CHAPTER ONE

1.1 Introduction

Bacteria that attach to surfaces aggregate in a hydrated polymeric matrix of their own synthesis to form biofilms. Formation of these sessile communities and their inherent resistance to antimicrobial agents and immunity are at the root of many persistent and chronic bacterial infections (Costerton *et al.*, 1999). The number of organisms present at the site of infection could overwhelm the host defense system (Gardner *et al.*, 2004; Sibbald *et al.*, 2003) and to a large extent communication or QS, possession and expression of virulence factors by the organisms often determine the progression of microbial colonization to infection (Jensen *et al.*, 2007; Williams *et al.*, 2000) even in immune competent individuals. The genetic units that are responsible for the virulence capability of an organism are usually located within the genome at the pathogenic islands which can be shared by organisms within a biofilm (Schmidt and Hensel, 2004; Hansen-Wester and Hensel, 2002).

Staphylococci are gram-positive cocci that occur in grape-like clusters and were first observed in human pyogenic lesions by von Recklinghausen in 1871. *S.aureus* is one of the most significant pathogens in both hospitals and the community and can cause numerous syndromes in humans. Staphylococcal infections are among the most common of bacterial infections such as cutaneous, deep infections and toxin-mediated diseases. More than 60% of *S.aureus* isolates are resistant to methicillin, and the emergence and spread of *MRSA* have become a worldwide challenge. Both *MRSA* and *MSSA* can cause chronic wound infection (Gary *et al.*, 2017).

Honey is a nutritional product and sweetener produced by bees from nectar which they collect from plants as source of food. The uses of honey in treatment

of infected wounds and topical infections have been shown through several studies (Robson *et al.*, 2009).

In the Holy Quran, Almighty Allah mentioned the special ability of honey to heal and cure disease. Allah said "And your Lord revealed to the bee: Make hives in the mountains and the trees in what they build. Then eat all the fruits and walk in the ways of your lord submissively. There comes forth form their bellies a beverage of many colours, in which there is healing for mankind.Verily in this, sign for those who give thought." (surah Al-Nahal; verses 68 and 69).

Ibne Sina, the prince among muslim physicians listed several beneficial uses of honey in his monumental work of "The Canon of Medicine" and Hippocrates, the father of western medicine, used honey to treat a number of diseases. Ancient users did not know its antibacterial properties; they only knew it as an effective remedy. During the twentieth century, it was reported that honey have good antimicrobial properties together with therapeutic potential in wound healing. Honey as an alternative antimicrobial has been explored in the treatment of infections and as a result the antimicrobial effects of honey have been widely investigated on various microorganisms (French *et al.*, 2005; Cooper *et al.*, 2002) including biofilm (Alandejani *et al.*, 2009).

1.2 Rationale

Honey is a natural antibacterial, available and relatively cheap remedy that can be used to treat wound infections. Antimicrobial susceptibility testing is crucial for the guidance of clinical management, nowadays isolates from many parts of the world are multidrug-resistant for example *MRSA*, beside the unavailability, high cost and harmful side effects of some antibiotic types. For this reason, we want to determine the antibacterial activity of natural *Acacia* honey against *S. aureus* biofilm and eradication of such infections from wounds.

The formation of biofilm in wounds by pathogenic organisms has been associated with impairment of healing processes due to combined effects of the virulence of organisms within the biofilm. The effect of attenuation of the organism through inhibition of biofilm and QS signaling molecules by honey will allow the body defense system of the host (patient) to easily eliminate the organism from the site of infection, therefore we need to prove this activity using scientific standard microbiological methods.

1.3 Objectives

1.3.1 General objectives

To detect the antimicrobial activity of *Acacia* bee honey on *S. aureus* biofilms.

1.3.2 Specific objectives

1. To reidentify *S. aureus* isolates from wound swabs using biochemical methods.
2. To detect *MRSA* bacteria using disc diffusion technique.
3. To detect the invitro antibacterial activity of Sudanese *Acacia* bee honey against biofilm forming *S. aureus* using microtiter plate method.
4. To correlate between antibacterial activity of different concentrations of *Acacia* bee honey and control antibiotics in microtiter plate culture method.

