metabolism or after phagocytosis (Murray *et al*., 2003). Different toxic forms of oxygen are produced as inadvertent by-products in during the reduction of O2 to H2O in respiration. the reduction of oxygen intermediates has many deleterious effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids. Bacteria have evolved enzymes that destroy toxin oxygen products. The most common enzyme in this category is catalase, which attack H2O2 (Janssen *et al*., 1993 ; Madigan *et al*., 2003).

2-4-2-3. Hyaluronidase

The hyaluronidase enzyme acts on hydrolyses of hyaluronic acids, the acidic mucopolysaccharides hold together certain cells of the body, particularly cells in the connective tissue, this digesting action is thought to be involved in the tissue blackening of infected wounds and to help the microorganism spread from its initial site of infection (Ibbotson *et al*., 2014).

2-4-2-4. Fibrinolysin

Fibrinolysin, also called Staphylokinase, is produced by virtually all *S. aureus* strains and can dissolve fibrin clots. Staphylokinase is distinct from the fibrinolytic enzymes produced by streptococci (Hentges and Smith, 2018).

2-4-2-5. Lipases

Lipases, all strains of *S. aureus* and more than 30% of the strains of coagulase negative staphylococci, produce several different lipases, as their names imply, these enzymes hydrolase lipids, an essential function to ensure the survival of staphylococci in the sebaceous areas of the body (Cadieux *et al*., 2014).

2-4-2-6. Nuclease

A thermostable nuclease (TNase) that enzyme is produced by nearly all strains of *S. aureus* and has been used as a diagnostic criterion for this species. TNase hydrolyses single and double stranded DNA and RNA at the 5' position of phosphodiester bonds by a calcium dependent mechanism. The main function of this protein may be to convert local host tissues into nutrients required for bacterial growth (Cotar *et al*., 2010).

2-4-2-7. Proteases

Proteases are enzymes which separate proteins, motivating peptide chain analysis. The proteinases have many and different functions inside and outside the cell which organizes many biotic process. Furthermore, the microorganism uses the protease, however excreted or connected with cell surface to weak and invade the host. Therefore, the protease considered target for many drug (Mc Aleese *et al*., 2001). It have been discovered that the *S. aureus* can produce four main types of exocellular proteases:

1. Staphylococcal serine protease (V8 protease) (SspA).

2. Cysteine protease (SspB).

3. Aureolysin (Metalloprotease) (Aur).

4. Staphopain (Scp).

(McAleese *et al*., 2001). The *S. aureus* protein A (SpA), as important virulence factor, is encoded by *spa* gene, which contain variable polymorphic X region)Afrough *et al*., 2013). The molecular characterization of X region of *spa* gene is documented as an exact method for typing of *S. aureus* strains)Ruppitsch *et al*., 2006).

2-4-2-8. Penicillinase

More than 90% of Staphylococcal isolates were susceptible to penicillin in 1941, the year the antibiotic was first used clinically. However, resistance to penicillin quickly developed, primarily because the organisms could produce penicillinase (β-lactamase). The enzyme cleaves the β-lactam ring of the

penicillin molecule. The widespread distribution of this enzyme was ensured by its presence on transmissible plasmids (Murray *et al.*, 2003).

2-4-2-9. Deoxyribonuclease (DNase):

The ability of an organism to break down deoxyribonucleic acid (DNA) by means of Deoxyribonuclease has been used clinically to differentiate between groups of microorganisms and to aid in determining the potential pathogenicity of staphylococci. (Kloos and Bannerman, 1999).

S. aureus produce two types of this enzyme: the first is heat labile DNase and the second is heat stable (Collee *et al*., 1996).

2-4-2-10. β–Lactomase Enzyme:

β-lactomase is a group of enzymes capable of hydrolyzing the four member β-lactam ring of beta-lactam antibiotics. (penicillins, cephalosporins, monobactam and carbapenems) (Rosdahi, 1973). Based on serological test, beta-lactamase enzyme can be grouped into four major classes A to D (Rosdahi, 1973).

2-5-1. Biofilm Formation

Biofilm is an important tool of pathogenic bacteria, and composed of bacterial communities and the polymeric matrix produced by them (Del Pozo, 2018 ; Saeed *et al.*, 2019). *S. aureus* have ability to adhere to surfaces of medical devices and host tissue leading to the formation of biofilm (Naimi *et al.*, 2003). Infections caused by biofilm producing organisms are chronic in nature and mostly occur in hospitals. *S. aureus* has been described as a major cause biofilm-associated infection.(Römling and Balsalobre, 2012).

Biofilm-associated infections are challenging for anti-infectious therapy, because the biofilm structure provides an ideal shelter for the bacteria to survive from antimicrobial killing and clearance of immune system (Saeed *et al*., 2019 ; Kumar, 2017). The formation of biofilm reduces the rate of penetration of antibiotics, thereby complicating treatment of infections caused by these bacteria (Abdelhamid and Yousef, 2023).

Biofim formation involves series of processes to include initial attachment to surfaces, Accumulation of bacterial population and maturation of complex biofilm layer and subsequent dispersal in case of change in environmental condition (Mustapha *et al*., 2020). *S. aureus* initially adheres to each other and then widen to structurally dynamic biofilm structures during the later phases of adherence. The maturation of the biofilm matrix into multi-layered patterns is initiated by the polysaccharide intercellular adhesin (PIA), synthesized from β-1,6-linkedN-acetyld-glucosamines (PNAG) (Periasamy et al., 2012). The synthesis of PIA is mediated by the intercellular adhesin (ica) locus, which comprises four core genes, namely icaA, icaD, icaB, and icaC, as well as a regulatory gene icaR ,These genes encode the corresponding proteins ICAA, ICAD, ICAB, and ICAC (Yu, 2016)..

The production of slimeis facilitated by the coexpression of icaA and icaD genes (Atshan *et al*., 2012). In addition to biofilm formation, S. aureus use vast array of virulent determinants to overcome host defence mechanism such as extracellular toxins (hemolysin, leukotoxins), enzymes such as coagulases and proteases and, surface proteins (clumping factor, adhesins) as virulent factors (Kong *et al*., 2018). The combinations of these virulence factors and ability of S. *aureus* to form biofilm increase challenge of treating biofilm associated infections cause by *S. aureus* (Paharik and Horswill,

2016). The clinical forms of *S. aureus* infections range from mild superficial skin infections to toxin mediated and severe lifethreatening systemic presentations (Kong *et al.,* 2018). In recent years the increasing incidence of diseases caused by biofilm-associated organisms has been noted globally. Biofilms pose a serious problem for public health. Because biofilm-producing microorganisms exhibit dramatically increased resistance to both antimicrobial agents and host immune response. Of note, the increase in the incidence of MDR bacterial and fungal strains makes many public creies (Ghaly *et al.,* 2020).

2-5-2. **Antibiotic Susceptibility of *S. aureus***

There is a wide variety of antimicrobial drugs that have been used to treat *S. aureus* infections, and most of these are still available, some of these (such as the Sulfa drug) were in development at the same time as the early Penicillin's (Zankari *et al*., 2012). Antimicrobial resistance in *S. aureus* is a major veterinary, as well as public, health concern worldwide since multi-resistant strains present a severe challenge to effective treatment (Garcés , 2012 ; Neopane *et al*., 2018*).*

In human medicine, :antimicrobial multi-resistance is frequently encounter and Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant :CNS (MRCNS) strain are among the most threatening bacteria involved in nosocomial infections, the type as well as severity of an *S. aureus* infection and its response to antibiotic treatment are dictated by the specific suite of virulence and antibiotic resistance associated genes carried by the strain of *S. aureus* causing the infection (Peacock *et al.,* 2002 ; Syed *et al*., 2011).

There are over 40 virulence associated genes identified among various strain of *S. aureus*, many of which are encoded by :mobile genetic element, However, within a few years, the *S. aureus* began to show resistance to Penicillin, :as an estimation 80% of all *S. aureus* isolates are Penicillin-resistant (David and Danm, 2010). Other antimicrobials related to Penicillin β-lactam drugs) were then developed: such as Methicillin, Oxacillin, and Ampicillin, A few years after the development of Methicillin, resistant strains were observed (MRSA), and eventually Methicillin was removed from the market. MRSA strains are considered to be resistant to all the Penicillin and (most β-lactam) drugs, and since Methicillin is no longer produced, Oxacillin is used for susceptibility testing, because of this, the name ORSA (Oxacillin-resistant *S. aureus*) is sometimes used instead of MRSA, but refer to the same strain (Blair *et al*., 2015).

Compound with β-lactam structure, but weak antimicrobial :activity, were discovered, These compounds were not useful as antimicrobial when used alone, but were found to be β-lactamase (an enzyme produced by the microorganisms to fight the β-lactam drugs) inhibitors, three of these inhibitors have been used in combination with Penicillin drugs; Amoxicillin / Clavulanic acid Ampicillin/sulbactam, and Piperacillin / tazobactam, These combination drugs improved the ability of the penicillin drugs: to kill the microorganism, but did not completely alleviate the resistance problem (Toussaint *et al*., 2015). The bacteria were not stymied for long, they acquired (from a still unknown source) a new PBP, named PBP2a or (PBP2') that had much lower affinity for methicillin and most other β-lactam drugs (Pu *et al*., 2016).

The gene that encodes the PBP2a protein is *mecA*, this gene is acquired through a horizontal transfer of a mobile genetic element known as the

staphylococcal cassette, these genetic elements contain two :required components; the *mecA* gene complex, and the *ccr* gene complex (which contains site-specific recombinase genes), the SCCmec elements have been classified into eight types (I-VIII) base on the structure and combination of *mecA* and *ccr* genes complex present (Paterson *et al*., 2014). These elements also differ in what other antimicrobial resistance genes are carried on them, types I, IV, V, VI, and VII generally do not carry other resistance genes, types II, III, and VIII may contain one or more other resistance genes, such as *ermA* (Erythromycin), *aadD* (Tobramycin), and *tetK* (Tetracycline), strains carry types IV, with some carrying types V and VII (Liu *et al*., 2016). Another group of drugs that has activity against cell wall synthesis is the glycopeptide ; Vancomycin being the main member of this group that has been used for *S. aureus* infections, with the. increase in antimicrobial resistant *S. aureus* strains (such as MRSA), Vancomycin has: been considered the drug that could be counted on to control these infection. However , in the last 8 years, strains of *S. aureus* that are resistant to Vancomycin have emerged (McDanel *et al*., 2015). The resistance of Vancomycin-resistance *S. aureus* (VRSA) is mediated through acquisition of the *vanA* gene from closely associated *enterococci*, the expression of this gene allow modification to the peptidoglycan precursors which decreases the binding affinity for Vancomycin. (Gardete and Tomasz, 2014).

There are several groups of antimicrobial drugs that affect :*S. aureus* infections inhibit protein synthesis, these groups are; Tetracycline's (Tetracycline, Minocycline, Tigecycline); the aminoglycosides: (Amikacin, Gentamicin, Tobramycin); Chloramphenicol (sole drug in this group); macrolides (Clarithromycin, Azithromycin, Erythromycin); and

Oxazolidinones (linezolid); Instances of antimicrobial resistance have been reported for all of these drug groups,:the resistance mechanism is similar for all aminoglycoside drugs; production of an aminoglycoside-modifying enzyme (AME) that chemically modifies the drug and decrease the drug's ability to bind to the 30S ribosomal subunit, the possible genes involved are the *aac*, *ant*, and *aph* genes, these genes are thought to be acquired via a plasmid (Magiorakos *et al*., 2012).

2.6. Vaginitis

Vaginitis is the most common women's health problem, and has been increasingly linked to a growing array of serious health risks. A vaginal infection is known medically as "vaginitis"(Alam *et al*., 2020) .It is an inflammation of the vagina presents with discharge, odor, irritation, or itching (Stöppler,2015).

Regarding women in reproductive age ,the vagina creates its own environment and maintains a balance among the normal flora found there with the hormonal changes. Vagina and its microflora form a balanced ecosystem in which dominated bacteria are vaginal *Lactobacilli*. There are dynamic changes in this ecosystem having structure and composition depending on many factors. Such a change in the quantity and quality of the respective microbial balance is associated with risk and requires correction and recovery (Kovachev, 2014).

The vagina stays moist as part of its self-cleansing mechanism, so it is normal to have some vaginal discharge . The normal moist discharge removes dead cells and bacteria from the vagina. It is devoid of glands and the secretions comes mainly from glands in the cervix, and is slightly acidic,

which helps to keep infections away. The acidity results from lactic acid, formed by dominant species of *Lactobacilli* (Petrova *et al*. ,2015).

The most common symptom of vaginitis is a vaginal discharge which may be excessive in amounts or abnormal in color (such as yellow, gray, or green),association with irritation and/or itching in the genital area ,inflammation (irritation, redness, and swelling caused by the presence of extra immune cells) (Sobel ,2016).

There are different causes of vaginitis like change in pH balance or the presence of foreign bacteria in the vagina that can lead to infectious vaginitis. Physical factors that contribute to the development of infections include the following: constantly wet vulva due to tight clothing, chemicals coming in contact with the vagina via scented tampons, antibiotics, contraceptive pills, or a diet favoring refined sugar and yeast .Improper hygiene, which may introduce bacteria or other irritants from the anal region to the vaginal area. (Rice,2015).

Hormonal vaginitis or atrophic vaginitis usually found in postmenopausal or postpartum women, is the thinning of the walls of the vagina caused by decreased estrogen levels . Sometimes it can occur in young girls before puberty. In these situations the estrogen support of the vagina is poor (Rice,2015).

Non – infectious vaginitis or Irritant vaginitis can be caused by allergies to condoms, spermicides, soaps, perfumes, douches, lubricants, semen and topical medications (Sobel, 2022).

Bacterial vaginitis is usually an aerobic vaginitis caused by bacteria like *Staphylococcus aureus, Escherichia coli*, Group B *Streptococci* (GBS), *Listeria, Mycoplasma* and *Ureaplasma* species (Sgayer *et al*. ,2020).

The main three kinds of Vaginitis according to the causative agent are Bac terial vaginosis (BV), Vaginal Candidiasis (Vulvovaginitis Candidiasis ,VVC), and Trichomoniasis or *Trichomonas vaginalis* infection show this in tabal (2-1) and (2-2)(Chris and Colebunders,2014).

Table (2-1) Signs and Symptoms of Vaginitis

Signs and Symptoms of Vaginitis				
Diagnosis	**Etiology**	**Symptoms**	**Signs**	**Other risks**
Bacterial vaginosis	Anaerobic bacteria *(Prevotella, Mobiluncus, Gardnerella vaginalis, Ureaplasma, Mycoplasma)*	Fishy odor; thin, homog-enous discharge that may worsen after intercourse; pelvic discomfort may be present	No inflammation	Increased risk of HIV, gonorrhea, chlamydia, and herpes infections
Vulvovaginal candidiasis	*Candida albicans*, can have other *Candida* species	White, thick, cheesy, or curdy discharge; vulvar itching or burning; no odor	Vulvar erythema and edema	—
Trichomonias is	*Trichomonas vaginalis*	Green or yellow, frothy discharge; foul odor;	Inflammation; strawberry cervix	Increased risk of HIV infection Increased risk

		vaginal pain or soreness		of preterm labor Should be screened for other sexually transmitted infections
Atrophic vaginitis	Estrogen deficiency	Thin, clear discharge; vaginal dryness; dyspareunia; itching	Inflammation; thin, friable vaginal mucosa	—
Irritant/allergic vaginitis	Contact irritation or allergic reaction	Burning, soreness	Vulvar erythema	—
Inflammatory vaginitis	Possibly autoimmune	Purulent vaginal discharge, burning, dyspareunia	Vaginal atrophy and inflammation	Associated with low estrogen levels
HIV = human immunodeficiency virus.				

Table(2-2) Type of vaginitis and Risk factors

Type of vaginitis	Risk factors
Bacterial vaginosis	Low socioeconomic status, vaginal douching, smoking, new or multiple sex partners, unprotected intercourse, women who have sex with women
Vulvovaginal candidiasis	Recent antibiotic use, pregnancy, uncontrolled diabetes mellitus, AIDS, corticosteroid use, other immunosuppression
Trichomoniasis	Low socioeconomic status, multiple sex partners, other sexually transmitted infections, unprotected intercourse, drug use, smoking
Atrophic or inflammatory vaginitis	Menopause, lactation, oophorectomy, radiation therapy, chemotherapy, immunologic disorders, premature ovarian failure, endocrine disorders, antiestrogen medications
Irritant vaginitis	Soaps, tampons, contraceptive devices such as condoms or diaphragms, sex toys, pessaries, topical products, douching, fastidious cleansing, medications, clothing
Allergic vaginitis	Sperm, douching, latex condoms or diaphragms, tampons, topical products, medications, clothing, atopic history

2.7. Bacterial Vaginosis (BV) :

It is a clinical syndrome, previously known as non-specific vaginitis that is characterized by disturbed vaginal ecosystem. The term "non-specific" was chosen to illustrate its poorly understood etiology, diagnosis and treatment, and the term "vaginitis" used to describe infection of the vulva (De Seta *et al*., 2020).

Bacterial vaginosis have other synonyms like anaerobic vaginositis , vaginal bacteriosis, *Gardnerella* vaginitis (Morrill *et al*., ,2020).

The conditions as having a characteristic discharge, increased vaginal pH and bacterial vaginosis manifests when the normally high lactobacilli concentrations in the vagina become reduced and replaced by commensal Gram-negative anaerobic micro-flora such as *Gardnerella vaginalis*, *Mobiluncus* species, *Bacteroides* species, *Prevotella* species, *Mycoplasma* species and more recently, *Atopobium vaginae* (Yasin *et al.*, 2021).

Bacterial vaginosis (BV) is caused by other organisms like *Ureaplasma* species , *Megasphera* spp., *Eggerthella* spp., and *Leptotrichia* spp. (Frølund *et al* ,2019)

The most common cause of abnormal vaginal discharge affecting women in reproductive age is the bacterial vaginosis (Sobel, 2016).

BV is generally diagnosed by using Spiegel or Nugent scoring that involve Gram stain examination and/or clinical symptoms often referred to as Amsel's criteria (Ruffier *et al*. ,2022).

The criteria involved the identification of three out of four clinical signs and/or symptoms including the identification of a grey, homogenous discharge, pH more than 4.5, amine (fishy) odor with the presence of 10% potassium hydroxide(KOH) and the microscopic observation of clue cells of vaginal epithelial cells (van *et al.*, 2018).

Amsel's criteria in its entirety is useful in clinical settings , the diagnosis by Amsel's criteria depend on clinical symptoms, but the identification of these components is autonomous (Shen *et al.*, 2021).

A thin homogenous vaginal discharge may not always be indicative of bacterial vaginosis especially in cases of asymptomatic bacterial vaginosis in which there is no discharge present, the remnant three criteria can be analyzed to make a diagnosis ,but the presence of vaginal discharge has the lowest sensitivity and is non-specific (Ajayi *et al.*, 2016).

Vaginal pH reach to 4.5 has good sensitivity for diagnosing BV. Disclosures of organic acids in vaginal secretions is useful for the presumptive identification of anaerobes in vaginal flora. Previously , gas liquid chromatography (GLC) was used to directly measure the characteristic organic acids in vaginal secretions produced by BV causing bacteria (Berus *et al.*, 2022).

The characteristic odour due to trimethylamine is produced by bacteria in BV, which can be tested by combining the detection of amine production with pH testing (Coleman and Gaydos ,2018).

where Paladineas *et al.* (2018) believe that using criteria such as pH and amine odour only are insufficient to diagnose BV, as well as microscopic examination for the presence of "clue cells" (particularly if ≥20% of vaginal epithelial cells) is considered as the most specific predictor of BV .

Microscopically, normal vaginal micro-flora consists largely of *Lactobacillus* morph types, while BV is characteristically dominated by *G. vaginalis.* This ignited the development of the spiegel criteria for quantifying bacterial morph types in vaginal smears (Stemmet, 2012).

This system is weighted so the lack of lactobacilli yields the highest score. As with the Spiegel criteria, *lactobacilli* and *G. vaginalis* are identified as large Gram-positive bacilli and small Gram-variable bacilli respectively(Martínez *et al.*,2011).

Culture techniques have long been the first approach in identifying the cause of infection. Several organisms associated with BV are difficult to cultivate, therefore their presence may be missed when using culture techniques and thus, it is not recommended as a diagnostic tool for BV (Sobel,2016).

Genital tract of women in reproductive age is commonly dominant by *Lacotbacills spp*. (Abdool Karim *et al*.,2019).

The most frequently isolated species are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jenseniiand*, *Lactobacillus iners* residing at the port of entry of bacterial and viral pathogens, the vaginal *Lactobacillus* species can create a barrier against pathogen invasion since mainly products of their metabolism secreted in the cervicovaginal fluid , help to maintain the vaginal pH (3.8 to 4.2) by producing lactic acid, which balances the vaginal ecosystem, and hydrogen peroxide, which suppresses the growth of Gram-negative and Gram-positive facultative and obligate anaerobes ;this can play an important role in the inhibition of bacterial and viral infections (Petrova *et al.*,2015).

This balance can be rapidly altered during processes such as menstruation, sexual activity , pregnancy and various infections. An abnormal vaginal

microbiota is characterized by an increased diversity of microbial species, leading to a condition known as bacterial vaginosis (Petrova *et al*. ,2015).

Lactobacilli-induced cytolytic vaginosis results from *Lactobacillus* overgrowth is characterized by a transformation in the length of lactobacilli (Korenek *et al*. ,2013).

2.8.Vulvovaginitis Candidiasis(VVC):

It is, also known as Vulvovaginal Vaginitis, vaginal thrush, and Vaginal yeast infection. It is typically caused by the yeast species *Candida albicans* , a common fungus in vagina without causing adverse symptoms. The causes of excessive Candida growth are not well understood (Watson *et al*.,2012). Predisposing factors include misuse of antibiotics, pregnancy, diabetes, HIV/AIDS and eating a diet high in simple sugar may play a role . A type of underwear, and personal hygiene do not appear to be factors (Farrokhi *et al*.,2021).

Women with vaginal yeast infection present with vulval itching, vulval soreness and irritation, pain or discomfort during sexual intercourse (superficial dyspareunia), pain or discomfort during urination (dysuria) and vaginal discharge, which is usually odourless ,this can be thin and watery, or thick and white, like cottage cheese(Velji *et al*. , 2022).

In order to confirm the infection , the physician should test vaginal pH, and should treat a vaginal specimen with 10% KOH. The alkaline pH of KOH does not affect the chitinous components of the fungi, whereas all non chitinous elements in the specimen (white blood cells, bacteria, epithelial cells) are dissolved (Pushpavani, 2020).

Microscopic reveals the characteristic architecture of fungal organisms (yeast buds and hyphae). Another clue is the presence of a rash with a prominent border, as it is seen in candidally infected diaper rash. The rash may spread outward from the vulvar area to involve the groin. The patient may also have satellite lesions outside the visible border ,these may also experience excoriations, formation of pustules, and fissures of the labia (Alter *et al*.,2018).

2.9. Candida

Is a form genus of incomplete yeast that is classified within the Kingdom of fungi and currently contains 150 species of what he said (Al-mamoory, 2014).

The *Candida* species of opportunistic fungi are found on the skin, mucous membranes and in gastrointestinal tracts or coexist naturally, ie, they are not originally pathogenic. However, when there is any change in the cellular immunity of the host, *C. albicans* was the most common species, although other species (eg, *C. tropicalis ,C. glabrata, C. krusei*) were less commonly identified and may cause infection, particularly in immunosuppressed and neutropenic patients (Epstein *et al.,* 2015). *Candida* can also cause various systemic diseases including disease pneumonia, Arthritis, osteomyelitis and endocarditis show this in table(2-1)(Chen *et al.,* 2022).

Sharma and Nonzom (2021). they pointed out that the humidity; the warm atmosphere and the health of the patients are the factors that help to infect the various areas of the body with the types of *Candida*.

The phenotypic characteristics of the species of *Candida* produce different forms of variability according to the different environmental conditions these shapes include budding yeast cell (blast conidia), pseudo mycelia and chlamydospores (Hunter and Barnett, 2019)

C. albicans is capable of forming well structured, three dimensional biofilm which contains yeast,hyphae, pseudohyphae and exopolysaccharides which prevents the entry of antifungal and protect the organism from host immune(Muthamil *et al.,* 2020)

system (Shafreen *et al.,* 2014)While most of the medical device related infections are caused by the biofilm forming *C. albicans,* few non *C. albicans Candida* species (NCAC) such as *C. tropicalis*, *C. glabrata* have also been reported for their capability to cause urinary tract and blood stream infections (Rathna *et al.,* 2016). *C. albicans* and *C. tropicalis* yeasts are responsible for a number of major diseases as well as recent cases of resistance to the main antifungal. Therefore, new substances should be research as an alternative to combat such resistance (Cornistein *et al.,* 2013; Oluwafemi *et al.,* 2013).

C. glabrata is not polymorphic, growing only as blastoconidia (yeast), A main distinguishing genetic characteristic of it was is that it has haploid genome, in contrast to the diploid genome of *C. albicans* and several other NCAC species (Silva *et al.,* 2011) it was generally considered a species of low virulence but with a higher mortality rate than *C. albicans* and is the most common non-*albicans Candida* isolated species (Rossoni *et al.,* 2017).

C. krusei to be less virulent than *C. albicans* in terms of its adherence to both epithelial and prosthetic surfaces, proteolytic potential and production of phospholipases Furthermore, it would seem that *C. krusei* is significantly different from other medically important *Candida* spp(Rodríguez-Cerdeira *et al.*,2019).

Its emergence may relate to an ability to colonize the skin, proliferate in glucose-containing solutions, and adhere to plastic. When clusters appear, determination of genetic relatedness among strains and identification of a common source are important. Its virulence appears associated with a capacity(Righini, 2020)

To produce biofilm and production of phospholipase and aspartyl protease. Further investigations of the host-pathogen interactions are needed (Hernando-Ortiz *et al.*, 2021).

2.9.1Classification of *Candida*

Candida is a eukaryotic organism, and it is described as an organism similar to yeast. The yeast follows the kingdom of fungi and is placed within the imperfect fungi section, order moniliales, family cryptococcaceaae and genus *Candida* (Cameron *et al.*, 2016).

The species of *Candida* has 150 species, of which 20 are pathogens and the most important of these species *C. glabrata, C. krusei, C. parapsilosis, C. dublieniesis, C. tropicalis, C. kefyr and C. guilliermondii, C. famata, C. lusitaniae and C. viswanathii* (Ng *et al.*, 2015).

2.9.2 Virulence Factors in *Candida*

Gut *et al.*, (2019) pointed out that some types of *Candida*, which have been tested on laboratory animals, have high levels of virulence compared with other non-pathogenic types of *Candida*, such as the ability to adhere to the production of enzymes and the ability to decompose blood

The process of adhesion of yeasts to the host surface and adhesion of the *Candida* cells to several types of host cells such as epithelial cells, endothelial and phagocytic cells such as *C. albican* by a protein Aspartyl proteinas that possesses the characteristic of pathogenesis and composition biofilm(Antorán Díaz, 2018).

Some types of yeast produce several enzymes linked to pathogens, including protein Aspartyl, phospholipase, lipase and hexoamaedyase (Spadaro and Droby, 2016) For example enzyme secretion *C. Albicans* Aspartyl proteinas which is produced by these types of yeast, is often produced at high levels. This enzyme acts on the decomposition of host proteins that include defense against infection and is followed by entering into the host's host tissue (Araújo, 2021).

2.10 *Zingiber officinale* plant

2.10.1 History *Zingiber officinale* or *Zingiber tuber*

Is one of the most common types of ginger of the Zingiberaceae family and is a common spice for food and drink. Ginger has a long record of medicinal uses, dating back to 2500 BC in China and India. It was used to

treat headaches, nausea, rheumatism, as well as colds (Rachkeeree *et al.*,2018).

It believes that the original home of ginger is India, where various varieties of ginger grow that we do not find anywhere else. Historical documents mention that the Roman Empire was importing ginger from India more than two thousand years ago, where the Romans used it for medicinal purposes more than they used to. To give food the distinctive flavor of ginger, and in traditional Chinese medicine, ginger is described as one of the stinging and hot spices. Ginger was used to raise body temperature, treat cold cases, improve weakness, slow heartbeat, improve pale skin, and tighten the body after blood loss (Vidya *et al*. ., 2021).

Figure (2-1): *Zingiber officinale* plant(Kaushik *et al*., 2020)

2.10.2 The chemical composition of *Zingiber officinale* plant

2.10.2.1 Nutrient Composition

Ginger contains 8.9% water, 2.3% protein, 0.9% fat, 1.2% minerals, 2.4% fiber and 12.3% carbohydrates. Of the minerals present is iron and calcium phosphate. Ginger also contains vitamins such as thiamine, riboflavin, niacin and vitamin C. The nutritional content of ginger varies according to the type, variety and season, as well as according to the method of drying and storage (Taşkın& Büyükalaca, 2017).

2.10.2.2 Active Components

Ginger contains a group of active substances. Ginger oil contains a high percentage of sesquiterpene hydrocarbons, in which zingiberene predominates. Studies on the fat-loving extracts of ginger have shown that it contains an active substance, gingerols, which can turn into shogaols. And zingerone and paradol (Munda *et al*., 2018). The substance 6-gingerol is responsible for the distinctive taste of ginger. Also, studies showed that the phenolic substances present in ginger have a role in the pharmacological and physiological activity of ginger (Gao *et al*., 2022). 6-gingerol has antioxidant properties (Gan *et al*., 2016). Zingerone and shogaols are present in small amounts in fresh ginger, but they are present in greater quantities in dried or extracted ginger (Li *et al*.,2016).

2.10.3 *Zingiber officinale* Classification

Kingdom: Plantae

Division: Angiosperma

Class: Monocotyledoneae

Order: Zingiberals

Family: Zingiberaceae

Genus: Zingiber

Species: officinal (Rosc) (Townsend and Guest, 1974)

2.10.4 Rhizome Mascolo *et al.* 1989.

figure(2-2) Rhizome *Zingiber officinale* (Mao *et al*.,2019)

2.10.5 Botanical description

Botanical description a perennial tuberous herbaceous plant with a height of up to 90 cm in appropriate conditions. The tubers are aromatic, thick and many-lobed, their color is pale yellow. The plant grows multiple side buds in the form of groups, which begin to dry out when the plant matures. The leaves are long, 2-3 cm wide with bases covered (coated) and the blade tapers gradually to a point shape. Single flowers (Adhikari and Bhandari, 2022).

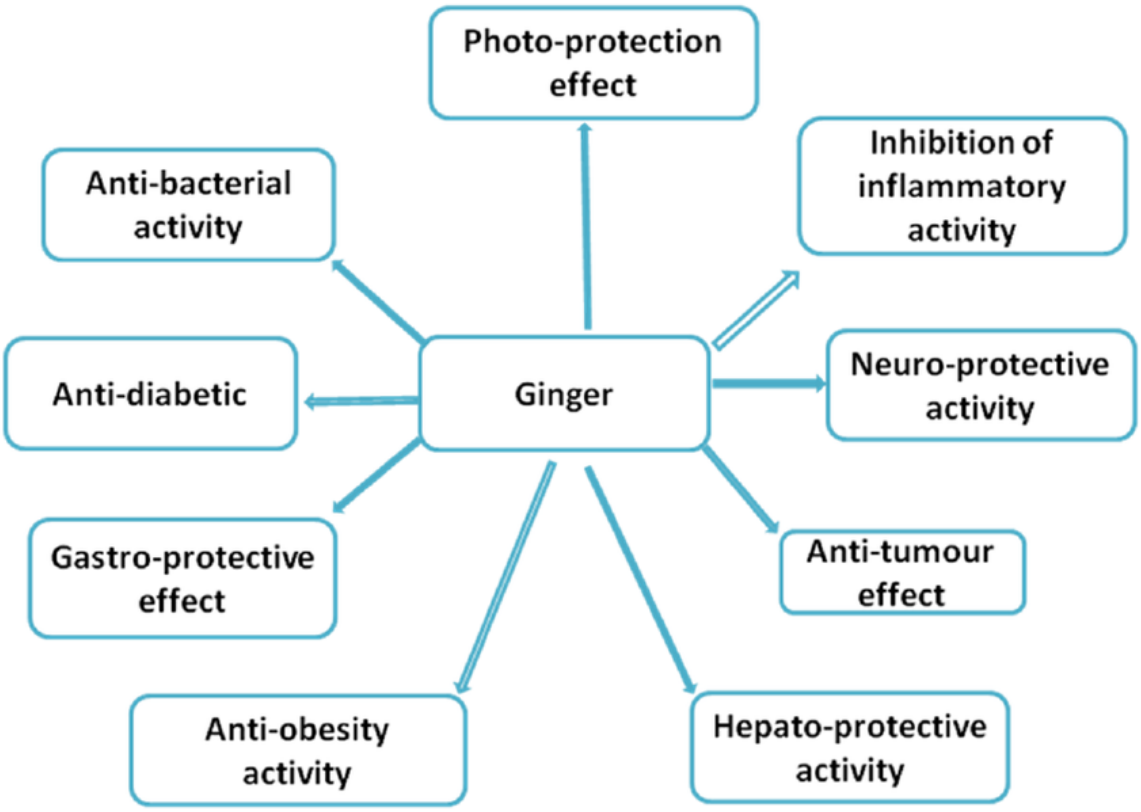
2.10.7 Geographical distribution

The plant is cultivated in large areas of India, Bangladesh, Taiwan, Jamaica and Nigeria and has a permanent growth in areas with warm climates (Ángeles-Hernández *et al*., 2020).

2.10.6 Treatment with plant extracts

Medicinal plants are the key elements in the traditional systems of medicine .They are used in various forms of preparations as powder, decoctions, oil etc., as a single herb or a mixture of herbs in therapeutic applications. Effectiveness, low cost, affordability and safety of the ethno medicinal preparations have made them popular among general public (Amarasiri *et al*., 2020). Medicinal plants contain several phytochemicals such as flavonoids, alkaloids, tannins, and terpenoids, which possess antimicrobial and antioxidant properties. The antimicrobial activities of some plant species have been widely researched. For example, the crude extracts of cinnamon, garlic, basil, curry, ginger, sage, mustard, and other herbs exhibit antimicrobial properties against a wide range of Gram-positive

and Gram-negative bacteria. In addition, it has been reported that the extracts from Chinese chives and cassia can effectively reduce the growth of Escherichia coli and other bacteria during storage of meat, juices, and milk (Maphetu *et al*., 2022). investigated the effect of some plant extracts on the growth of Candida albicans, the results indicated that the alcoholic extract of curry leaves effectively inhibit the growth of C. albicans with 24.05 ± 0.07 after 48 h. reported that thyme oil extract could decrease the growth of *C. albicans* and Pseudomonas aeruginosa(Gonelimali *et al*., 2018).



Figrure (2-3)shows uses *Zingiber officinale* (Rahmani and Aly, 2014)

2.10.7 Antibacterial and Antifungal compound of *Zingiber officinale* that were demonstrated by HPLC analysis

2.10.7.1 Gingerol

Gingerol is one of the active compounds found in ginger with several biological activities. Of these, its antioxidant and antimicrobial effects as reported before are against periodontal bacteria (Tang *et al*.,2020). The mechanisms behind gingerol-antibacterial activities are still unclear. In addition, the ameliorative effects of gingerol on the microbial status and formation of BA in the meat had scarcely investigated.

Phytochemicals are important sources for antimicrobial and antibiofilm agents against drug resistant microorganisms (Ali and Neelakantan, 2022). Recently, several studies have demonstrated ginger components have antibiofilm activities against pathogenic bacteria, such as, ginger water extract against Pseudomonas aeruginosa (Alam *et al*.,2020).

The antifungal activities of 6-gingerol and 6-shogaol were investigated by measuring minimum inhibitory concentrations (MIC), and for 6-gingerol and 6-shogaol MICs were 1000 μg/ml and > 2000 μg/ml, respectively, against *C. albicans* DAY185. These results support the notion that biofilm formation by *C. albicans* was effectively inhibited by the antibiofilm activities of 6-gingerol and 6-shogaol and not by their fungicidal activities. Furthermore, the observed biofilm inhibition in the absence of any effect on planktonic cell growth suggests that unlike conventional fungicides, 6-gingerol and 6-shogaol may less prone to the development of drug resistance(Lee *et al*,2018).

Many studies were conducted on ginger and its constituents and a wide array of bioactivities were reported, e.g., antioxidant, anti-inflammatory, antiemetic, and anticancer activity. Most of these had been correlated to gingerols and shogaols, the most abundant secondary metabolites in ginger(Ahmed *et al*.,2021).

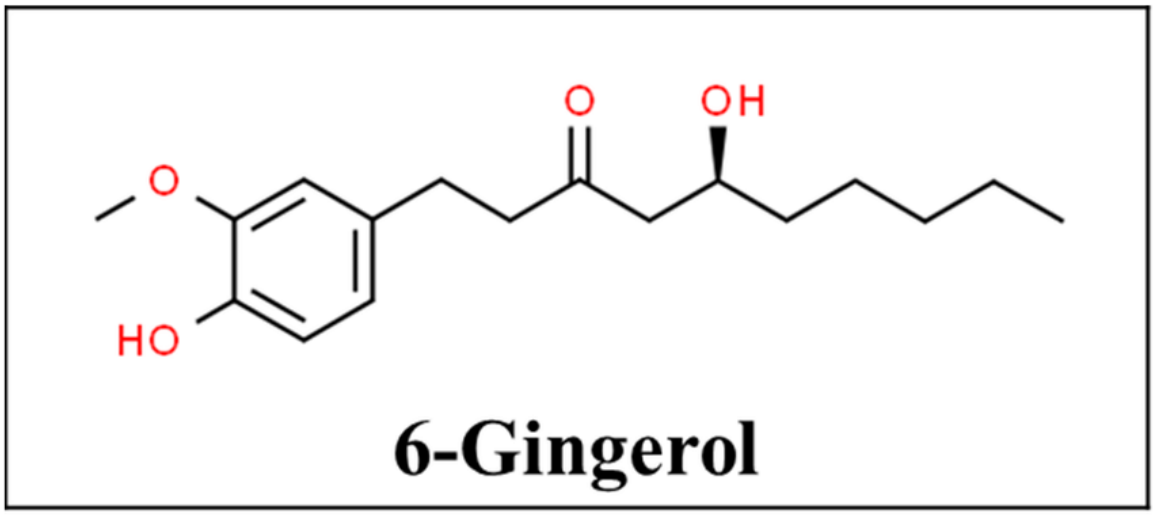


Figure (2-4): The chemical structure of gingerol (Jahromi *et al*., 2020)

Chapter Three
Materials and Methods

Materials and Methods

3.1: Materials

3.1.1: Laboratory equipment and instruments

The laboratory equipment and instruments used in the present study are listed in the Table (3-1).

Table (3-1): Laboratory Equipment's and Instruments

No.	Equipment and Instrument	Manufacturer/Origin
1.	Autoclave	Hirayama (Japan)
2.	Bench centrifuge	Memmert(Germany)
3.	Benzes burner	Amal(Turkey)
4.	Compound light microscope	Zeiss (Germany)
5.	Disposable and glassware	Cito (China)
6.	Electric sensitive balance	Denver (USA)
7.	Gel documentation system	Vilber (France)
8.	Gel electrophoresis system	Cleaver Scientific (UK)
9.	Hot plate with magnetic stirrer	Heidolph (Germany)
10.	HPLC	Japan
11.	Biological safety cabinet	Korea Denmark
12.	flasks and beakers	Germany
13.	Gel electrophoresis	USA
14.	Incubator	Germany
15.	Loop, wood sticks	China
16.	Medical injection Syringes	UAE
17.	Micro centrifuge	India Germany
18.	Micropipettes	USA
19.	Medical cotton	Turkey
20.	Oven	Germany

21.	PCR system/Conventional	USA
22.	Platinum wire loop	Germany
23.	Paraffin film	India
24.	PCR system/Conventional	Cryste (Korea)
25.	Micropipette	Capp (Denmark)
26.	pH-meter	WTW (Germany)
27.	Real time PCR	Germany
28.	Refrigerator	Concord (Lebanon)
29.	Sensitive electron balance	Japan
30.	Vortex	Taiwan
31.	Water bath	Germany
32.	Tips	Syria
33.	filter tips	USA
34.	Micro-centrifuge tube	Korea

3.1.2: Biological and chemical materials

The biological and chemical materials used in this study are listed in Table (3-2).

Table (3-2) Biological and Chemical Materials

No.	Biological and Chemical Materials	Manufacturer(Origin)
1.	6X DNA Loading buffer Blue	Eurx (Poland)
2.	Agarose	Condalab (spain)
3.	Barium chloride	CDH (India)
4.	DNA loading dye	Promega/USA
5.	Ethanol 70%	BDH, UK
6.	Glucose	CDH (India)
7.	Glycerol ($C_3H_8O_3$)	Merck(England)
8.	gram stain	CDH (India)
9.	McFarland's standard solution	Biomerieux(France)
10	Nuclease Free Water	Bioneer (Korea)
11	PCR pre mix (master mix)	Bioneer, Korea
12	Phosphanuclease free water tablet	Himedia (India)
13	Red safe nucleic acid staining solution 1ml	BDH, England
14	TBE buffer	Condalab (spain)
15	Tris borate TBE buffer (loading buffer)	Promega ,USA

3.1.3 : Marketable kits

Kits used in the present study are listed in Table (3-3).

Table (3-3): kits

No.	Type of Kit	Company(Origin)
1.	DNA extraction Kit to fungi and bacteria	Promega (USA)
2.	DNA ladder	Promega (USA)
3.	Primers	Macrogen, Korea
4.	Master mix PCR – Kit	Promega, USA
5.	Green master mix	Promega (USA)
6.	SYBR Green	Promega (USA)

3.1.4: Culture media

Table (3-4): Culture media

No.	Culture Media	Manufacturer and origin
7.	Brain heart infusion broth,	India
8.	Nutrient broth,	
9.	Chromogenic agar for candida	India
10.	Luria broth	India
11.	Potato dextrose agar medium (PDA)	India
12.	Sabouraud Dextrose Agar medium (SDA)	India
13.	CHROM agar	India
14.	Blood agar	Accumix (India)
15.	Urea agar base	Himedia (India)
16.	Czapek Dox Agar	India
17.	Potato dextrose broth medium	India
18.	Tobacco Agar Medium	India

3.1.5Antibiotics and antifungals Disks

The antibiotics and antifungal used in this study are listed in Table (3-5)

Table (3-5): Antibiotics and Antifungal Disks

No.	Antimicrobial Agent	Assembly	Disk Content (mcg)	Company/ Origin
1.	Tobramycin	TOB	10	Condalab
2.	Ciprofloxacin	Cip	5	Condalab
3.	Kanamycin	Kana	30	Condalab
4.	Doxycycline	Do	30	Condalab
5.	Itraconazol	Itra	30	Condalab
6.	Nystatin	Nys	50	Condalab

3.1.6. Primer Pairs for bacteria and fungi

Table (3-6) Show The primer pair (Macrogen/Korea)

Table (3-6) Primer Sequencing for *S.aureus*

Gene	Symbol	bp.		PCR primer for virulence factor of *S.aureus*	Ref
Nuclease	*nuc*	537	R	5'-AAGAGGTTTTTCTTTTTCGCTACTAGTTGC-3'	Lin *et al* .,2021
			F	5'-CTCCAAATATTTAATTTCTGTTGTTTAGCTTT-3'	
Lipase	*lip*	921	R	5'-GTAGATTATGGTGCAGCACATGCAGCAAAATATGG-3'	Lin *et al* .,2021
			F	5'-AGCTTTTCAGTTTTCACTAAATCGTCTGCT-3'	
Staphylokinase	*sak*	492	R	5'-ATGCTCAAAAGAGGTTTATTATTTTTAACTGTTTT-3'	Lin *et al* .,2021
			F	5'-ATTTCTTTTCTATAACAACCTTTGTAATTAAGTTG-3'	
Hyaluronidase	*hysA*	936	R	5'ATGACATATAGAATGAAGAAATGGCAAAAATTATCCACC 3'	Lin *et al* .,2021
			F	5'-TAATTCAAAGCGCACGCCGGATTCATTAGA-3'	
16s rRNA	*16s rRNA*	756	R	5'-AACTCTGTTATTAGGGAAGAACA-3'	Rasheed and Hussein,2020
			F	5'-CCACCTTCCTCCGGTTTGTCACC-3'	
Penicillin binding protein 2a	*mecA*	310	R	5'-GTAGAAATGACTGAACGTCCGATAA-3'	Rasheed and Hussein,2020
			F	5'-CCAATTCCACATTGTTTCGGTCTAA-3'	
Intercellular adhesion A	*icaA*	188	R	5'-ACACTTGCTGGCGCAGTCAA-3'	Haddad *et al*.,2018
			F	5'-TCTGGAACCAACATCCAACA-3'	
Intercellular adhesion D	*icaD*	198	R	5'-ATGGTCAAGCCCAGACAGAG-3'	Haddad *et al*.,2018
			F	5'-AGTATTTTCAATGTTTAAAGCAA-3'	
Cysteine protease	*sspB*	493	R	5'-TGAAGAAGATGGCAAAGTTAG-3'	Haddad *et al*.,2018
			F	3'-TTGAGATACACTTTGTGCAAG-5'	
Toxic shock syndrome toxin-1	*Tst*	326	R	ACCCCTGTTCCCTTATCATC	Haddad *et al*.,2018
			F	TTTTCAGTATTTGTAACGCC	
Exfoliative toxin A	*Eta*	119	R	CTAGTGCATTTGTTATTCAA	Derakhshan *et al* .,2021
			F	TGCATTGACACCATAGTACT	
Exfoliative toxin B	*etb*	226	R	ACAAGCAAAAGAATACAGCG	Derakhshan *et al* .,2021