CHAPTER TWO

2. Literature Review

2.1 Definition of biofilm

Biofilms are accumulations and attachment of microorganisms within a complex matrix that adhere to a surface, they are closely linked with the etiology of diverse chronic and recurrent human infections, device-related infections and treatment failures (Davey & O'Toole, 2000).

2.2 Biofilm formation:

Biofilm is a complex, heterogeneous and integrated community of surface attached microorganisms of either single or multiple species that are encased within the extracellular polymeric matrix produced by them (Chang *et al.*, 2007). They have been found attached to solid (abiotic) surfaces including industrial water systems as well as medical environments and devices (Jacobsen *et al.*, 2008) and mucosal surfaces in humans (biotic) (Hall-Stoodley *et al.*, 2004). Most bacteria grow in a free-living planktonic state, but some are able to exhibit different phenotypes which differ in physiological characteristics including structural and metabolic changes. The main stages of biofilm formation are attachment, maturation and dispersion (Figure 1). These various stages of biofilm formation are initiated when the planktonic organisms transform to the sessile form (O'Toole *et al.*, 2000). Each of the stages has distinguishing characteristic features and requires regulation by QS molecules (Sauer *et al.*, 2002).

2.2.1 Attachment

Planktonic bacteria transform to the sessile form prior to biofilm formation as they adhere to a favourable surface; such as a medical device or the host tissue. In some cases initial adhesion of biofilm forming microorganisms is achieved by means of adhesins located on specialised organelles such as fimbriae (pili)

and production of auto-inducers (Lasaro *et al.*, 2009). There are two stages, the reversible and irreversible attachment.

2.2.2 Formation of microcolonies

The cells aggregate as they divide on adhesion to a surface but the daughter cells multiply at the point of attachment to form cell clusters. The dividing cells produce QS molecules and EPS, or polymer matrix. The matrix houses the aggregating cells in microcolonies and attaches the biofilm to the surface on which it is formed, with flagellum driven movement increased number of bacteria and signaling it grows larger (Watnick and Kotler, 1999).

2.2.3 Detachment and dispersal of biofilm

The biofilm environment is innately regulated , and population density within a mature biofilm induced programmed detachment of bacteria from biofilm through the secretion of chemical substances by the organisms. Detachment occurs when the organisms respond to chemical substances secreted by them such as signalling molecules (Stoodley *et al.*, 2005), proteins, degradative enzymes and oxidative or nitrosative stress-inducing molecules such as nitric oxide (NO) produced as a result of metabolic processes within a biofilm also organisms cleaves the polymer matrix into short oligosaccharides (Wood *et al.*, 2007).

The presence of optimal amounts of nutrients has been observed as an inducing factor for dispersal of biofilm organisms by increasing the growth of organisms and production of autoinducers which usually aid the dispersal processes within the biofilm. Nutrient deprivation causes single bacteria to reversibly switch to a state of metabolic arrest but at these very low metabolic rates in biofilms, bacteria will survive a wide range of environmental threats, in addition temperature shifts and extreme pH changes, as well as exhibiting decreased sensitivity to antimicrobial agents (Rice *et al.*, 2005).