Table (3-7) Primer Sequencing for *C.albicans*

primers	bp.		DNA sequence (5-3)	Ref.
ITS	354	R	TTT ATC AAC TTG TCACACCAG A	Asadzadeh and Khan, 2019
		F	GGT CAA AGT TTG AAG ATA TAG GT	

3.2. Study design

The main methods included in this study are show in the figure (3-1)

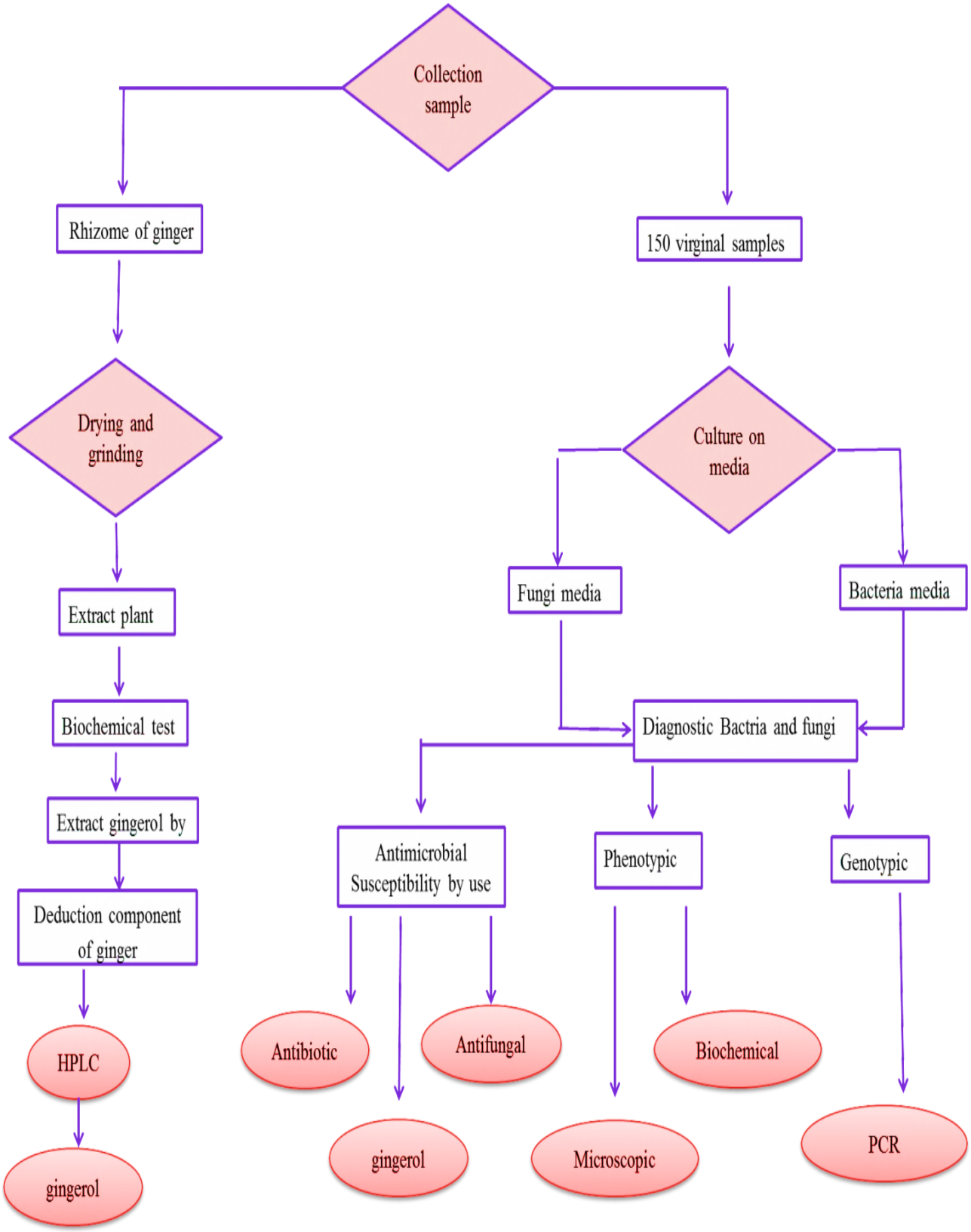


Figure (3-1) Design study

3.2.1.Sample Collection

The research period lasted from 2021 to 2023, as the period of sample collection included the third and fourth months, and the rest of the period included practical work and writing.

One hindered fifty clinical samples were taken from female patients using a sterile swab. Bacteria and fungi culture media Samples were collected with the assistance of a Gynecologist. all of the women in the study were married women between the ages of 18 to 68 years old.

3.2.2 Solutions used in the study and methods of preparation

3.2.2.1 Saline Normal

Ready to use, sterile normal saline (NS) was use for the preparation of culturesuspension.

3.2.2.2 Phosphanuclease free water Saline (PBS)

One tablet of ready to use phosphanuclease free water saline (PBS) was dissolved in 100 ml of D.W. the PBS was sterilized by Millipore filters, 0.22 µm.

3.2.2.3 McFarland's Turbidity Standard No. 0.5

Obtained ready and used according to the manufacturer's instructions

3.2.2.4 TBE Buffer (Tris-Borate-EDTA)

Obtained ready and used according to the manufacturer's instructions

3.2.3. Reagent and culture media

The way to work according to the manufacturer

Table (3-8): Reagent, cultural media and their purpose

No.	Reagent and culture media	purpose
1	Nutrient Agar Medium	supports the growth of various types of bacteria and fungi, and contains many of the nutrients necessary for the growth of bacteria
2	MacConkey Agar Medium	for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non- fermenting gram-negative bacteria
3	Manitol Salt Agar Medium	isolating pathogenic staphylococci from clinical samples, food and other materials of sanitary importance
4	Blood Agar Medium	grow fastidious organisms and to differentiate bacteria based on their hemolytic properties
5	Eosin Methylene Blue (EMB) Agar	selective for gram-negative bacteria against gram-positive bacteria
6	Kligler's Iron Agar	tests for organisms' abilities to ferment glucose and lactose to acid and acid plus gas end products
7	Peptone Water Medium	used as a diluent and nutrient enrichment broth for the resuscitation and growth of a wide variety of microorganisms
8	Simmon’s Citrate Medium	determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of Enterobacteriaceae.
9	Urea Agar Media	used to detect urease activity in a variety of microorganisms
10	Potato dextrose agar medium (PDA)	detecting the presence of yeasts and molds in product samples
11	CHROM agar	To differentiate organisms on the basis of the specific color colonies
12	Catalase Reagent	for the detection of microorganisms that possess the catalase enzyme
13	Oxidase reagent	to detect the presence of oxidase enzymes produced by a variety of bacteria
14	coagulase test	useful for differentiating potentially pathogenic Staphylococci such as Staphylococcus aureus from other Gram positive, catalase-positive cocci

3.2.4 stains

3.2.4.1Lacto phenol stain

Used for stain chlamydo spore and to germ tube

3.2.4.2 Gram's stain

Gram stain was prepared by (Elgohary *et al*.,2021).

3.2.5. Isolation and Diagnosis Microorganism

After taking samples, they were cultivated on culture media for fungi and bacteria, and the species were diagnose by several methods, including phenotypic, which included biochemical and microscopic examinations, and differentiation media. After that, they were diagnosing using a genetic method.

3.2.6.Plant Collection and drying

The rhizomes of ginger were taken, washed well, cut into small pieces, dried at room temperature for 7 days, then ground in an electric mixer and a hot water extract was made for biochemical tests (Ogori *et al*.,2021).

3.2.7.Detection of photochemical compounds by a chemical method

3.2.7.1. Detection of Glycosides

The method of (Pitura and Arntfield, 2019) was followed by mixing two parts of the Fehlink Detector and the plant extract, then left in boiling water bath for 10 minutes and indicated the positive detection through the appearance of red color

3.2.7.2.Test of Tannins and Phenols

A solution of ferric chloride 1% Prepared by dissolving 1 g of FeClj2 in 100 ml of distilled water and indicate the positive detection by appearance of the bluish green color when mixed with an equal amount of plant extract and used to detect tannins and phenols (Alwan, 2018).

3.2.7.3.Saponin

According to (Vyn *et al*, 2020) the Foam test was performed to detect about the presence of Saponin compounds as the following: 1ml of the extract was diluted with distilled water and made up to 20ml.The solution was shaken vigorously in a graduated cylinder for about 15Minutes. A one cm layer of foam formed confirms the presence of saponins in the sample**.**

3.2.7.4. Test of Flavonoids

Prepare by adding 4 ml of ethanol 95% to 1 ml of extract, leave in a water bath for 25-30 minutes at boiling point then add drops of 50% Potassium hydroxide to 5 ml of the solution, the appearance of yellow color evidence of flavonoids (Sudharsan *et al*., 2022).

3.2.7.5. Test of Terpenes and Steroids

One gram of extract was diluted in a small amount of chloroform with a drop of concentrated sulfuric acid. The brown color indicates containing the extract Terpenes, and if after a period (5 minute) of dark blue color, appearance refers that the extract contains steroids (Dilshad and Batool, 2022).

3.2.8. HPLC analysis

HPLC used to Detection gingerol in rhizome of Zingiber officinale

3.2.8.1.Sample preparation to HPLC

Twenty gm of ginger were grind and extracted with (250 ml) of methanol. The methanol extract was filtered, transferred to a 500 ml round-bottom flask and concentrated in vacuous at 40^{o}C using a rotary evaporator. The residue was re-suspended in methanol, vortexed for 1 min, then filtered using 0.22 µm membrane filter, and transferred to a 1.5-ml micro centrifuge tube. All extracts were stored at 4^{o}C until used (Cafino *et al*.,2016). Fpe 22

3.2.8.2 HPLC condition:

Sample were analyzed by high performance liquid chromatography HPLC model (SYKAM) Germany . the mobile phase was methanol :distilled water (80:20, v/v) , the column separation was (C18 – ODS (25cm * 4.6 mm) and the detector = UV – 282 nm at flow rate at 1.0 ml / min.

3.2.8.3 Calculation the concentration of HPLC analysis

The concentration of each phytochemical (gengerol) , Which was analysis by HPLC determine according to following equation depending on the area under the peak. (Tanweer *et al* .,2020).

conc. of sample (mg/ml)= $\frac{CON.OF\ STANDERD * A\ OF\ SAMPLE}{A\ of\ stander} * DF$

Where conc. = concentration DF= delusion factor , A = area

3.2.9. Biosafety and Hazard Material Disposing

Biosafety aspects were followe during the work that includes disposing of all swabs, petridishes, and all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol (70%) before and after the work. Simply safe were used instead of ethidium bromide to reduce biohazard . (Al-Allak,*et al*.,2019).

3.2.10. Molecular Study

3.2.10.1. Genomic DNA extraction

Favor PrepTM Genomic DNA Mini Kit was used to extract genomic DNA from isolates following the manufacturer's protocol (please see appendices no.)

3.2.10.2. Primer preparation

Primer pairs used in this study were dissolved using Nuclease free water, (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2. Firstly the primer stock tube prepared and then the working solution would prepared from primer stock tube.

According to the instruction provided by primer manufacturer (Macrogen / Korea) the Nuclease free water were added to get 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock by dilution with Nuclease free water to get 10 picomole/microlite (Lusser *et al.,* 2020).

3.2.10.3. Reaction mixture

Amplification of DNA was carried out in a final volume of 20 µl reaction mixture as mentioned in table (3-7).

Table 3-9: Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Green master mix	10 µl
2.	Upstream primer (10pmol/µl)	1µl
3.	Downstream primer (10pmol/µl)	1µl
4.	DNA template	2 µl
5.	Nuclease free water	6 µl
Total volume		20 µl

3.2.10.4. Polymerase chain reaction (PCR)

Conventional PCR were used to amplify the target DNA using specific primer pairs (table:3-6). It include three consecutive steps that repeated for specific number of cycles to get PCR product (amplicon) which could be finally visualized after agarose gel electrophoresis. The thermal cycling conditions mentioned in the table (3-6).

3.2.10.5.Agarose Gel Electrophoresis

The method Agarose includes the following steps:

1. Place the gel-casting tray in plastic tray, check that the teeth of the comb are approximately 0.5mm above the gel bottom.

2. Prepare 500ml of TBE (1X) by adding 50ml of TBE (10X)stock solution to a final volume of 500ml of deionized water.

3. Place 100ml of the buffer into a 500ml flask and add 0.8g of agarose. Melt the agarose by heating (microwave) the solution on hot plate for approximately 10 min. Carefully swirl the agarose solution to ensure that the agarose is dissolved, that is no agarose particles are visible.

4. Cool the agarose solution to approximately 50°C and added 2-3 µl of safe red stain. Slowly pour the agarose into the gelcasting tray. Remove any air bubbles by yellow tip.

5. Position the comb approximately 1.5cm from the edge of the gel. Let the agarose solidify for approximately 20–30 minutes. After the agarose has solidified remove the comb with a gentle back and forth motion, taking care not to tear the gel.

6. Remove the gel-casting tray and place the tray on the central supporting platform of the gel box.

7. Add electrophoresis buffer to the buffer chamber until it reaches a level of 0.5–1cm above the surface of the gel.

8. Load the samples into the wells using a yellow tip. Place the tip under the surface of the electrophoresis buffer just above the well. Expel the sampleslowly, allowing it to sink to the bottom of the well. Take care not to spill the sample into a neighboring well. Note: samples must be loaded in sequential sample wells. When loading fewer samples than the number of wells it is preferable to leave the wells nearest the edge of the gel empty.

9. First load 5µl of ladder molecular weight marker to each side of the gel(flanking the sample line) and 20 µl of DNA specimen in the other well.

10. Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the well. Turn on the power supply.

11. Continue electrophoresis until the tracking dye moves at least 10

cm of the gel length

3.2.10.6 PCR Cycling Conditions for bacteria

PCR mixture was set up in a total volume of 25 µL, included 12 µL of PCR premix, 2 µL of each primer and 5µL of extracted DNA have been used table (3-3). The rest volume was completed to 25 µL of Dnase free water, then vortexes. Negative control contained all material except template DNA, so instead that distilled water was added. PCR-reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermo cycler PCR that programed as (table 3-4).

Table (3-10): PCR program that apply in the thermo-cycler for *S.aureus*

		No. of cycles 25				
Gen	Primary Denaturation c/t	Denaturation Temperature c/t	Annealing Temperature c/t	Extending Temperature c/t	Final Extending c/t	Cooling c/t
Nuc	98/2m	98/10s	72/20s	72/1m	72/1m	4ċ
Lip	98/2m	98/10s	72/20s	72/1m	72/1m	4ċ
Sak	98/2m	98/10s	72/20s	72/1m	72/1m	4ċ
Hysa	98/2m	98/10s	72/20s	72/1m	72/1m	4ċ
16s rRNA	94/4m	94/55s	60/40s	72/30s	72/5m	4ċ
mecA	94/4m	94/55s	60/40s	72/30s	72/5m	4ċ
icaA	94ċ /5m	94ċ /5m	60ċ/30s	72ċ/45s	72ċ/10m	4ċ/
icaD	94ċ /5m	94ċ /5m	60ċ/30s	72ċ/45s	72ċ/10m	4ċ/
sspB	94ċ /5m	94ċ /5m	46.6ċ/30s	72ċ/30s	72ċ/10m	4ċ

Tst	94ċ /5m	94/1m	46.6ċ/30s	72/1m	72/5m	4ċ
Eta	94ċ /5m	94/1m	46.6ċ/30s	72/1m	72/5m	4ċ
Etb	94ċ /5m	94/1m	46.6ċ/30s	72/1m	72/5m	4ċ

3.2.10.7: PCR Cycling Conditions for *Candida albicans*

A loopful of yeast colony was suspended in 1 mL of sterile water in a micro centrifuge tube containing 50 mg Chelex-100 (Sigma-Aldrich Co., St. Louis, MO, USA).

The contents were heated at 95°C for 20 min and then centrifuged. The supernatant was transferred to a new tube and then used as source of genomic DNA in PCR. species-specific primer pairs derived from the internally transcribed spacer (ITS) region (comprising ITS-1, 5.8S rRNA and ITS-2) of ribosomal DNA (rDNA) were designed for differentiation of *C. albicans* (Asadzadeh and Khan, 2019).

Table (3-11): PCR program that apply in the thermo-cycler for *C. albicans*

		No. of cycles 25				
Gen	Primary Denaturation c/t	Denaturation Temperature c/t	Annealing Temperature c/t	Extending Temperature c/t	Final Extending c/t	Cooling c/t
ITS	95°C /2m	95°C /10s	58°C /10s	72°C /15s	72°C/15 s	4ċ

3.2.11: Antibiotic Susceptibility Test (Disk Diffusion Method)

It was perform according to Clinical Laboratory Standard Institute (CLSI, 2023) by using a pure culture of previously identified bacterial organism.

1. With a sterile wire loop, the tips of 4-5 isolated colonies of the organism to test tube containing 5 ml of sterile normal saline in a cell density equivalent to turbidity of McFarland tube No. (0.5) which approximately equal to bacterial cells density of $1.5x10^8$ cells/ml

2. A sterile cotton swab was dippe into the standardized bacterial suspension. The excess fluid was removed by rotating the swab firmly against the inside of the tube above fluid level. The swab was then streaked onto the dried surface of a Muller-Hinton Agar plate in 2 different planes to obtain an even distribution of the inoculums.

3. The plate lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undisturbed for 3-5 min to allow absorption of excess moisture.

4. With the sterile forceps, the selected discs were place on the inoculated plate and pressed gently into the agar. Within 15 min the inoculated plates were incubated at 37 ºC for 18 -24 hr in an inverted position.

5. After incubation, the diameters of the complete inhibition zone were note and measured using reflected light and a ruler. The end, measured to the nearest millimeter, was take as the area showing no visible growth.

6. The results interpreted according to (CLSI,2023), the critical diameters and to the leaflet of antibiotics manufactures.

3.2.12: Preservation of Bacterial Isolates

3.2.12.1. Short Term Preservation

The bacterial isolates was preserved after identification by transferred single pure isolate colony to nutrient agar slant in screw–capped tube, incubated over night at 37˚C, and then store in refrigerator at 4˚C for daily used (Balakrishnan *et al.,* 2022).

3.2.13.2. Glycerol at -20˚C Method

Overnight bacterial isolates on Brain heart infusion agar were suspend in screw-capped tubes or vials containing sterile 15-20% glycerol and Brain heart infusion broth, stored at -20˚C (Mahmood,2021).

3.2.13: Statistical analysis

Data were analyzed statistically using SPSS program through an ANOVA. Means were comparing by (5%) LSD test to determine the significant difference between the different treatments assayed. (Etemadifar and Masoudi, 2018).

Chapter Four
Results and Discussion

4. Results and Discussion

4.1 Isolation and diagnosis of the bacteria and fungi in vaginitis

4.1.1 Isolation and diagnosis of the bacteria

The samples were culture on different medium, where 141 bacterial isolates were found.

After that, it was diagnosed whether it was gram-positive or gram-negative by culturing it on special culture media. Then a microscopic examination was performed for each sample where the following types appeared that are positive for a gram stain (*Staphylococcus aureus and Staphylococcus epidermidis*) and negative for a gram stain (*Escherichia coli, Klebsiella pneumonia pneumonia*).Figure (4-1) shows Gram positive and gram-negative bacteria isolated from vaginal swap and Table (4-1) shows type of bacteria isolated from vaginal samples .

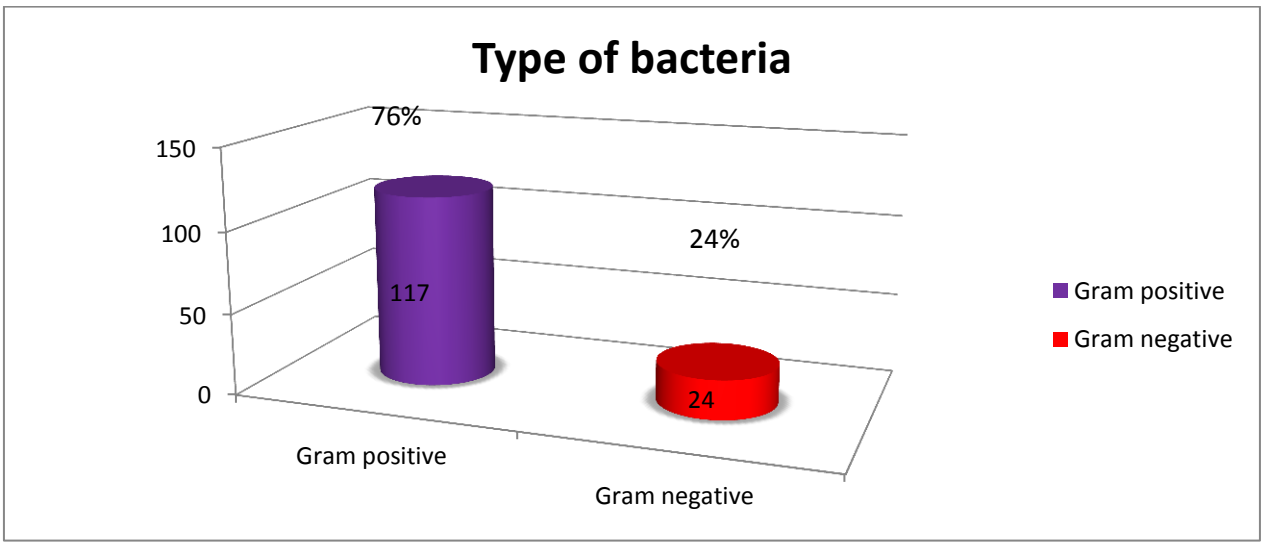


Figure (4-1) percentage Gram positive and gram-negative bacteria isolated from vaginal swap

Table (4-1) Type of bacteria isolated from vaginal samples

Type bacteria	number	Percentage (%)
Gram positive		
S. aureu s	95	81.2%
S. epidermidis	22	18.8%
Total	117	100%
Gram negative		
Klebsiella pneumonia spp.	9	37.5 %
Escherichia coli	15	62.5%
Total number	24	100%

4.1.1.1: Biochemical test for bacteria

Many tests have been performing to diagnose bacteria. Figure (4-2) Show the ability of bacteria (*E coli , Klebsiella pneumonia and S. aureus* to hemolysis blood This result is in agreement with Rémi *et al.*,2021.