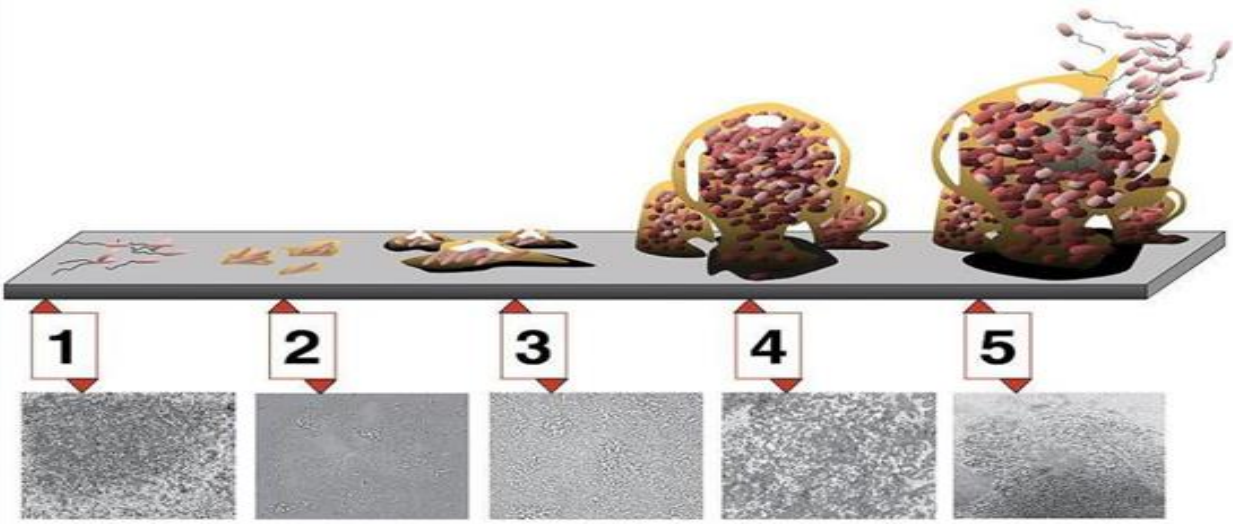

Figure (2.1) the stages of biofilm formation. The diagram and the corresponding microscopic examination of biofilm growth showing (1) Initial attachment (2)irreversible attachment (3) biofilm maturation I (4) biofilm maturation II (5) dispersion. Each stage of development in the diagram is paired with a micrograph of a developing biofilm (Monroe, 2007).

2.3 Biofilm physiology

Biofilm has a complex physiology due to the collective effect of the activities of the various organisms within the biofilm. The knowledge about biofilm physiology was highly important in understanding the activities of biofilm organisms for effective control and has become available through the use of various methods to examine the ultra-structure including electron microscope, staining and examination with light microscope, epifluorescent microscope (Davis *et al.*, 2008; Tolker-Neilsen *et al.*, 2000) confocal laser scanning microscope (CLSM) (Percival *et al.*, 2008) and detection of chemical products such as the QS molecules (Nakagami *et al.*, 2008). The architecture is mainly

influenced by the EPS produced by the individual residents and have been used in the study of biofilm. The EPS is often composed of polysaccharides (alginate and levan), MVs proteins, lipids, enzymes and nucleic acids. Although EPS aid the adhesion of biofilm to surfaces and tissues but the main function is to serve as the niche within which biofilm organisms inhabit. The EPS form the bulk of the biofilm architecture (Chang *et al.*,2007; Laue *et al.*, 2006) forming 75 -90 % of biofilm while only 10 - 25% are cells.

In general, biofilms are usually highly hydrated with the open structures comprising of up to 73 to 98% extracellular materials and space (channels). Chemical gradients (pH, redox potential, and ions) are known to occur usually within the biofilm due to the degrees of diffusion of nutrients, metabolic products, and oxygen in all parts of the biofilm (Hunter and Beveridge, 2005). The rate of growth and development of biofilm organisms are influenced by chemical gradient, the organisms at the biofilm-liquid interface having the fastest growth as against the much slower rate of those in the interior of the microcolonies. The cells within biofilm are able to maintain intracellular pH homeostasis thus enhancing their physiological condition and acid tolerance (McNeill and Hamilton, 2004).

Biofilms constituents are diverse as the microbes within them especially the multispecies biofilms. Production of QS molecules sometimes depends on the site of infection and QS dependent phenotypes vary according to the site where the organisms are isolated. Various differences have been observed amongst the biofilms from the wound, the dental plaque and those from the natural ecology like rocks or water especially in their microbial population (Reardon *et al.*, 2004) as well as their extracellular matrix and production of virulence factors. There are some effects of chemical and physical agents on biofilm such as: indole, lactoferrin, electrical current and water current (Favre-Bonte *et al.*, 2007).

2.4 Quorum sensing

Microorganisms are capable of communicating with each other by QS to coordinate complex activities like multicellular organisms in addition to the basic role of nutrients metabolism, growth and multiplication. Adaptation to an environment by microorganisms involves response to a series of changes via signal transduction systems, comprising of protein kinase that phosphorylates itself using adenosine triphosphate and response regulator which accepts the phosphoryl groups so QS is a process by which organisms monitor and respond to the presence of other organisms within the environment through the production of signalling molecules. In biofilm regulation of metabolic activities and population density is controlled by cell density-dependent genes expression as a result of accumulation of signalling molecules in the medium. The autoinducer molecules produced by individual microorganisms diffuse through the cell and accumulate in the medium and once a threshold level is attained due to large numbers of organisms the population of microorganisms within the environment respond to the critical cell mass via transcriptional regulation of various target genes (Sauer *et al.*, 2002).

Because of the role of QS in the regulation of virulence factors including biofilm formation, blockage of QS in pathogenic organisms has therefore been suggested as a treatment strategy especially in the control of biofilm infections (Bjarnsholt and Givskov, 2007).

2.5 Resistance of biofilm to immune system and antimicrobial agents

Biofilm organisms are known to cause persistent infections due to their inherent resistance to immune mechanism and antimicrobials (Leid *et al.*, 2002 and 2005). Biofilm organisms also grow at a slow rate which is a factor that enhances their ability to resist host immune mechanisms and antimicrobial interventions. The components of EPS of biofilm prevent phagocytosis of biofilm organisms. The older biofilm are more resistant to phagocytic actions

of PMNs than the younger biofilm (Günther *et al.*, 2009). Some chemicals and enzymes produced by *P.aeruginosa* biofilm phenotypes such as proteases are virulence factors that damage host tissues and interfere with host antibacterial defence mechanisms (Ołdak and Trafny, 2005) although human leucocyte was able to penetrate and responded to the biofilm of *S.aureus* (Leid *et al.*, 2002) but generally phagocytic and immune resistances of organisms are high in biofilm and have been linked to the large molecules which pose problems to immune recognition and signal molecules control (Jensen *et al.*, 2006; Bjarnsholt *et al.*, 2005) and protection by the EPS (Leid *et al.*, 2005).

Organisms within biofilm are able to resist antimicrobials. The mechanisms of resistance are either innate due to the physiology and the architecture of biofilm and may be due to genetic acquisition of resistant genes (Anderson and O'Toole, 2008; Fux *et al.*, 2005). The resistance of biofilm organisms to antimicrobial agents starts from the attachment phase and increases with the development of the biofilm (Patel, 2005). The extracellular matrices act as barrier which tends to physically restrict the diffusion of antimicrobial agents into the biofilm niche thus reducing the availability of antimicrobial inside the biofilm. Nutrient and oxygen depletion within the biofilm environments with consequent slow rate of growth of the organisms within the biofilm hence the organisms are less susceptible to growth-dependent antimicrobial killing. The heterogeneous environment within biofilm such as the pH, oxygen tension and other chemical substances have been shown to reduce the activities of antimicrobials hence the effect of antimicrobials will vary with the location within the biofilm (Borriello *et al.*, 2004). The ability of biofilm organisms to rapidly mutate has been shown to influence the rate of antimicrobial resistance (Driffield *et al.*, 2008).

The regulation of genes by QS molecules including antimicrobial resistance genes but QS deficient strains infections have shown reduced susceptiblity to antimicrobials (Karatuna and Yagci, 2010). Also age of biofilms tends to affect

the resistance of the biofilm to antimicrobials. It has been shown that older biofilms are more resistant to antibiotics (Donlan, 2001).

2.6 Biofilm detection methods

Various methods have been used to determine the presence of biofilm in cultures invitro and tissues. These procedures have been used singly or in combinations to detect biofilm in wounds and they include:

2.6.1 Light microscopic examination of stained cultures and tissue samples: this will provide information about the intact physiology of the biofilm. Stains such as Congo red in Tween 80 can be used to stain biofilm EPS which often appear pinkish-orange while the organisms appear purplish-red when stained with Ziehl carbol fuchsin. The stains reveal the structures of the organisms within the biofilm matrix which usually provide information regarding the arrangement of the organisms as well as the size of biofilm and quantity of EPS (Harrison- Balestra *et al.*, 2003). Basic information about the organisms such as shape and size can be obtained and can be used for pure and mixed cultures. Examination of tissue samples may show the location of the biofilm within the tissue and host components such as the PMNs (Dongari-Bagtzoglou *et al*., 2009).