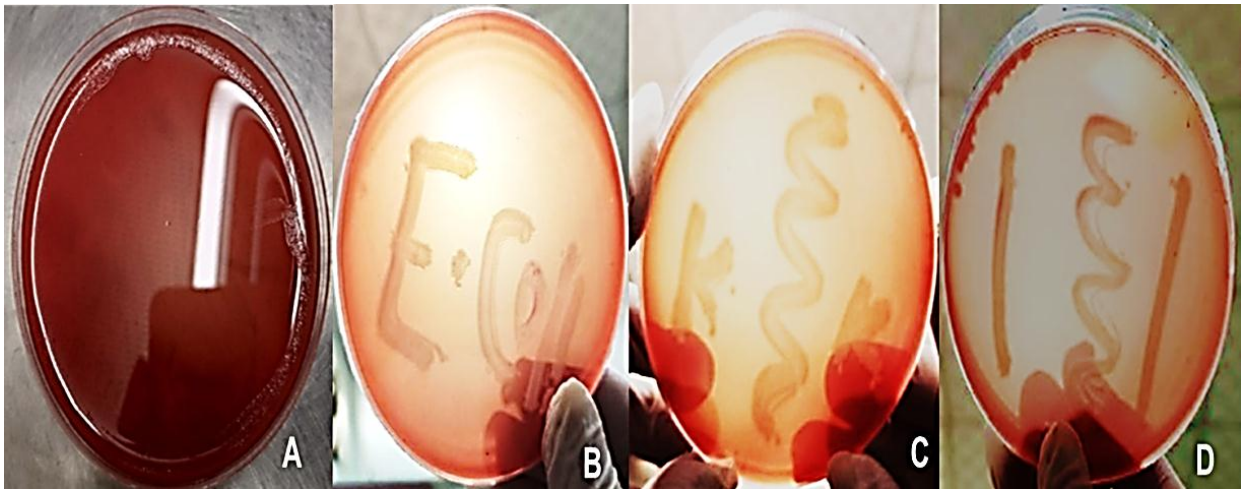
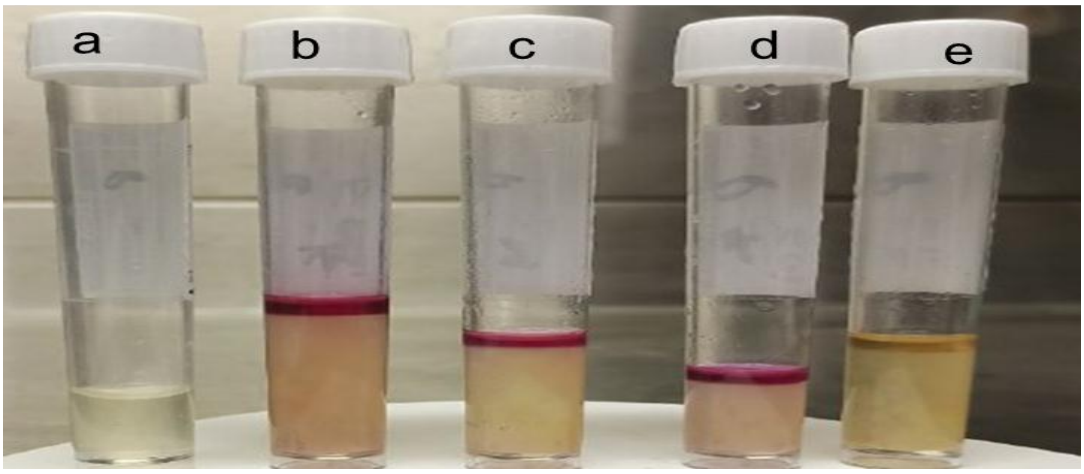


Figure (4-2)Hemolysis test (A) control (B)*E. coli* (C) *Klebsiella pneumonia*(d) *S. aureus*

Through research, the different bacterial species that were isolated differed in their ability in producing the enzyme Tryptophanase that is why we found *E.coli* and *s.aureus* to be positive for indol test while *Klebsiella pneumonia* are negative for indole test. As shown in figure(4-3).

An indole test is a biochemical test performed on a bacterial species to determine the ability of an organism to convert tryptophan to indole. This division is performing by a cascade consisting of a number of different intracellular enzymes, a system commonly referred to as 'tryptophanase'.The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures,abattery of tests designed to distinguish among members of the family Enterobacteriaceae. Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme(Vignesh *et al*.,2016).



Figer(4-3) Indol test (a)control (b) *s.aureus* (*c)E. coli* (d) *S.epdermides* (e) *klebsieala*

Through the samples that were isolated and diagnosed, it was found that some bacterial species *Klebsiella pneumonia* and S. aureus positive for simmon citrate and *E.coli* negative for this test, as shown in Figure (4-4).

Simmons citrate agar tests the ability of organisms to utilize citrate as a carbon source. Simmons citrate agar contains sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen, other nutrients, and the pH indicator bromthymol blue. This test is part of the IMViC tests and is helpful in differentiating the Enterobacteriaceae .Organisms, which can utilize citrate as their sole carbon source use the enzyme citrase or citrate-permease to transport the citrate into the cell. These organisms also convert the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide, which creates an alkaline environment in the medium (Hamiduzzaman *et al*., 2018).

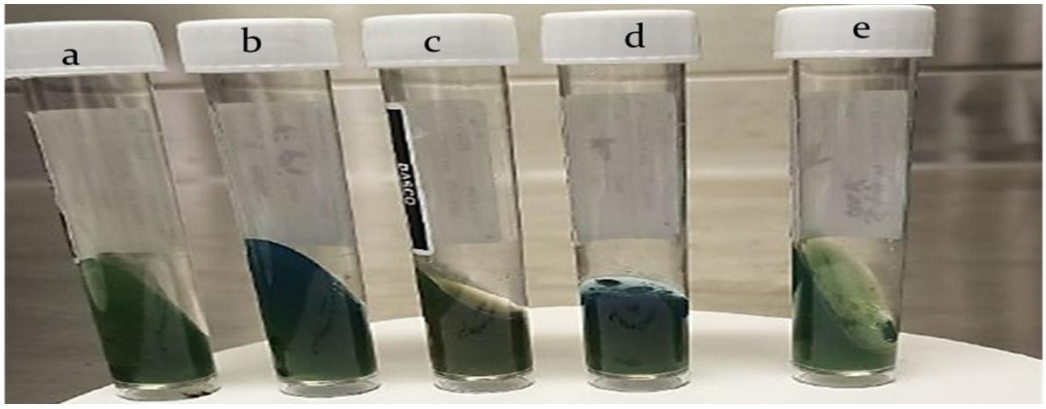
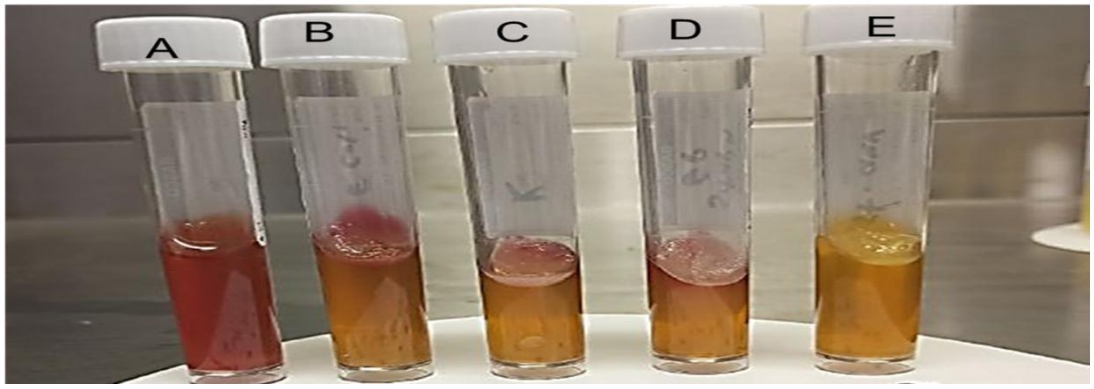


Figure (4-4) Simmon citrate test (a)control (b)*S.aureus* (c)*E.coli* (d) *klebsieala* (e)*S. epdermides*

Figure (4-5) shows kligler iron test all bacteria is positive (*S.aureus ,E. coli ,klebsieala and S.epdermides*) . This test shows the susceptibility of bacteria to produce H2S and gas on Kligler iron agar and fermentation tests for nine different saccharides namely glucose, sucrose, lactose, mannitol, xylose, rhamnose, mannose, dextrose and maltose (Ali and Maaroof, 2020)



Figure(4-5) kligler iron test (A)control (B)*S.aureus* (C)*E. coli* (D)*klebsieala*(E) *S.epdermides*

The samples were diagnosed and all bacterial species that were isolated and diagnosed were positive for catalase test, such as *E.coli*, *s.aureus*,*Klebsiella pneumonia*, as shown in Figure (4.6) **found in appendix**.

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (such as bacteria, plants, and animals) which catalyzes the decomposition of hydrogen peroxide to water and oxygen It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second(Halim and Halim,2019).

The samples were diagnosed and all bacterial species that were isolated and diagnosed were positive for Coagulase test, such as *E.coli*, *s.aureus*,*Klebsiella pneumonia*, as shown in Figure (4.7) **found in appendix.**

Coagulase is an enzyme produced by *Staphylococcus aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin. Other *staphylococci* do not produce coagulase, thus this test can distinguish *S. aureus* from other *staphylococci* (Nanjundaswamy *et al*.,2022) Coagulase is of two types; free coagulase and bound coagulase, each of which is detected by different methods. The bound coagulase is call the clumping factor and is detect rapidly by a slide test. The free coagulase, in turn, is detected in the test tube as a result of the formation of a clot (Hamad and Alrawi,2022).

The diagnosis of *s.aureus* was done using the coagulase test, and this test was found to be positive only for *s.aureus*, while the other samples were negative for this test, such as *E.coli*, *Klebsiella pneumonia*, as shown in figure (4-8) **found in appendix**.

Table (4-2) Biochemical test for some pathogenic bacteria

Bacteria	Indol	citrate	Ureaes	Catalase	Oxidase	Coagulase	H2S
E.coli	+	-	+	+	+	-	+
Klebsiella pneumonia	-	+	+	+	+	-	+
S.aureus	+	+	+	+	-	+	-

4.1.2: Isolation and diagnosis of the fungi

The samples were cultured on fungal media, 114 yeasts were obtained, which were later grown on chromo-agar media, in which it was found that there are several types of *Candida* including C. *albicans,C.glabrata* and *C. krusie*. Show in figure (4-9) and tables (4-3) .

Morphology and color of colonies on CHROM agar, according to the color key that putted by the differentiation in colors belong to the containing this agar a chromosomal material interacts with an enzyme hexosaminidase that secreted by the same species, this leads to a quick diagnosis, depending on the color and appearance of the colony (Mulet Bayona *et al*., 2022).

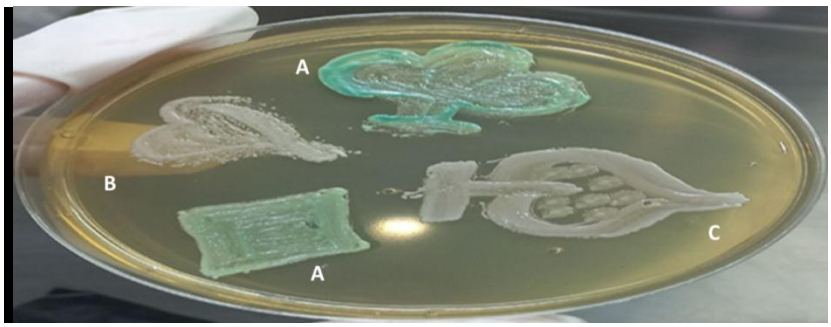


Figure (4-9) chromo agar (A) *C.albicans* (B) *C. krusie* (C)*C.glabrata*

Table (4-3) Type of Fungi isolated from vaginal sample

Type fungi	Number	Percentage %
C. krusie	27	23.7%
C. glabrata	27	23.7%
C. albicans	60	52.6%
Total number	114	100%

Number of studies, *C. albicans* is more common and causes more cases of vaginitis,*C. albicans* is thought to be a normal flora organism, but there are variations in the digestive and reproductive tracts, as well as exogenous factors such as pregnancy situations, lacteal phases of the menstrual cycle, hormone changes to oral estrogen with high doses, antibiotic side effects, and different types of diabetes mellitus in monarchal women who are predisposed to acute vulvovaginitis candidiasis. (Mashatan *et al*.,2023).

4.1.2.1 Diagnostic tests for Candida(found in appendix)

Hemolysis test

Figure (4-10) show ability to produce enzymes, such as hemolysin, is an important virulence factor for the genus *Candida*, and the aim of this examination was to compare the hemolytic activity between *Candida* species, where a positive result appeared for all species(*C.albicans, C. krusie and C.glabrata*). *C.albicans, C. krusie and C.glabrata* have the ability to analyze blood.

Hemolytic capacity is an important virulence factor, that allows fungi of the genus Candidato acquire iron from host tissues, which then is used by the fungus for

metabolism, growth and invasion during host infection Iron is an essential element for almost all organisms, both unicellular and multicellular(Rossoni *et al*., 2013).

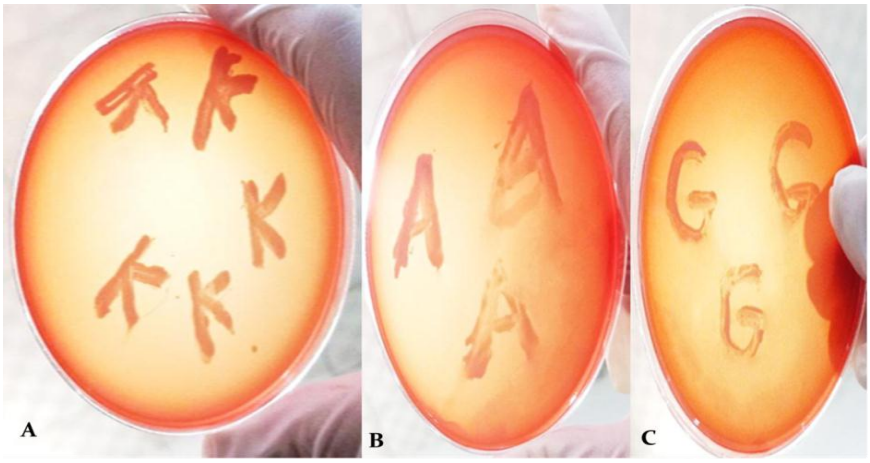


Figure (4-10) blood agar (A) C. krusie (B)C.albicans (C) C. glabrata

Figure (4-11) shows All isolates are positive for Catalase test this result is agreement with Gonçalves, 2022 The results revealed by using the hydrogen peroxide detector showed that all *Candida* positive.

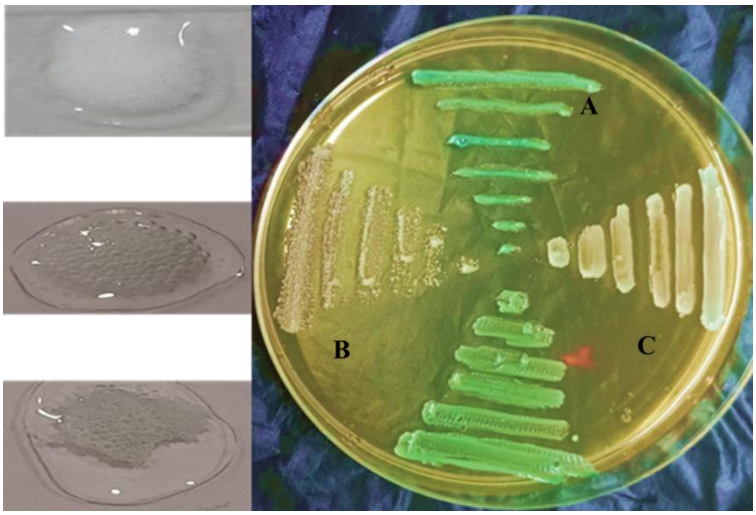


Figure (4-11) Catalase test (A) *C.albicans* (B) *C. krusie* (C) *C.glabrata*

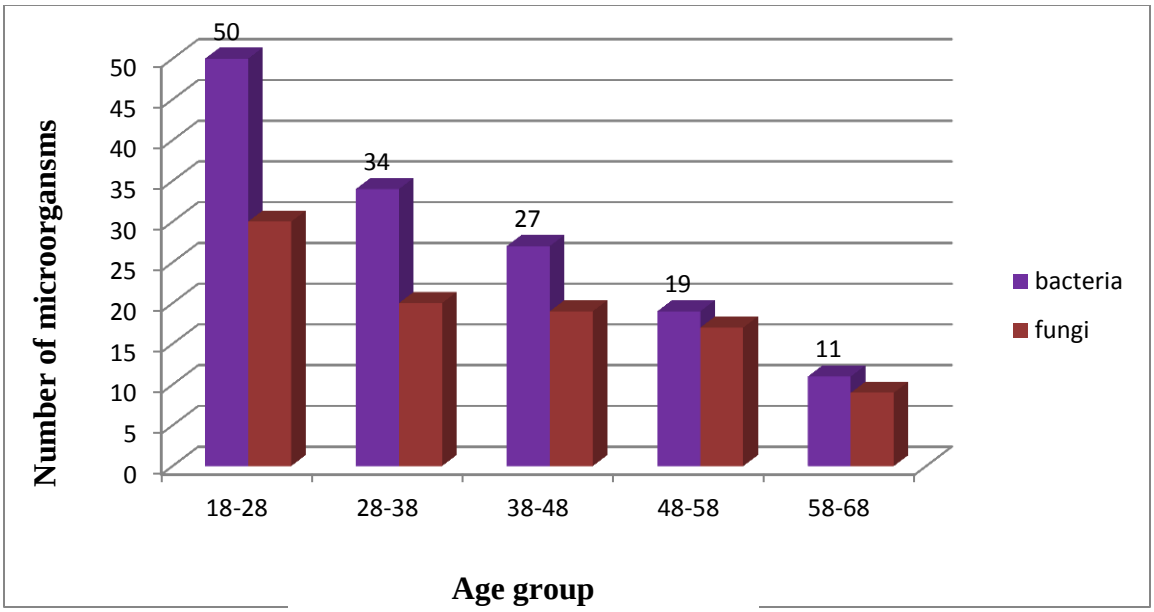
Figure (4-12) shows All isolates are negative for testing Oxidase **found in appendix**.

The oxidase test is a technique for detecting the presence of the terminal enzyme system in aerobic respiration called cytochrome C oxidase or cytochrome a3(Rotko and Kunz, 2022). All yeast isolates were negative for oxidase production(Afreen *et al*., 2016).

Table (4-4) Biochemical test for some *Candida* spp.

Candida	Ureaes	Catalase	Oxidase	Germ tube	Blood hemolysis
C.albicans	-	+	-	+	+
C. krusie	-	+	-	-	+
C.glabrata	-	+	-	-	+

By comparing the numbers of bacteria and fungi according to age groups, it was found that the age group (18-28)is more susceptible to infection than the rest of the groups, and it was revealed shown that infection with bacteria is more than fungi, as shown in Figure (4-13).



A figure (4-13) showing the comparison between the numbers of bacteria and fungi according to age groups

4.2. Molecular study

4.2.1 16 s RNA

PCR results showed compatibility with the phenotypic results of bacteria. The *16 s RNA* gene ratio reached (99%) of what could be adopted as a positive indication of the presence and spread of the *16 s RNA* gene, according to the results of the current study figure (4-14).

Staphylococcus aureus is the most predominant and important pathogene in clinical microbiology. A DNA amplification assay using the polymerase chain reaction (PCR) was designed to identify *S. aureus* through a single-base-pair mismatch in the sequences of staphylococcal 16S ribosomal RNA (16S rRNA) genes(Church *et al.*,2020 and Nesaraj,2021).

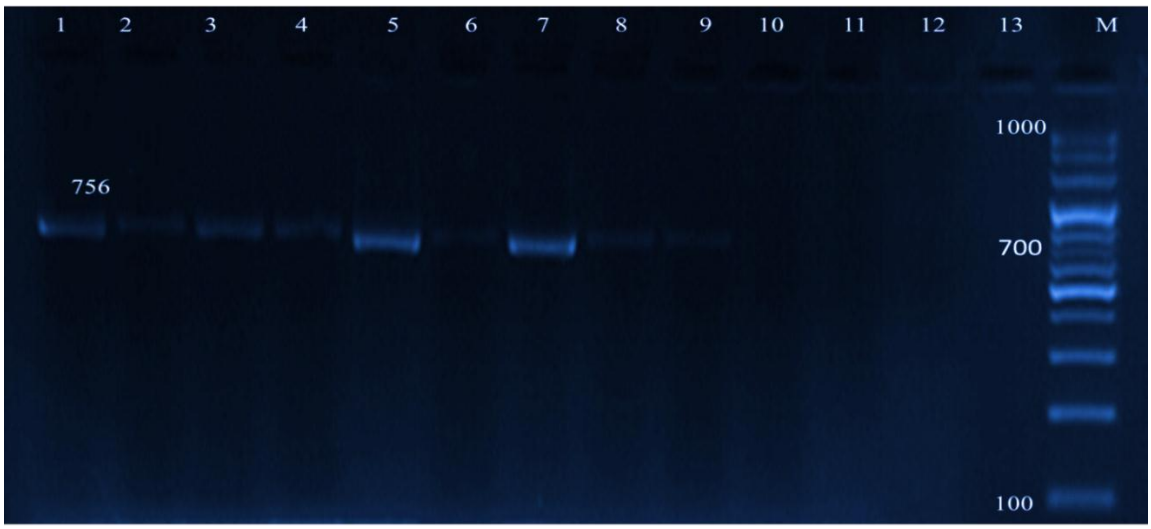


Figure (4-14): Electrophoresis Pattern of *16RNA* gene . L lane contain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V. all Lanes Negative results except positive Lanes results for *16RNA* gene (5,6,10-14,17,20,25-30,33,34,36-44) Lanes .