2.6.2 Confocal laser scanning electron microscopic examination of biofilm: provides the opportunity for live visualisation of biofilm to observe viable and non-viable organisms on continuous basis. Activities such as multiplication of organisms, production of EPS or interaction among the organisms within the biofilm can be observed also be stained with dyes (Tolker-Nielsen *et al*., 2000).

2.6.3 Atomic force microscopy (AFM) of biofilm: It can be used to observe biofilm at high magnification and can provide measurement of each organism within the biofilm (Surman *et al*., 1996).

2.6.4 Scanning electron microscopic examination of tissue samples (SEM): It reveals detailed structures of the biofilm components especially arrangement

of the organisms within the biofilm as well as the adherence of the organisms to the matrix (Davis *et al*., 2008).

2.6.5 Epifluorescent microscopy: It is employed when the organisms are stained with fluorophores which absorb light emitted at specific wavelengths to emit lights of longer wavelength and the stained preparations are examined with an epifluorescent microscope (Davis *et al.*, 2008) .

2.6.6 Fluorescent in situ hybridization (FISH) or Peptide nucleic acid-based fluorescence: It is the utilisation of fluorescence technique to stain specific components of the cell such as the deoxyribonucleic acid (DNA). The fluorescent probes bind to the specific component with genetic similarity for species identification of organisms within a biofilm (Kirketerp-Moller *et al.*, 2008; Lefmann *et al.*, 2006).

2.6.7 Sonication or vortex mixing and estimation of colony forming units (cfu): The numbers of organisms provide indirect information about the size of biofilm relative to time or an indication of the effect of substances such as antimicrobials on biofilm but does not provide information about the morphology of the biofilm or the interaction amongst the organisms within it (Sun *et al.*, 2008).

2.6.8 Detection of QS molecules: Biofilm markers including QS molecules has been used for the detection of biofilm formation by a particular class of organisms. This was applied to catheters, sputum and biopsy specimens from rats. In polymicrobial infections other tests are required to identify each of the organisms in such group (Nakagami *et al.*, 2008).

2.6.9 Detection of EPS components: such as alginate can be used to determine the presence of biofilm in cultures or materials and medical devices such as catheters. The detection can be achieved by means of SEM, staining and examination with light microscope (Harrison–Balestra *et al.*, 2003) or chemical methods and antibodies (Kives *et al*., 2006).

2.6.10 Biofilm adherence assay (in microtitre plate or on slides): This method is mostly used for screening organisms for biofilm invitro. Although the morphology of the biofilm can not be seen but the ability of organisms to form biofilm can easily be determined in a simple and reproducible analysis. Biofilm culture on slide can be achieved in a batch or continuous culture and biofilm can be stained and examined with light microscope or by determination of viable counts after releasing the organisms by sonication or vortex (Sun *et al.,* 2008).

2.7 Medical importance of biofilm

The physiological nature of biofilm particularly the protection of the organisms from immune and antimicrobial strategies by the EPS and the slow growth rate of organisms arising from distribution gradient of air and nutrients greatly enhanced the ability of biofilm organisms to further resist antimicrobials thus resulting in chronic infections (Borriello *et al.,* 2004). The effect of QS molecules on the up-regulation of virulence factors (Sauer *et al.,* 2002) and the effects of the expression of these factors by the large numbers of organisms within biofilm could aggravate pathogenic consequences. The proximity of the organisms within biofilm enhances the transfer and acquisition of virulence genes such as antimicrobial resistance, toxin and enzyme production amongst biofilms of mixed organisms (Donlan and Costerton, 2002).

The cumulative factors of resistant strategies employed by biofilm organisms has resulted in huge resistance to antimicrobials which could range between10 to 1000 times that of the planktonic counterparts (Lewis, 2001). The cells from biofilm detach to initiate infections at other sites within a host including the blood stream and the internal organs (Wood *et al.,* 2007; Rice *et al.,* 2005). In certain circumstances the biofilm slough could be transported through the veins and can lead to embolism which could be life threatening (Wenzel, 2007). Biofilm organisms may colonise medical devices and equipment such as heart

valves catheters, joint prostheses and the central nervous system devices where they serve as reservoirs for the organisms and sources of nosocomial infections particularly in immunocompromised patients. Many species of bacteria implicated in diseases form biofilms (Dowd *et al.*, 2008; Donlan, 2001); including *Streptococcus species* (Lembke *et al.*, 2006), *Staphylococcus species* (O'Gara and Humphreys, 2001; Donlan, 2001), *Enterococcus species* (Mohamed and Huang, 2007), *P.aeruginosa* (Wagner and Iglewski, 2008), *enteric* bacteria (Gaetti-Jardim *et al.*, 2008) and *Candida albicans* (Douglas 2003). Most biofilms are usually polymicrobial and the synergistic effect of the virulence factors of the organisms, consequently results in complications and chronicity of infections (Dowd *et al.*, 2008). Physiological concentrations of bile or bile acids stimulate in vitro biofilm formation in several lung-colonizing pathogens for example *S. aureus.* In natural environments biofilm organisms can influence each other in a synergistic or antagonistic manner depending on species-specific physical interactions between cells or on extracellularly secreted factors which are less specific interactions. Studies have shown that *Candida species* produce certain chemical substances which are inhibitory to QS in *P.aeruginosa* (Rasmussen *et al.*, 2005). In humans it has been shown that biofilms are responsible for over 60-80% of chronic infections as well as 65% of nosocomial infections with associated high cost of treatments (Wilson, 2001). Biofilms are implicated in a wide variety of microbial infections including urinary tract (Jacobsen *et al.*, 2008) and catheter infections (Fux *et al.*, 2004), otitis media and other ear infections, dental infections (Thomas and Nakaishi, 2006), endocarditis (Manetti *et al.*, 2007), cystic fibrosis (Hassett *et al.*, 2009) and human wounds (Dowd *et al.*, 2008).

2.8 Staphylococcus *aureus*

The genus *Staphylococcus* contains 33 defined species and 20 species found in man. They are gram-positive spherical cells, usually arranged in grapelike irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. Some are members of the normal microbiota of the skin and mucous membranes of humans others cause suppuration, abscess formation, pyogenic infections, and even fatal septicemia. Species of *Staphylococci* are initially differentiated by the coagulase test and are classified into two groups: coagulase-positive and coagulase-negative *staphylococci.*

Coagulase-positive staphylococci: *S.aureus* (formerly also called *S.pyogenes)* is coagulase positive. *Coagulase-negative staphylococci (CNS*): *S. epidermidis and S. saprophyticus* are the most clinically significant species in this group (Surinder, 2016; Gary *et al.,* 2017).

2.8.1 The antigenic structures of *S. aureus*

Cell-associated Polymers such as capsular polysaccharide surrounding the cell wall inhibits opsonization, peptidoglycan confers rigidity and structural integrity to the bacterial cell. It activates complement and induces release of inflammatory cytokines. Teichoic acid, an antigenic component of the cell wall, facilitates adhesion of the cocci to the host cell surface and protects them from complement-mediated opsonization (Rachna and Anshul, 2009).

Cell Surface Proteins such as Protein A which is a group-specific antigen unique to *S.aureus* strains. It has many biologic properties including chemotactic, anticomplementary and antiphagocytic and elicits hypersensitivity reactions and platelet injury. It binds IgG molecules, nonspecifically, through Fc region leaving specific Fab sites free to combine with specific antigen. Clumping factor (tube bound coagulase test) is component on the cell wall of *S.aureus* that results in the clumping of whole *Staphylococci* in the presence of

plasma which reacts directly with fibrinogen converts it to insoluble fibrin, causing the *Staphylococci* to clump or aggregate. Slide coagulase test is done to detect free enzyme (Anna and Donald, 2010).

Toxins and Enzymes such as cytolytic or membrane-damaging toxins are alpha, beta, delta, gamma, and P-V and leucocidin. Alpha (a) hemolysin is the most important among them. It is a protein and is inactivated at 60°C. The toxin lyses rabbit and sheep erythrocytes. It is leucocidal, dermonecrotic and lethal. These components act synergistically to damage PMN leukocytes and macrophages and to produce dermonecrosis (Bubeck Wardenburg *et al*., 2007). Enterotoxin is responsible for the manifestations of staphylococcal food poisoning nausea, vomiting and diarrhea 2–6 hours after consuming contaminated food containing preformed toxin. Eight serologically distinct staphylococcal enterotoxins (A-E,G-I). TSS is a severe and often fatal disorder characterized by multiple organ dysfunction. TSST-I is a super-antigen. Epidermolytic or exfoliative Toxins is responsible for the (SSSS). Extracellular Enzymes it produces a number of enzymes such as coagulase, catalase, hyaluronidase, fibrinolysin, lipases, nucleases and penicillinase (Rachna and Anshul, 2009).