4.2.2 Cysteine protease (SSPB)

The amplification results of PCR study for *SSPB* (493bp), revealed that (36.1 %) of *S. aureus* isolate gave positive for *SSPB* (36.1% from sample woman as show figure (4-15) .

A SSPB is a gene that encodes a cysteine protease,. Circulating neutrophils and monocytes constitute the first line of antibacterial defence, which is responsible for the phagocytosis and killing of microorganisms. Previously, we have described that the staphylococcal cysteine proteinase staphopain B (*SspB*) cleaves CD11b on peripheral blood phagocytes, inducing the rapid development of features of atypical cell death in protease-treated cells (Page, 2019 and Chen *et al*.,2023).

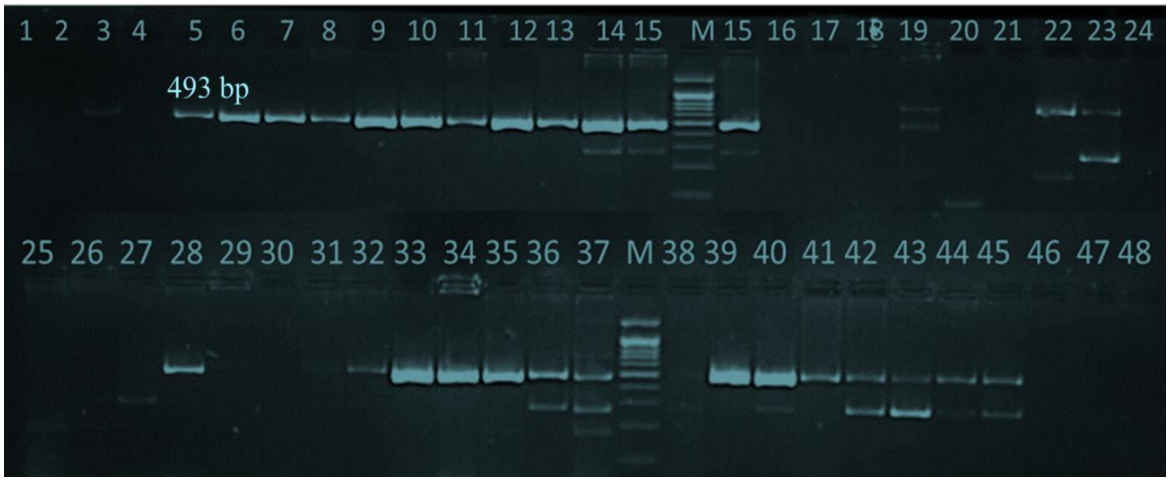


Figure (4-15): Electrophoresis Pattern of *SSPB* gene . L lane contain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V.

4.2.3: Enterotoxin B (*Seb*) gene

The amplification results of PCR study for *Seb* (164bp) revealed that of *S. aureus* isolate gave from sample woman as show figure (4-16).

Several previous studies have shown that *S. aureus* bacteria have *Seb* gene that was isolated from the vagina, and Staphylococcal enterotoxin B is one of the most potent bacterial super antigens that exerts profound toxic effects upon the immune system, leading to stimulation of cytokine release and inflammation (Al-Khafaji *et al*., 2019 and Mohammed, 2020).

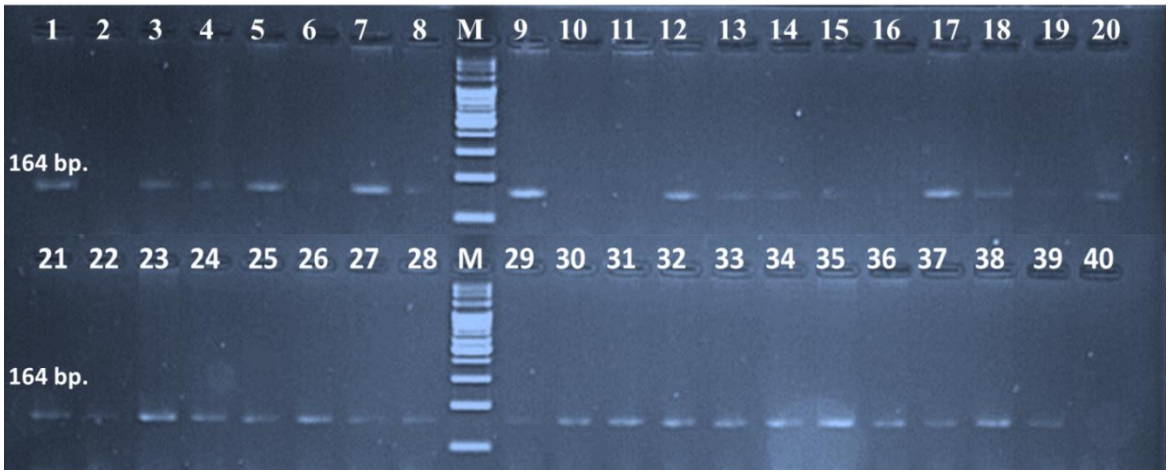


Figure (4-16) Electrophoresis Pattern of *Seb* gene . L lane contain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V.

4.2.4: Detection of Penicillin binding protein 2a (*MecA*) gene by PCR

Penicillin binding protein 2a is the major mechanism developed by MRSA to exhibit a broad clinical resistance to the β-lactam antibiotics. The current study included the molecular detection of genes 72 specimens, PCR results showed compatibility with the phenotypic results of bacteria. The *mecA* gene ratio reached (22.2%) of what could be adopted as a positive indication of the presence and spread of the *mec-A* gene, according to the results of the current study figure (4-17).

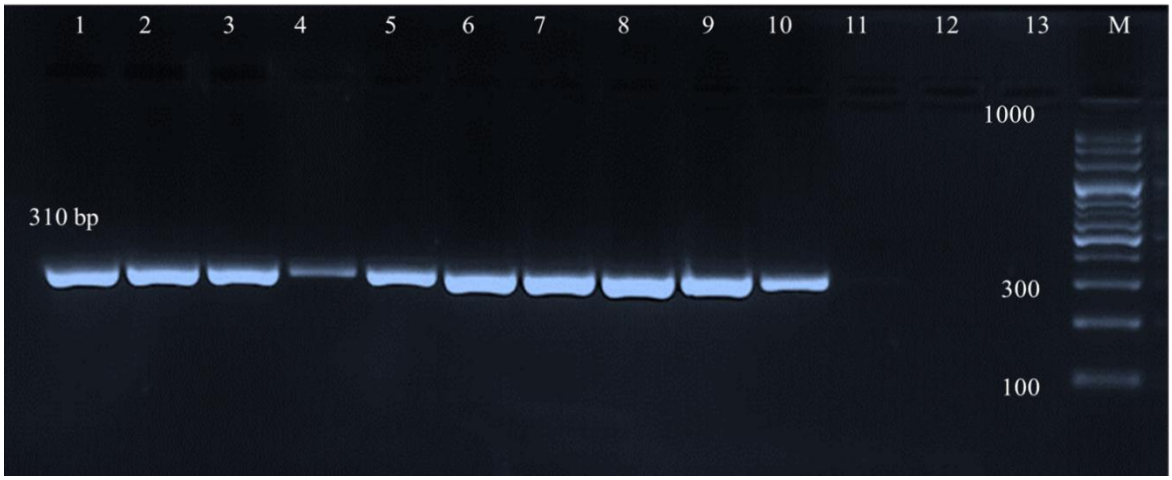


Figure (4-17): Electrophoresis Pattern of *MecA* gene . L lane contain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V.

Today, MRSA knows to be a subclass of *S. aureus* that produces increased stability against numerous kinds of medicines including drugs of beta lactams (Lee *et al*., 2018). A high incidence of *S. aureus* infection generated via isolates of MRSA remains a significant public health interest. MRSA infection is difficult in the therapy as well as usually has results that are more critical. MRSA infection is correlate with risen mortality and morbidity during compared into sensitive MSSA isolates (Kotey *et al*., 2022). Figure (4-7) revealed high rate of *mecA* among VRSA/VISA isolates. A study in India among 17 isolates observed 7 cases positive to *mecA* gene (Khan *et al.,* 2011). Also, a study done by Sajith *et al.*, (2012) they observed according data of PCR among 35 isolate of MRSA 33 (94%) were harbored *mecA* gene. Other study achieved by Zeinalpour *et al*., (2019) they reported that the gene of *mecA* was approved in 54.54% of the samples.

4.2.5: Detection of intercellular adhesion (*IcaA* and *IcaD*) genes

An *ICA* gene production Slime layer as the major part of biofilm formation plays a remarkable role in bacterial colonization of exterior surfaces (Nguyen *et al*., 2020)

The amplification results of PCR study for *IcaA* (188bp) and *icaD* (198bp), revealed that (77.7%) of *S. aureus* isolate gave positive for *icaA* (45.8 % Show in figer (4-18,4-19).

Kaur *et al*.,2021 and Nguyen *et al*., 2020 also explained that *S. aureus* possesses the Ica A and D gene, which has a role in the formation of slime, which is one of the virulence factors for this bacterium.

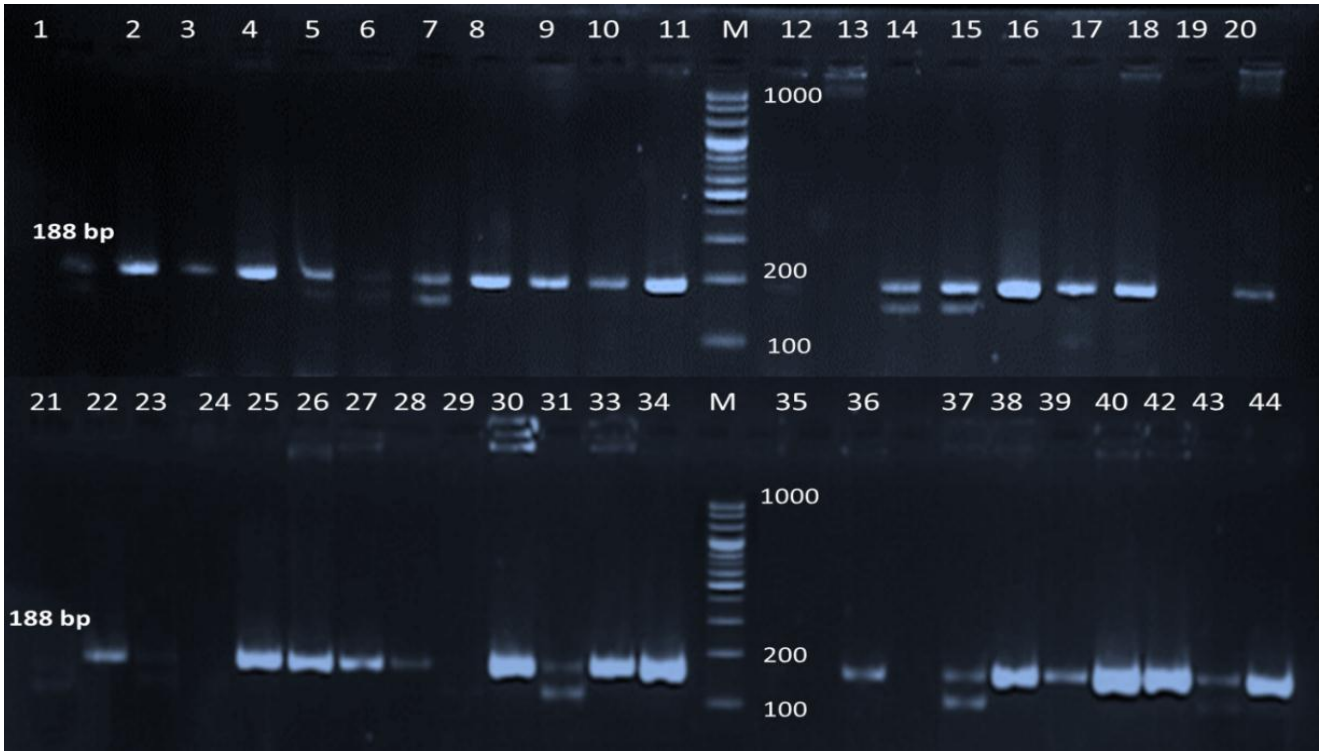


Figure (4-18): Electrophoresis Pattern of *icaA* gene . L lane obtain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V .

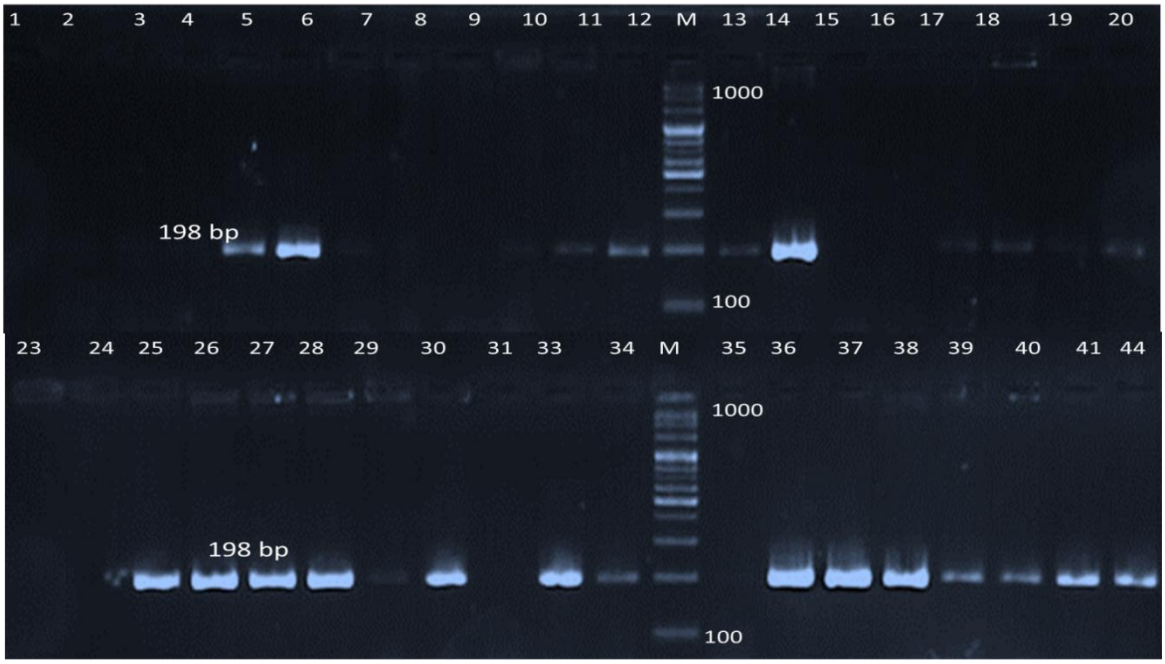


Figure (4-19): Electrophoresis Pattern of *icaD* gene . L lane contain the 100 bp DNA Ladder, 2 % agarose gel in 1X TBE buffer containing 5µl Safe red stain for 45min,70V.

The results also revealed that both *icaA* and *icaD* genes were either present or absent and no single strain had shown the presence of one gene. These results confirm the fact that both genes are part of one operon and so the entire operon was either present or absent. In addition, our results showed that both genes (*icaA* and *icaD*) were present in all biofilm producing strains, indicating the important role of *ica* genes as virulence markers in staphylococcal infections(El-Nagar *et al.*,2021).

findings are different to other reports in which the *ica* genes were detected in all isolates , However, agree with the proportion (4, 5.1%) of isolates possessing the *icaA* and *icaD* genes associated with slime formation was relatively low in this study, as compared with some previous studies (Wang *et al.*, 2018). These results suggest that

strains of *S. aureus* may present with different capacities to form biofilms based on their source and geographical origin. Further studies, including varied sources, are needed to fully clarify this assertion. Although none of the biofilm genes tested were detected in some of the isolates, the expression of biofilm formation in these isolates could be mediated by Usually, biofilm-producing isolates had a higher prevalence of the biofilm genes than non-biofilm-producing isolates. According to (Bissong and Ateba ,2020), these findings are consistent with earlier studies.

Initial attachment, which is a crucial stage in *S. aureus* biofilm development, is done by the expression of several Microbial Surface Components Recognizing Adhesive Matrix Molecules (Arciola *et al*., 2018).

S. aureus initially adheres to each other and then widen to structurally dynamic biofilm structures during the later phases of adherence. The maturation of the biofilm matrix into multi-layered patterns is initiated by the polysaccharide intercellular adhesin (PIA), synthesized from β-1, 6- linked N-acetyld-glucosamines (PNAG) (Periasamy et al., 2012). The synthesis of PIA is mediated by the intercellular adhesin (ica) locus, which comprises four core genes, namely *icaA, icaD, icaB* and *icaC*, as well as a regulatory gene, icaR (Chen, 2018). These genes encode the corresponding proteins *ICAA, ICAD, ICAB,* and *ICAC*. The production of slime is facilitated by the expression of *icaA* and *icaD* gene, It has been shown that strains harbouring the icaADBC cluster are potential biofilm producers (Bissong and Ateba, 2020).

4.2.6: Detection of thermostable endonuclease (*nuc* gene)

For molecular identification of *S. aureus* isolates, (Garcia Gonzalez and Hernandez, 2022) demonstrated molecular targeting of species-specific nuc gene of *S. aureus* coding for the extracellular thermo stable nuclease protein (TNase) of *S. aureus* and the same was utilized in the present study. PCR utilizing synthetic oligonucleotide primers

of 21 and 24 bases has been used to amplify a segment of the *nuc* gene that is specific for *S. aureus* (Madhu and Choi, 2022).

The findings of the molecular revelation of the *nuc* gene showed that (66.6%) of *S. aureus* isolate gave positive for *nuc* gene.

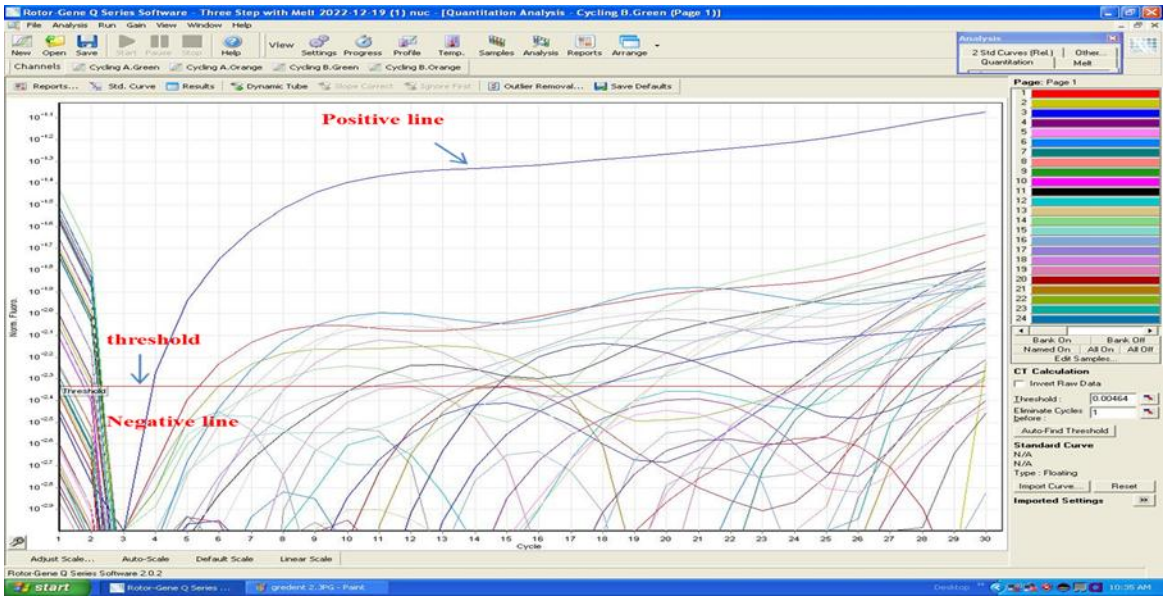


Figure (4-20): Detection of *Nuc* gene *S. aureus* from *vagina* by Real time-PCR

The *nuc* gene acts as a marker and also the presence of heat resistant nuclease gene (*nuc*) is strongly associated with the production of enterotoxin and it can be considered as an indicator of infection with enterotoxin producer *S. aureus* (Karimzadeh and Ghassab, 2022).

Vasileiou *et al*.,(2019) suggested that the rapid *nuc* PCR assay as a suitable and practical tool for the routine detection and differentiation of *S. aureus*, MRSA and CoNS; which can be easily applied in microbiology laboratory procedure,*Staphylococcus* is ability to produce a wide variety of exoenzymes, among these, nuclease is known to be an important virulence factor and are able to retain activity after incubation at 97˚C for 60 min. Nuclease is able to degrade nucleic acid of

the host by hydrolyzing phosphodiester bonds of DNA and RNA yielding 3′-mononucleotides.

4.2.7 Toxic shock syndrome toxin-1(*tst*)

Result of PCR was demonstrated 25% as positive as Figure (4-21). There was statistical found significant difference (0.03) among sample group. Supported by the presence of this gene Sharma *et al*.,2019 **,**Staphylococcal toxic shock syndrome (TSS) is a potentially lethal illness characterized by fever, rash, desquamation, organ dysfunction, and shock. The syndrome is attributed to super antigens produced by *S. aureus*, in particular, toxic shock syndrome toxin.

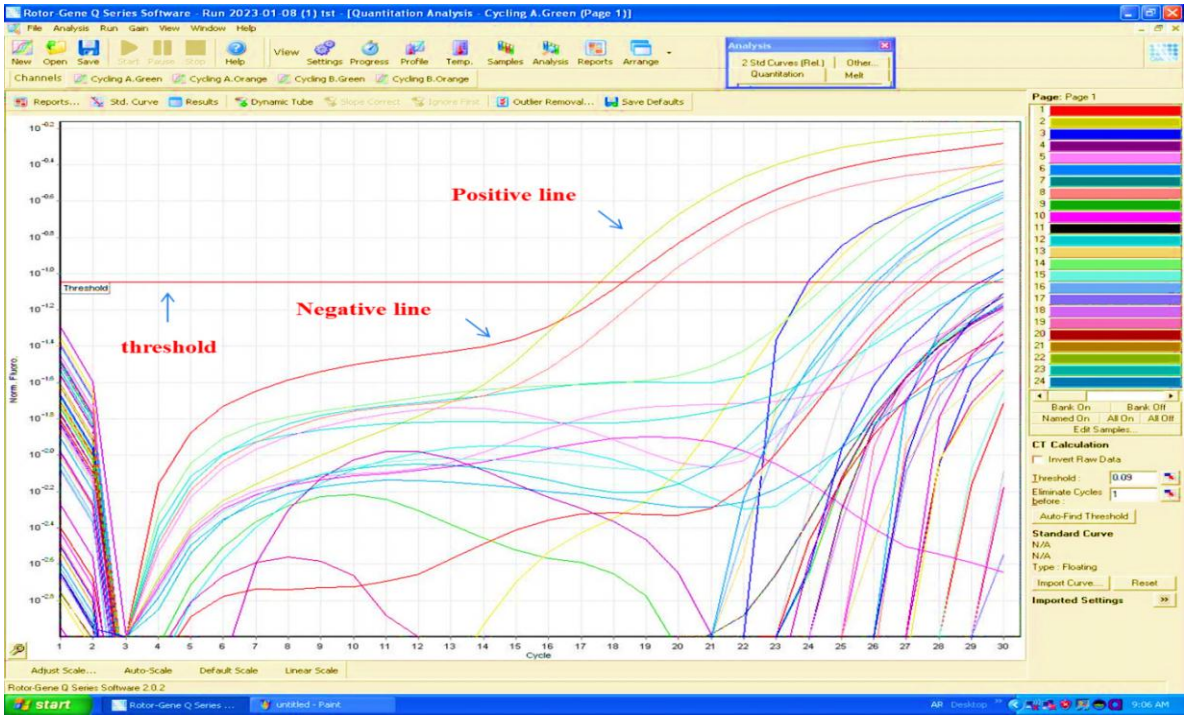


Figure (4-21): Detection of Toxic shock syndrome toxin-1(*tst*) *S. aureus* from *vagina* by Real time-PCR by real time PCR

4.2.8: Enterotoxin A(*Sea A*)gene

Result of PCR was demonstrated 84.7% as positive as Figure (4-22). There was statistical found significant difference (0.04) among sample group. Supported by the presence of this gene in *Staphylococcus aureus* (Chiaruzzi *et al*.,2020, Bhunia and Bhunia,2018) *S. aureus* is an opportunistic pathogene that expresses a variety of virulence factors. Among them, TSST-1 and staphylococcal enterotoxins (SEs) belong to the family of superantigenes.

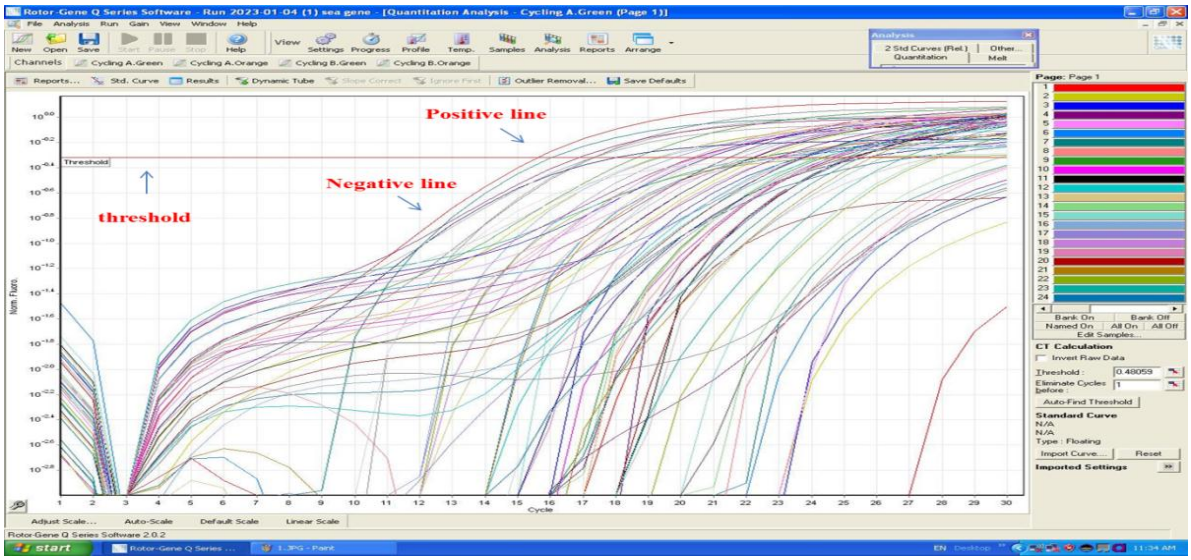


Figure (4-22): Detection of *sea S. aureus* from *vagina* by Real time-PCR by REAL TIME-PCR

4.2.9 Staphylokinase (*sak*)gene

Result of REAL TIME-PCR was demonstrated 79.2% as positive as well as Figure (4-23). There was statistical found significant difference (0.02) among sample group. One of virulence factors produced by *S. aureus* is staphylokinase (SAK), which enhances their proteolytic activity leading to tissue damage and improving bacterial invasiveness(Deepa *et al*.,2019).

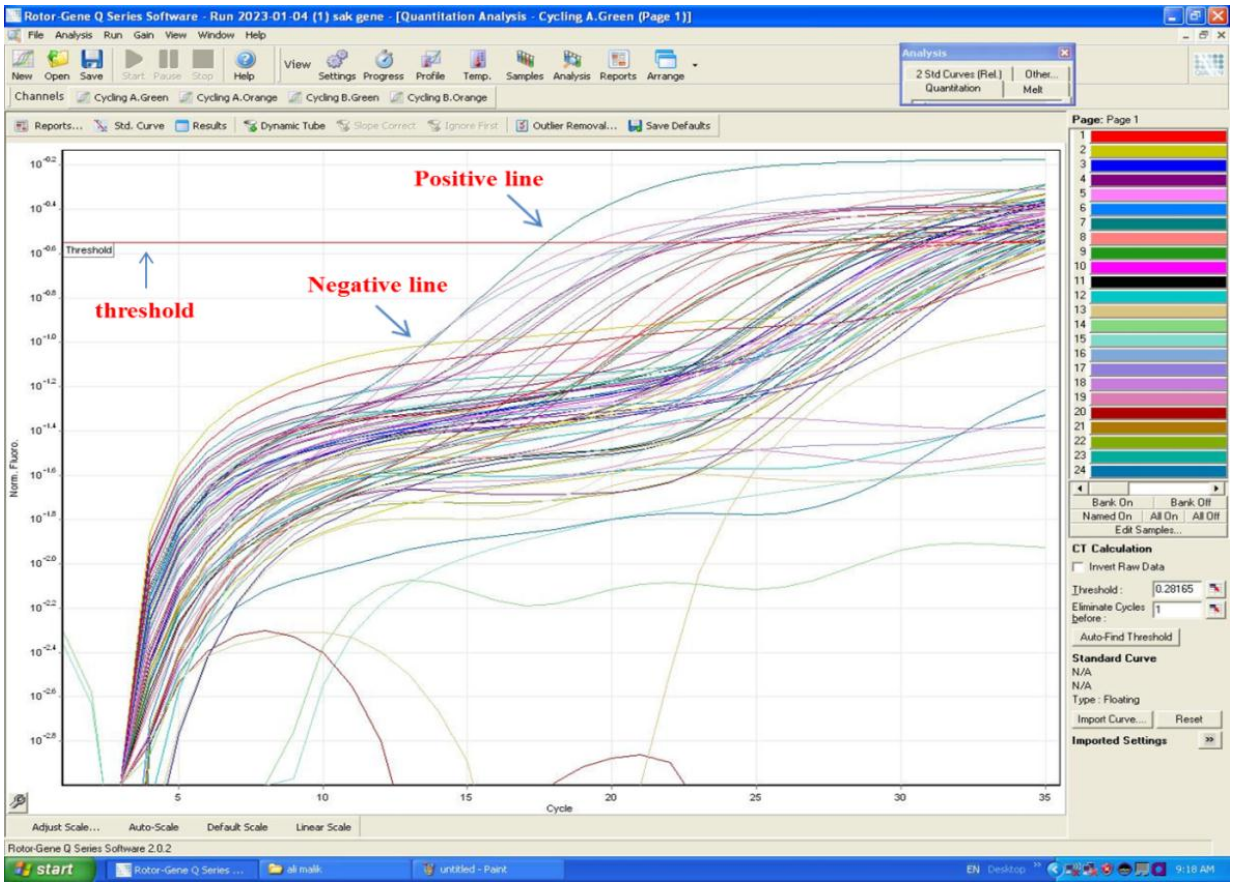


Figure (4-23): Detection of *sak* gene *S. aureus from vagina* by Real time-PCR by REAL TIME-PCR

4.2.10 *Exfoliative toxin A* (*Eta)* gene

Result of PCR was demonstrate 79.2% as positive as shown as Figure (4-24). There was statistically a significant difference (0.01) among sample group. The current study agreed with other studies on the existence of the *eta* gene in *S. aureus* bacteria such as(Elbargisy, 2022, Azarian *et al*.,2021).

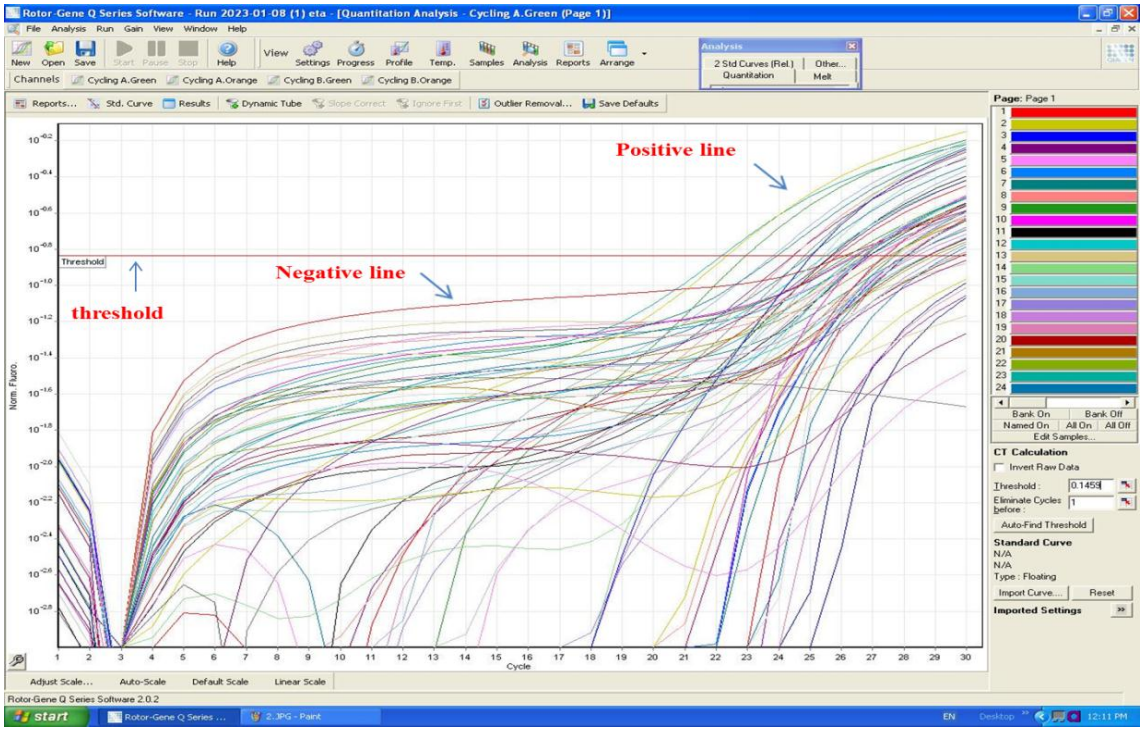


Figure (4-24): Detection of *eta A S. aureus* from *vagina* by Real time-PCR by Real time-PCR

4.2.11 *Exfoliative toxin B* (*etb*) gene

Result of PCR was demonstrated 62.4% as positive as shown as Figure (4-25). There was statistical found significant difference (0.02) among sample group. Depending on the percentages, we note that the *etb* gene is present in vaginal samples more than samples from different sources. Numerous researches have supported the presence of Exfoliative toxin B in *S. aureus* suh as(Azarian *et al*.,2022, Trifonova and Strateva, 2019).

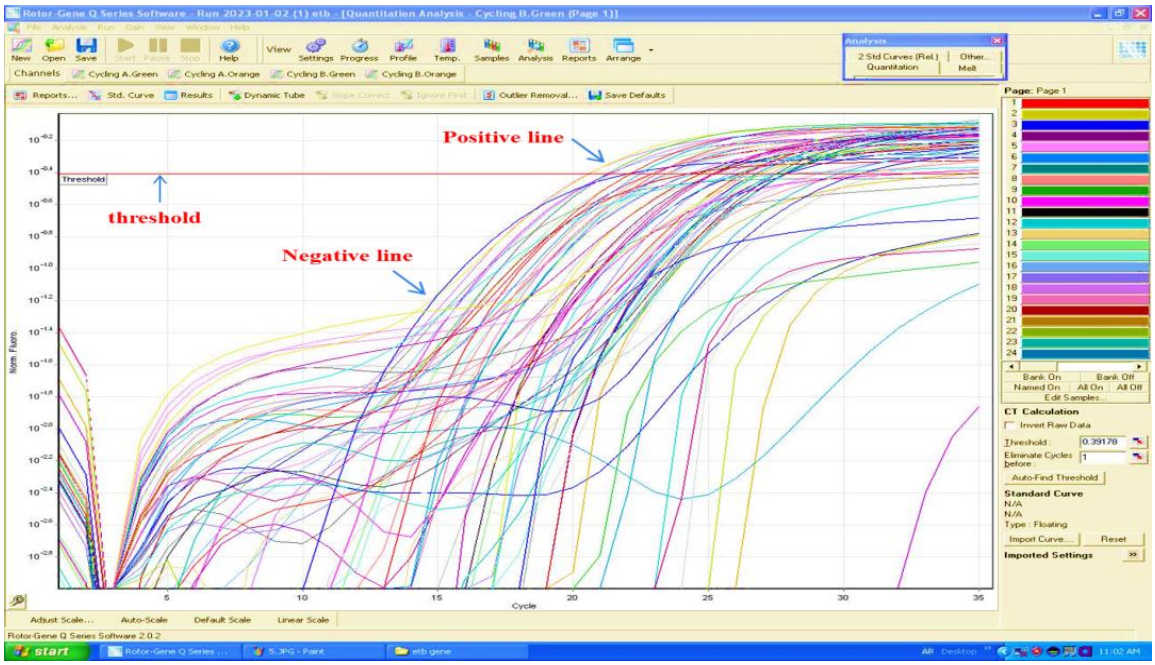


Figure (4-25): Detection of *etb* gene *S. aureus* from *vagina* by Real time-PCR by REAL Time-PCR rotergene using red safe dye

4.2.12 Hyaluronidase (*Hys A*) gene

Result of REAL TIME-PCR was demonstrated 26.3% as positive as shown in Figure (4-26). There was statistical found significant difference (0.02) among sample group. We notice through the percentage of the gene Hys has a higher percentage in different source than samples from Vagina. The presence of this gene in the S. aureus bacterium is consistent with what he explained Yuwen *et al*.,2021 .For a number of Gram-positive organisms, hyaluronidases have been shown to be essential virulence factors because of their ability to disseminate cells and virulence factors through tissue (Trifonova and Strateva, 2019).

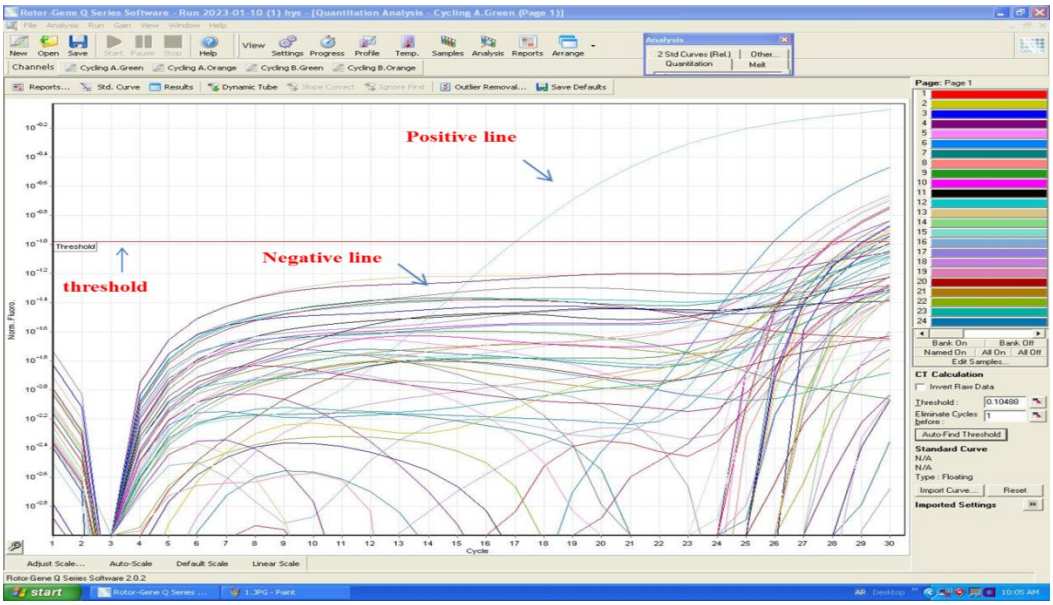


Figure (4-26): Detection of *hys* gene *S. aureus* from *vagina* by Real time-PCR by REAL TIME-PCR

4.2.13: *Staphylococcus aureus* lipase (*lip*) gene

Result of Real time-PCR was demonstrated 68.9% as as shown in Figure (4-27). There was statistical not found significant difference (0.399) among sample group. that the proportion of genes specific to the *S. aureus* in the vagina is more than the rest of the places. Staphylococcus aureus lipase (SAL), a triacylglycerol esterase, is an important virulence factor in *S. aureus* and may be a therapeutic target for infectious diseases caused by *S. aureus* and this is what he supported Kitadokoro *et al*.,2020.

Tigabu and Getaneh,(2021) capacity supported *Staphylococcus aureus* produces multiple enzymes that enable it to invade and destroy host tissues and metastasize to other sites. One such enzyme, lipase, has been recognized for its relationship in the virulence of *S. aureus*. However, a direct involvement of lipase in the pathogenesis of *S. aureus* remains to be demonstrated. Our prior study indicated that anti-lipase serum inhibits biofilm formation in *S. aureus* clinical strains. The aim of this study was

to further characterize the roles of lipase in the pathogenesis in *S. aureus*. We found that deletion of the lipase-coding gene reduced biofilm formation relative to the wild-type strain. This was shown by culture in 96-well plates coated with collagene to resemble the *in vivo* infection process. Intraperitoneal inoculation of mice with a lipase mutant strain showed defective formation of peritoneal abscesses, and bacterial loads in different organs were much lower compared with the wild-type. Importantly, active immunization with recombinant lipase protected mice against a lethal challenge with *S. aureus*. Altogether, our data provide evidence that *S. aureus* lipase plays important roles in the pathogenesis of *S. aureus*.

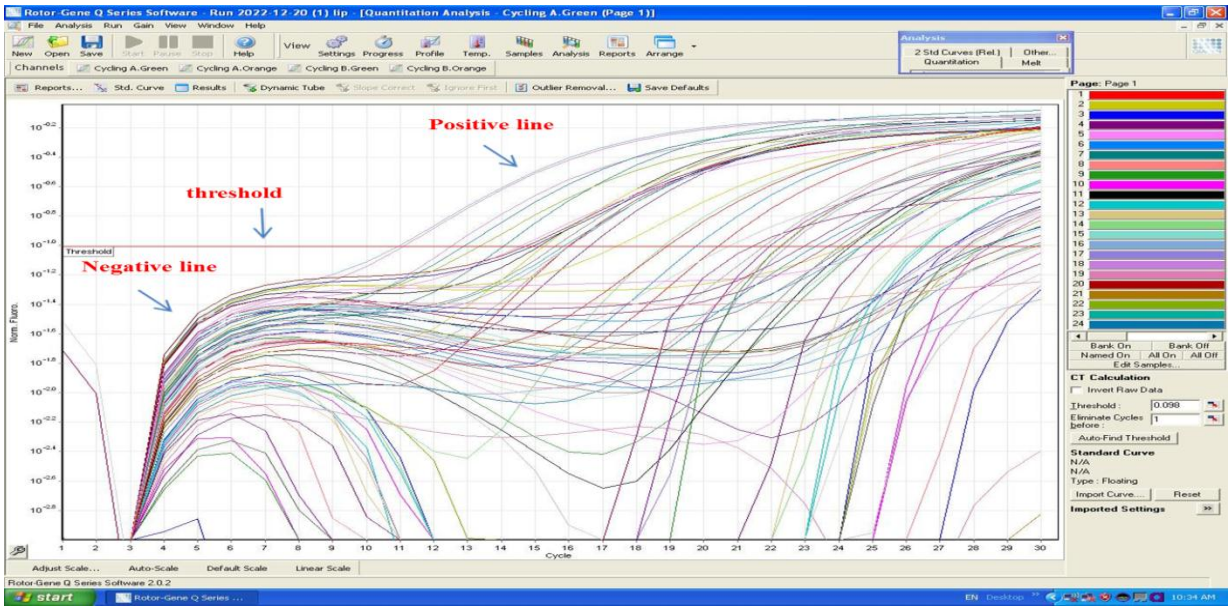


Figure (4-27): Detection of *lip* gene *S. aureus* from *vagina* by Real time-PCR by Real time-PCR

Table 4-5: Percentage of genes Detection from *S. aureus* isolated from vagina

Discretion	Gene	Percentage%
Nuclease	*Nuc*	48.1
Lipase	*Lip*	68.01
Staphylokinase	*Sak*	79.1
Hyaluronidase	*hysA*	26.3
Penicillin binding protein 2a	*mecA*	22.2
Intercellular adhesion A	*icaA*	66.7
Intercellular adhesion D	*icaD*	40
Cysteine protease	*sspB*	38.8
Toxic shock syndrome toxin-1	*Tst*	25
Exfoliative toxin A	*Eta*	79.1
Exfoliative toxin B	*Etb*	62.4
Enterotoxin A	*Sea*	84.7
Enterotoxin B	*Seb*	53.3
16s rRNA	*16s rRNA*	99

4.2.14: Conventional PCR for identification of *C. albicans*

Candida *albicans* was diagnosed using Conventional PCR technology and the of samples was positive 23 sample of *C. albicans* (69.7%), all these isolates were germ tube-positive. Two species-specific primer pairs derived from the internally transcribed spacer (ITS) region (comprising ITS-1, 5.8S rRNA and ITS-2) of ribosomal DNA (rDNA) were design for *C. albicans* strains by PCR show that in figure (4-28). This is confirming by studies that *C. albicans* possesses this gene, and from these studies, by germ tube formation and accurately reports results within 2 h. Detection of amplicons by agarose gel electrophoresis is also suitable for resource-poor settings PCR (Awandkar et al.,2021).

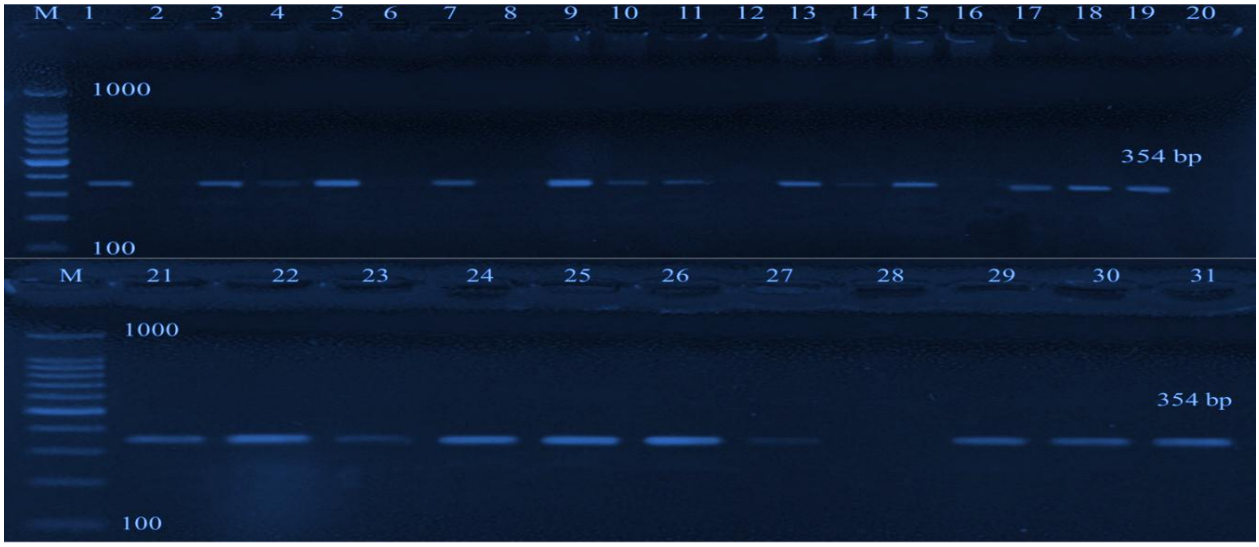
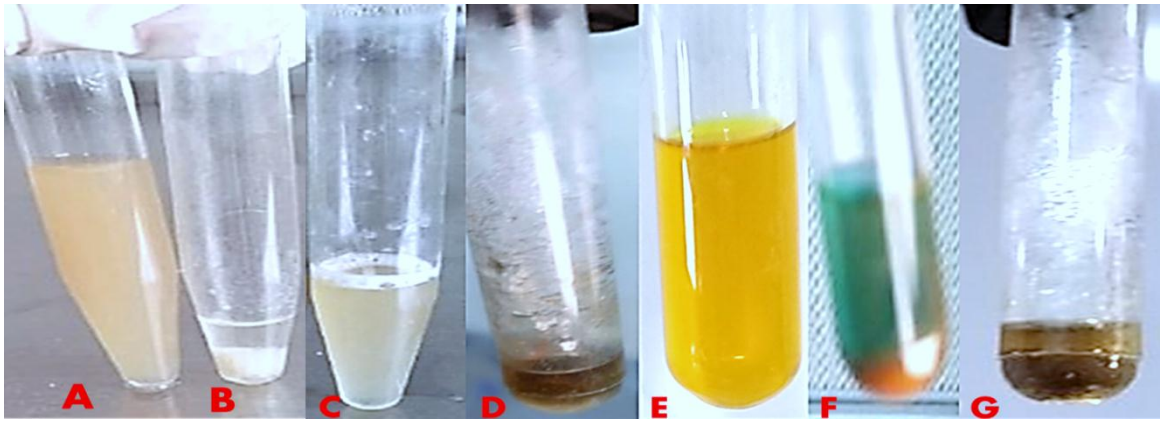


Figure (4-28) An 2% agarose gel of PCR amplified product with DNA from *C. albicans* isolates from from *vagina* (354 bp) . Lane M is a 100-bp DNA ladder.

2.3: *Zingiber officinale*

2.3.1: phytochemical compound

The results showed chemical detection of *Zingiber officinale* extracts by using reagents and solutions, presence of the compounds listed in figure (4-29). The results confirm all testing phytochemical compound which were Tannins, Saponins, Glycosides, Flavonoid, Terpenes and Steroids, This result is in agreement with the results of the following studies (Ashraf *et al*., 2017, Marrelli and Conforti, 2015) .



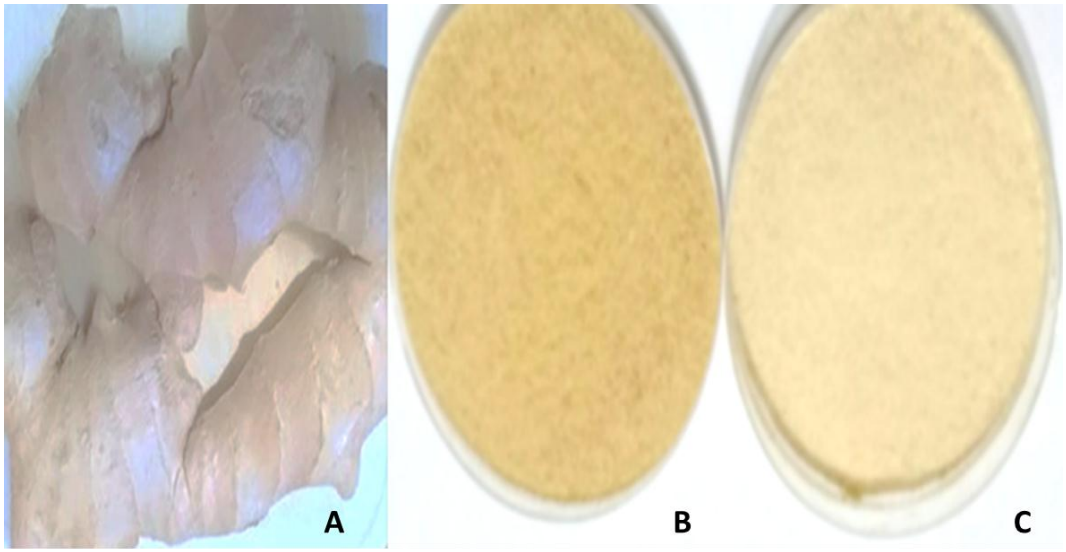
Figure(4-29) Analysis phytochemical compound :(A)Control (B)Tannins (c) Saponin (D)Steroids (E)Flavonoids (F)Glycosides (G)Terpenes

4.3.2 HPLC Analysis of *Ginger*

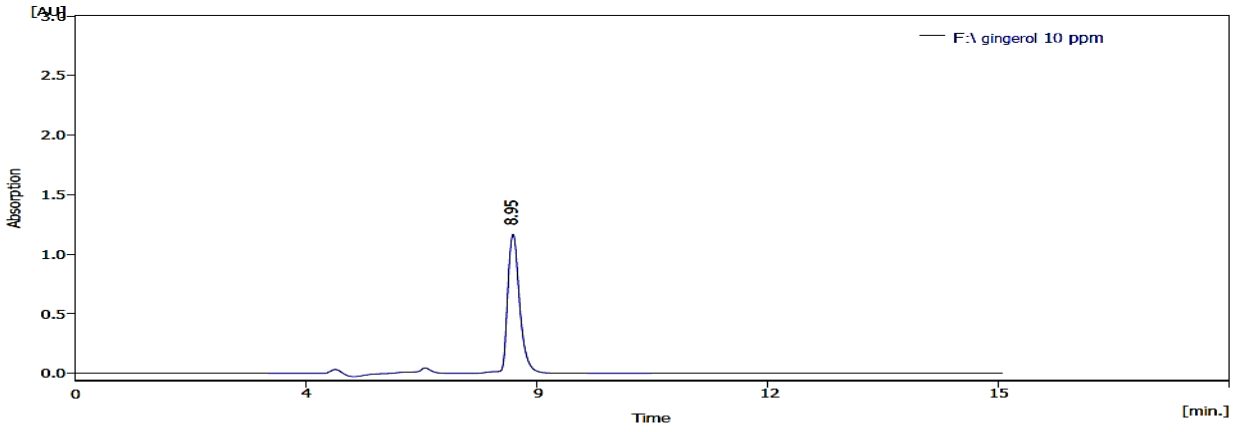
4.3.2.1: Quantitative and qualitative Identification of gingerol in *Ginger* by HPLC

Identify the presence and concentration of gingerol, appropriate chromatographic conditions from stationary phase and a step gradient polarity system of mobile phase was performed, as shown in Figure(4-32)where the retention time of *Zingiber officinale extract* was 8.96 min and area was 33147.58 in compared with gingerol

standard retention time and area which was respectively 8.95 and 578.58 . This result indicate the presence of gingerol in concentration 39.58 ppm .



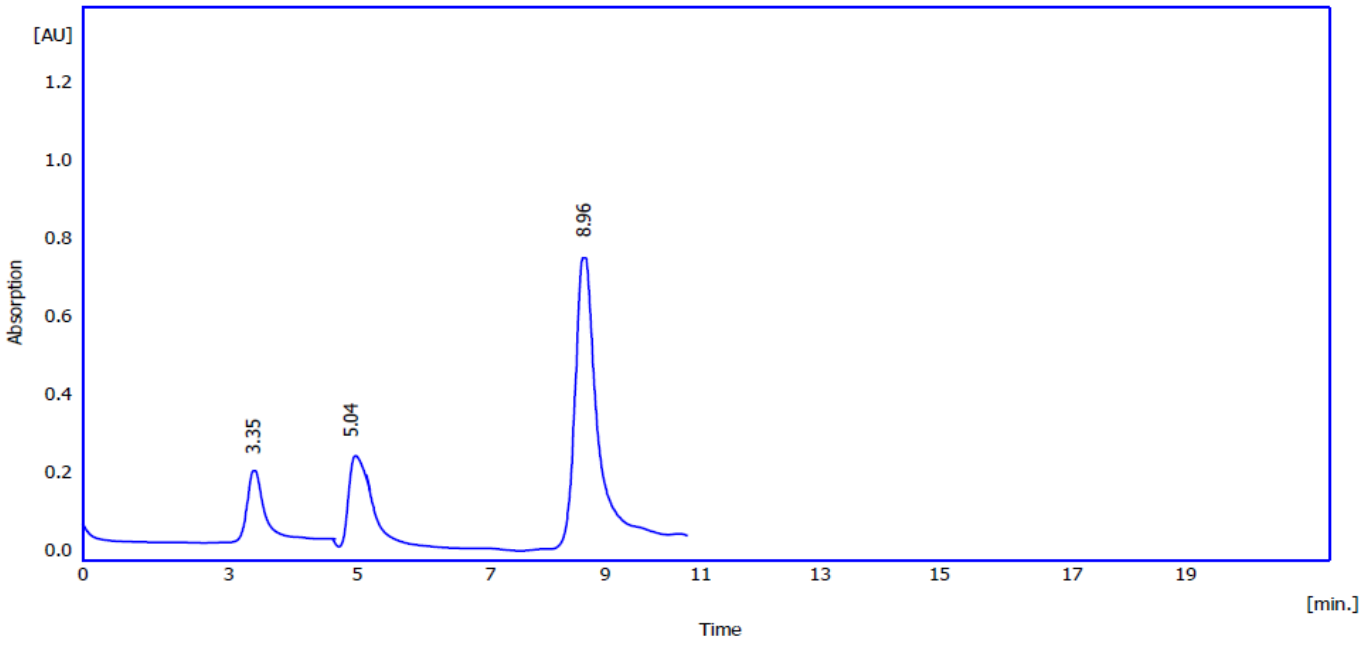
Figure(4-30) Stages of extracting gingerol from ginger:A(Ginger rhizomes)B(ginger powder)c(Gingerol)



Result Table (Uncal - F:\ gingerol 10 ppm)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	8.95	579.58	114.18	100.0	100.0	0.10	
	Total	579.58	114.18	100.0	100.0		

Figure (4-31) detection of gingerol in *Zingiber officinale extract* watery- methanol extract(Stander) by HPLC



Result chromatography Table (Uncal - F:\ Ginger SAMPLE)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	3.35	2145.89	364.19	25.21	25.14	0.14	
2	5.04	2365.29	228.97	26.64	26.46	0.36	
3	8.96	33147.58	895.28	40.56	43.81	0.04	
	Total	37658.58	1488.56	100.00	100.00		

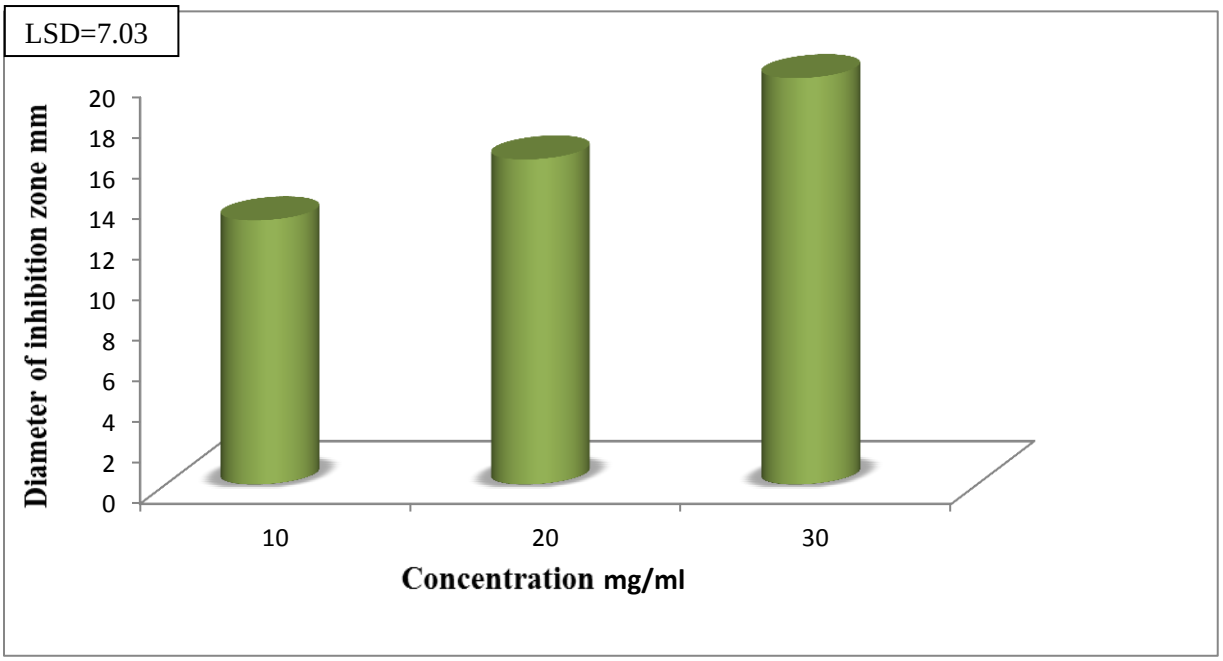
Figure (4-32) detection of gingerol in *Zingiber officinale extract* watery- methanol extract(sample) by HPLC

4.4 Antimicrobial Susceptibility Test :

4.4.1: Effects gingerol and antibiotic (Tobramycin and kanamycin) on *Staphylococcus aureus* growth

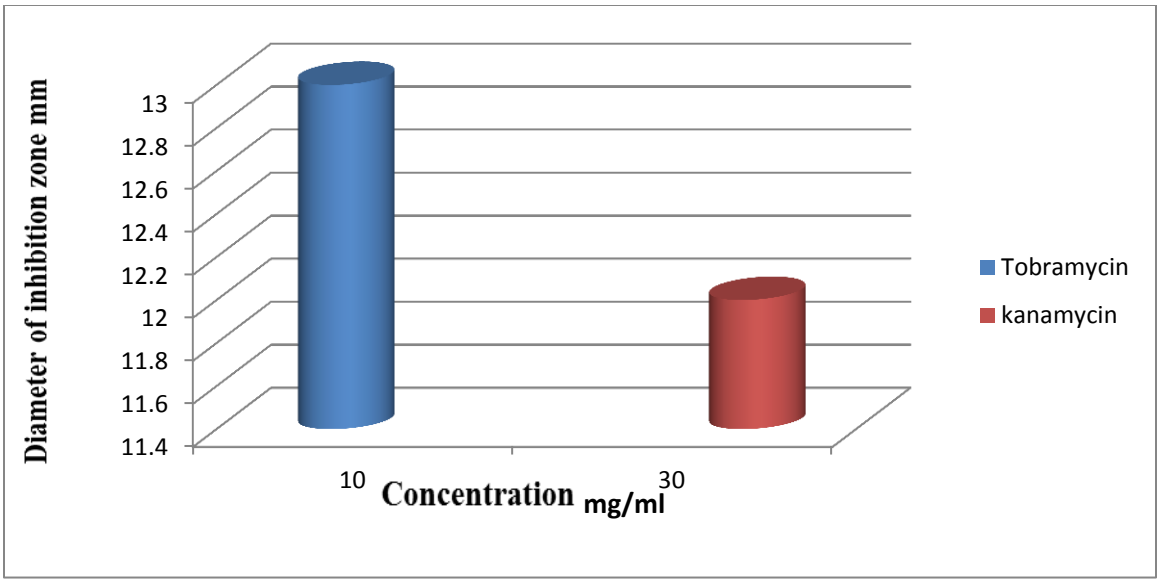
The current investigation demonstrated that there were variations in the concentration of gingerol on *staphlococcus* . Aurwas 30mg/ml was the concentration that caused the greatest inhibition at a rate of 20 mm, 20mg/ml caused the inhibition zone to be 16 mm in diameter, and 10mg/ml caused the least inhibition at a rate of 13 mm, That is, the greater the concentration, the greater the rate of inhibition. We notice through the statistical analysis that the third concentration (30) has a clear significant

difference unlike the first and second concentrations Statistically they do not have a significant difference. Figures (4-33) and (4-35) show that the diameter of the inhibition zone grows as the concentration does.. This result is in agreement with the result (Wadhwa and Haneef, 2019) that states that gingerol and shogaol have Many potential pharmacological activities like anti-oxidant activity,anti- inflammatory, anti-microbial, anti- cancerous, anti-bacterial and anti- analgesics activity are reported.

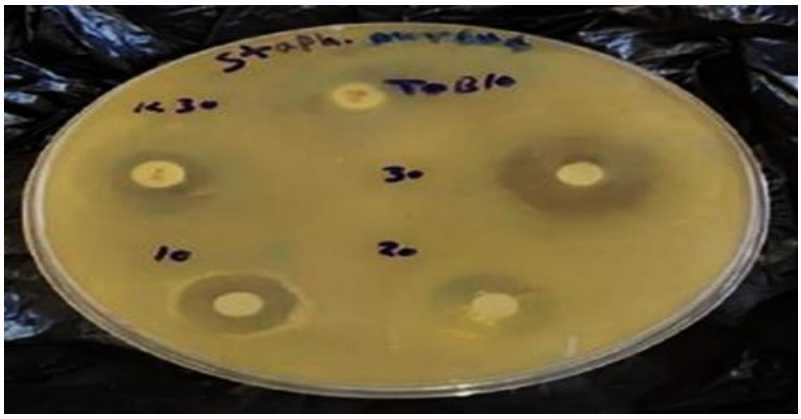


Figure(4-33) Effect different concentration of gingerol on *S. aureus*

As for the antibiotics(Tobramycin and kanamycin), it showed a lower effect compared to gingerol(13,12)mm, as well as the antibiotic Tobramycin, but it gave a better effect, even though it was less concentrated than the kanamycin antibiotics show in Figure(4-34) and (35).



Figure(4-34)Effect antibiotic(Tobramycin and kanamycin)on *S. aureus*



Figure(4-35) Effects gingerol and antibiotic (Tobramycin(TOB) and kanamycin(K) on *Staphylococcus aureus* growth

4.4.2: Effects gingerol and antifungal (Nystatin and and Itraconazole) on *Candida albicans* growth

The results of the study showed that there were concentration of gingerol at 30mg/ml concentration was the most concentrated inhibition the of 19 mm, while the concentration was 20 mg/ml the inhibition zone 10 mm, the concentration 10mg/ml

was don't appear inhibition, Statistically, the first concentration does not have a significant difference, while the second and third concentrations have a significant difference .In figure (4-36)and (4-38)which appear the effect gingerol on *C.albicanus* ,inhibition zone increase with increase concentration, Gharibpour *et al*., (2021) explained that ginger has components that act as anti-fungal, such as *C. albicans*, and this result was reached in the following research.

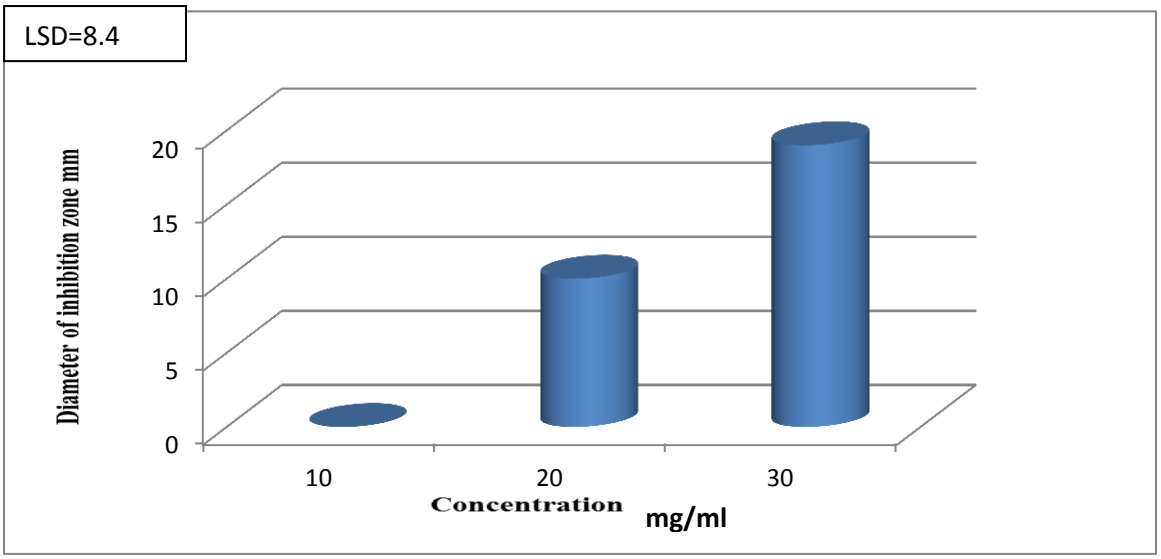


Figure (4-36) Effect different concentration gingerol on *C. albicans*

The result effect itraconazole on *Candida albicans* diameter inhibition zone as (15) mm. nystatin diameter inhibition zone. Statistically does not found a significant difference between itraconazole and nystatin . as(11) Figure 4-37 and 4-38 and show that.

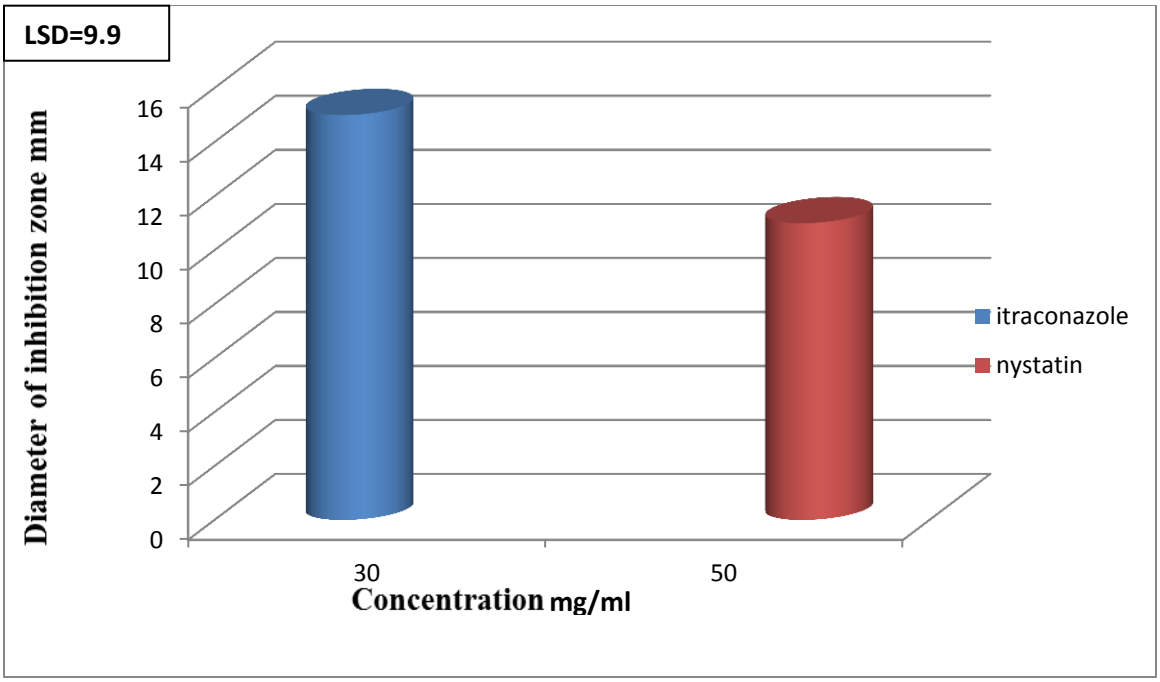


Figure (4-37) Effect itraconazole and nystatin on *Candida albicans*

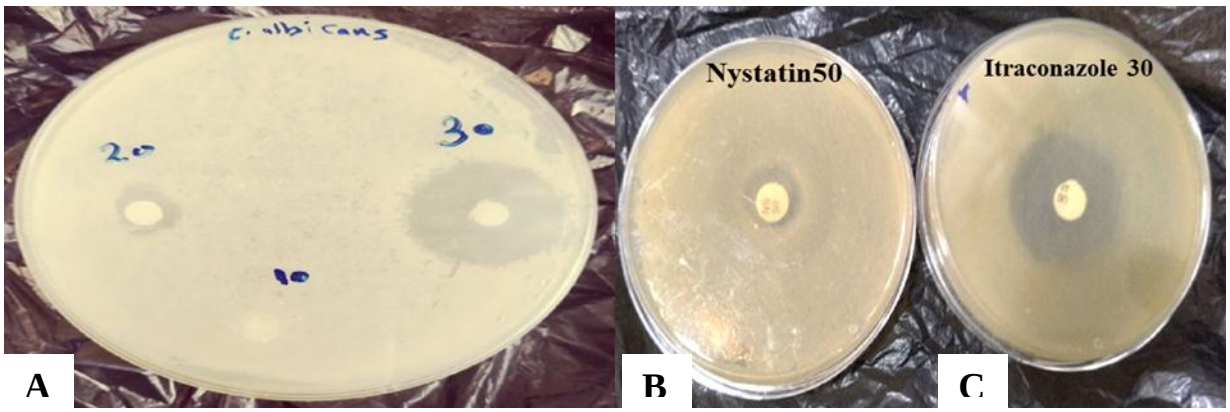


Figure (4-38) Effects (A) gingerol and (B) Nyystatin (C)Itraconazole on *Candida albicans* growth

Conclusions

And

Recommendation

Conclusions and Recommendation

Conclusions: -

1. Clinical isolates of S. aureus possess number of virulence factor associated with different infection such as adhesion factors (biofilm), capsular polysaccharide, protease, DNase, Gelatinase and Coagulase.
2. 16 sRNAgene the highest percentage, It is a diagnostic gene for Staphylococcus aureus, .
3. The isolates of *Staphylococcus aureus* were the dominant among the types of bacteria that appeared.
4. During the study, several types of Candida appeared, the most frequent of which was *Candida albicans*
5. Age group 18-28 years is more susceptible for different infection.
6. This study founded that the Gingerol has a better effect than antibiotics on bacteria and fungi growth.

Recommendations:

1. Used other compounds from the ginger plant extract and testing their effectiveness against microorganisms.
2. Testing the efficacy of gingerol compound on animals.
3. Carrying out a gene expression for the deleterious genes
4. Determining the reason for the presence of virulence genes in vaginal bacteria more than in other places.

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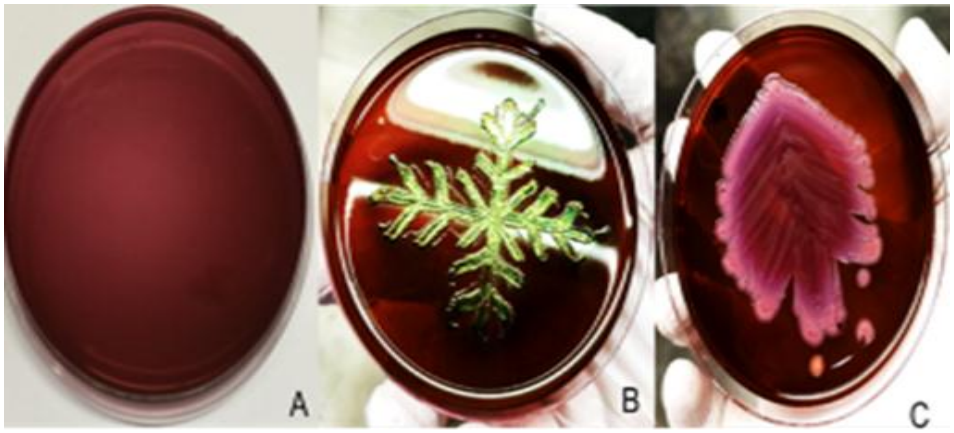
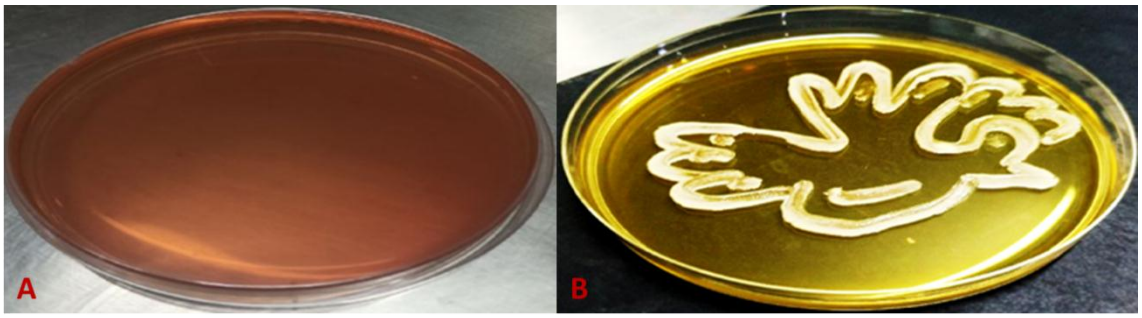
Appendix

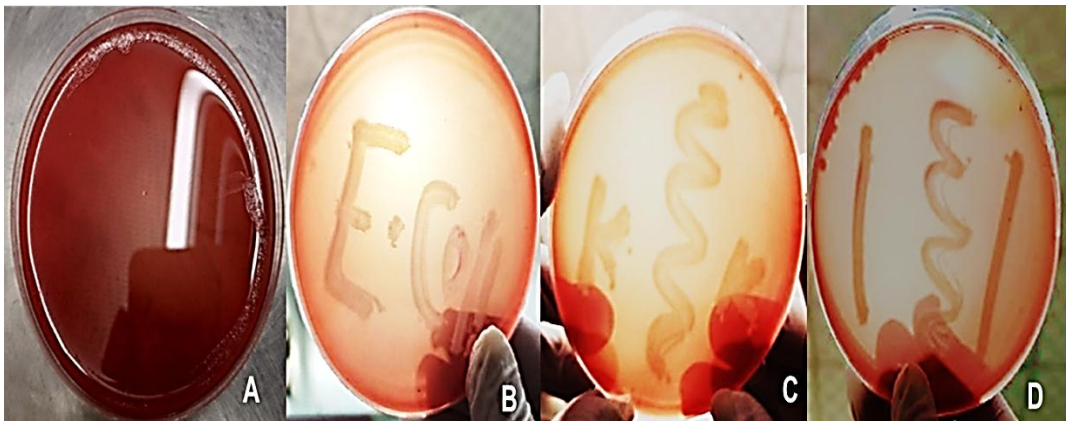
Appendix



Gram negative bacteria on EMB (a) control (b)*E. coli* (c) *klebsiella*

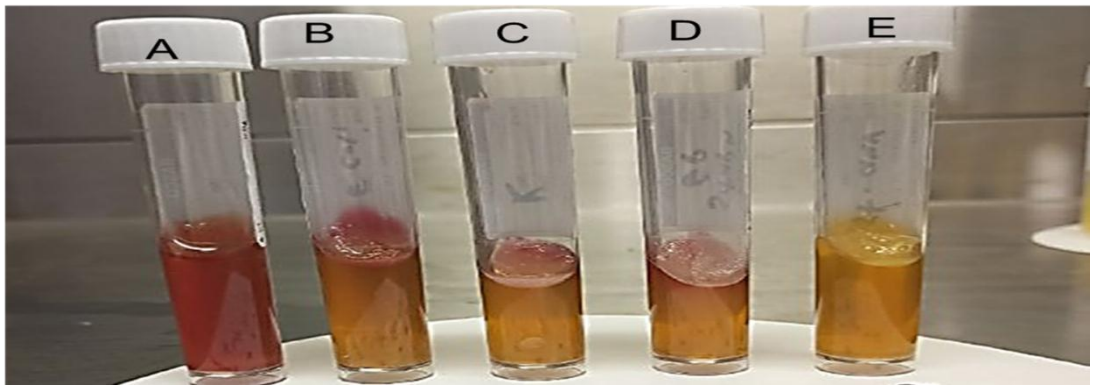


Mannitol salt agar (a)control (b) *s. aureus*



Figure(4-2)Hemolysis test (A) control (B)*E. coli* (C) *klebsiella*(d) *S. aureus*

Appendix



Figure(4-5) kligler iron test (A)control (B)*S.aureus* (C)*E. coli* (D)*klebsieala*(E) *S.epdermides*

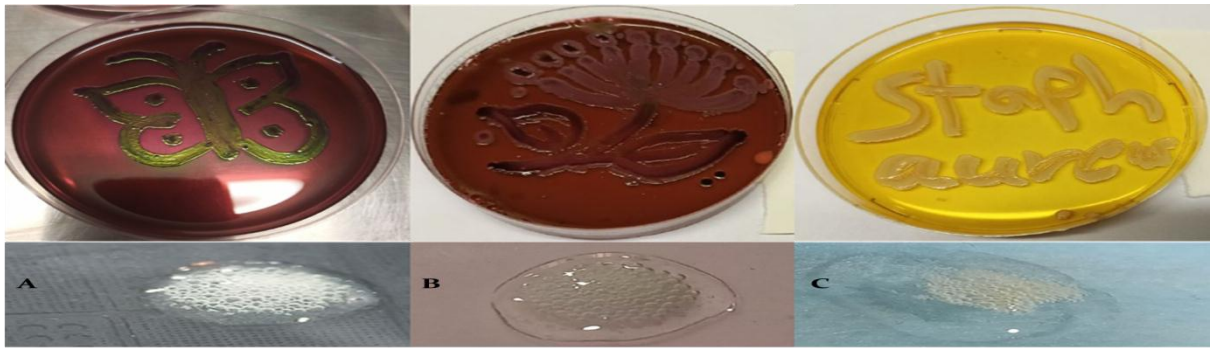
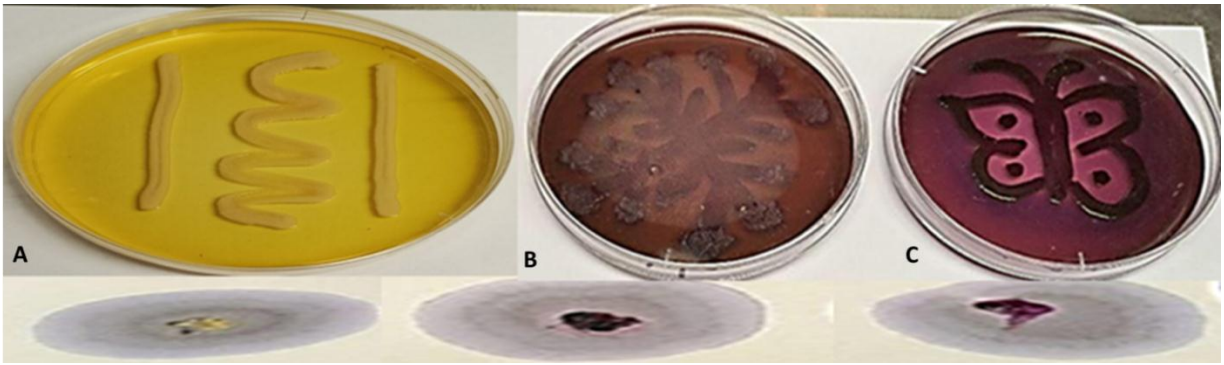


Figure (4-6) Catalase test (A) *E.coli* (B) *klebsiella* (C) *s.aureus*



b

Appendix

Figure (4.7) Oxidase test (a) *s.aureus* (*b)E. coli* (c) *klebsieala*

Figure (4-8) Coagulase test(a) *s.aureus* (*b)E. coli* (c) *klebsieala*

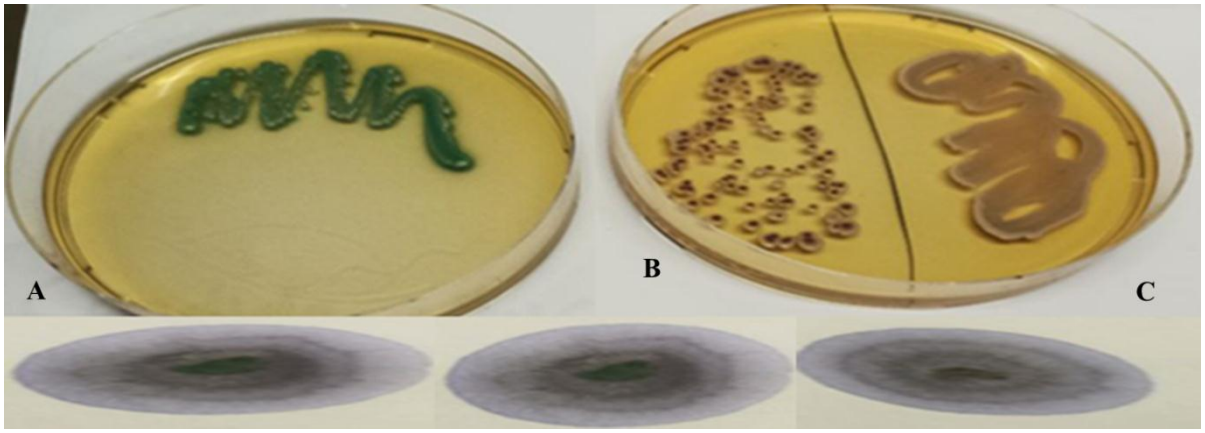


Figure (4-12) Oxidase test (A) *C.albicans* (B) *C. krusie* (C) *C.glabrata*

Appendix

Sample collection form

No.	Name	Age	Type Of Bacteria	Type Of Fungi	Notes

الخلاصة

التهاب المهبل هو التهابا يصيب المهبل عادة ما ينتج عن عدوى بكتيرية وكأذلك فطرية. تشمل الأعراض المحتملة الإفرازات والحكة والحرق وربما الألم.

تم أجراء الدراسة الحالية على التهاب المهبل في كلية العلوم –قسم علوم الحياة في مختبر عزل الفطريات والسموم الفطرية كذلك مختبر الأحياء الجزيئي كلية العلوم بنات –قسم علوم الحياة.

في هذه الدراسة تم جمع 150 عينة من منطقة المهبل لنساء متزوجات لا يحملن اي مرض كمرض السكري وغيره كذألك غير حوامل من العيادات النسائية الخاصة.

حيث زرعت على الأوساط الغذائية الفطرية والبكتيرية بعد الحصول عليها وجلبها الى المختبر ، وبعد زراعة البكتريا والفطريات المعزولة على عدد من الاوساط التخصصية التشخيصية اظهرت النتائج ان النسبة الكلية للبكتيريا المعزولة (55.3٪) اما النسبة المئوية للفطريات (54.7٪). وان اهم أنواع البكتيريا التي ظهرت هي *E.coli* بنسبة 24.3 ٪ ،*Klebsiella pneumoniae* 20٪ ، *S.aureus* 55.7٪ من خلال النتائج ظهرت بكتيريا *S. aureus* أنها أكثر تردد من بقية أنواع البكتيريا المعزولة. في حين كانت الفطريات هي *C. albicans* 52.6 % و *C. glabrata* 23.7% و *C. krusie* 23.7 % وهي فطريات معزولة في الحالات السريرية. تم إجراء اختبارات كيميائية حيوية لكل عينة سواء كانت بكتيرية أو فطرية وتضمنت فحص (إندول ، سترات ، يوريا ، كاتالاز ، أوكسيديز ، Coagulase و H2S)حيث أظهرت العزلات البكتيرية استجابة موجبة لأغلب تلك الاختبارات . أما العزلات الفطرية فقد كانت النتيجة موجبة لفحص كاتالاز واختبار تحليل الدم والنتيجة سالبة لفحص اليوريا و الأوكسيديز في حين كان اختبار انبوب الانبات Germ tube موجب فقط لعزلات النوع *C. albicans* . من خلال النتائج ظهرت بكتيريا *S. aureus* أنها أكثر تواجدا من أنواع البكتيريا الاخرى المعزولة وبنسبة 81.2% .

اما الفطريات في الحالات السريرية فان *C. albicans* أكثر شيوعًا من أنواع المبيضات الأخرى وبنسبة 52.6 % وقد كانت نسب الاحياء المجهرية المعزولة عالية في اعمار النساء بين 18-28 سنة .

تضمن العمل الحالي الكشف والتحري الجزيئي عن عوامل الضراوة للعزلات البكتيرية المعزولة من منطقة المهبل وكذلك تشخيص العزلات الفطرية . حيث تم فحص 14 جينًا مسؤول عن عوامل الضراوة باستخدام تقنيةReal Time PCR and PCR ، وأظهرت بيانات PCR أن نسبة الجينات كانت (*Nuc* 48.1 ،*Lip* 68.01 ، *Sak* 79.1 ، *hys*A 26.3 ، *mec*A 22.2 ،icaA 66.7 ،*icaD* 40

16s rRNA 99 ، *Seb* 53.3 ، *Sea* 84.7 ، *Etb* 62.4 ،*Eta* 79.1 ، *Tst* 25 ، *sspB* 38.8 ،) . كذلك تم استخدام زوج من البرايمرات ITS1 وITS2 في تشخيص الفطر *Candida* حيث اختبرت 32 عزلة فطرية.

اما بالنسبة للنبات المستخدم في الدراسة وهي رايزومات *Zingiber officinale* فقد أظهرت نتائج الكشف النوعي وجود العديد من المركبات الكيميائية النباتية التي تم اختبارها باستخدام الكواشف الكيمياوية وهي قلويدات ، فينولات ، فلافونويد ، تانينات ، كومارين ، جليكوسيدات ، صابونين ، راتنج ، كربوهيدرات ، تربين.

كذا تم استخدام تقنية HPLC التي أكدت وجود المركب الفعال الجينجيرول بتركيز 39.58 ppm المنقى بطريقه جزيئيه وهو يعتبر مضادة للالتهابات من خلال قدرته على تثبيط نمو البكتيريا والمبيضات. حيث أظهر مركب جينجرول وباستخدام ثلاث تراكيز (10 , 20, 30 ملغم \مل)تأثيراً واضحاً على البكتيريا وعازلات الكانديدا المعزولة من منطقة المهبل مقارنة مع المضادات الحيوية المستخدمة على العزلات البكتيرية والفطرية ، وكان التأثير التثبيطي الأعلى عند تركيز 30 ملغم/ مل وذلك لمركب جينجرول حيث كانت نسبة التثبيط 20 ملم لبكتريا *S. aureus* كذألك بينت نتائج التأثير التثبيط لمركب جينجيرول على نمو الفطريات ان نسبة التثبيط 19 ملم لخميرة . *C. albicans*

جمهـــــورية العـــــــــــراق
وزارة التعليم العالي والبحث العلمي
جامعـــــة بابـــل/ كلية العلوم
قســـــم علـــوم الحيـــاة

الفعالية الضد مايكروبية للجنجرول المنقى جزئياً على بعض الممرضات المشخصة جزيئياً والمعزولة من التهاب المهبل

أطروحة مقدمة إلى

الى مجلس كلية العلوم / جامعة بابل

جزءاً من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/علوم الحياة

من قبل

هبه حديد راشد عيسى

بكالوريوس علوم في الأحياء المجهرية/ جامعة بابل (2016)
ماجستير علوم الحياة / جامعة بابل (2019)

بإشراف

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2023 م