2.8.2 Staphylococcal Diseases

Cutaneous such as wound and burns, deep infections within organs, toxin mediated diseases (food poisoning and TSS) and exfoliative diseases (SSSS) (Surinder, 2016; Panizzi *et al*., 2011).

2.8.3 Morphology and identification

They are spherical cocci, approximately 1μm in diameter, they are arranged characteristically in grape-like clusters. Cluster formation is due to cell division or binary fission occurring in three planes, with daughter cells tending to remain in close proximity. They are non-sporing, non-motile and usually non-capsulated with the exception of rare strains (Patricia, 2017).

2.8.4 Laboratory Diagnosis:

2.8.4.1 Specimens

can be collected depend on the type of lesion, for example: pus, sputum, food remains and vomit from cases of food poisoning; nasal and perineal swabs from suspected carriers. Swabs of the perineum, pieces of hair and umbilical stump (Gary *et al.*, 2017).

2.8.4.2 Direct microscopy

Gram stained smears where cocci in clusters may be seen. This is of no value for specimens like sputum where mixed bacterial flora are normally present (Gary *et al.*, 2017).

2.8.4.3 Culture

They are aerobes and facultative anaerobes. Optimum temperature for growth is 37°C (range being 12–44°C). Optimum pH is 7.5. They can grow readily on ordinary media.

Nutrient agar: After aerobic incubation for 24 hours at 37°C, colonies are 1-3 mm in diameter and have a smooth glistening surface, an entire edge. Most strains produce golden-yellow pigment. These white-colonies strains of *S.aureus* are fully virulent. Pigmentation is enhanced on fatty media, such as tween agar, by prolonged incubation, and by leaving plates at room temperature.

Blood agar: The colonies have the same appearances as on nutrient agar, but may be surrounded by a zone of β-hemolysis.

MacConkey agar: Colonies are smaller and are pink due to lactose fermentation.

Milk agar: colonies are larger than those on nutrient agar and pigmentation is well-developed.

Phenolphthalein phosphate agar: This is an indicator medium and assists in the identification of *S.aureus* in mixed cultures. Colonies become bright pink when culture plate is inverted over the ammonia for a minute.

Selective salt media: is useful for the isolation and enumeration of *Staphylococci* from materials, such as feces, food and dust likely to contain a predominance of other kinds of bacteria. Therefore, 7–10% of sodium chloride may be added to mannitol salt agar containing 1% mannitol, 7.5% NaCl, and phenol red; and Ludlam's medium containing lithium chloride and tellurite; and salt cooked meat broth (10% NaCl) (Patricia, 2017; Surinder, 2016).

2.8.4.4 Biochemical reactions

Sugar fermentation*:* it ferments a range of sugars producing acid but no gas. Mannitol which is usually fermented anaerobically by *S. aureus* but not by other species.

Catalase test*:* Catalase positive which degrade hydrogen peroxide.

Lipolytic test: When grown on media containing egg-yolk, produce a dense opacity because most strains are lipolytic.

Phosphatase test*:* This is a useful screening procedure for differentiating *S.aureus* from *S.epidermidis* in mixed cultures, as the former gives prompt phosphatase reaction, while the latter is usually negative or only weakly positive. All strains of *S.aureus* produce phosphatase which liberates phenolphthalein from sodium phenolphthalein diphosphate. The culture plate, with the culture, is inverted over the ammonia for a minute or so. Colonies of *S. aureus* become bright pink because phenolphthalein is pink in alkaline pH. Most other *Staphylococci* form colonies that remain uncolored.

Deoxyribonulease *(DNase) test:* It produces a DNase, and a heat-stable nuclease (thermonuclease, TNAase).

Other biochemical tests*:* Indole negative, MR positive, VP positive, urease positive, hydrolyzes gelatin and reduces nitrates to nitrites (Patricia, 2017).

2.8.4.5 Bacteriophage typing

it might be done if the information is desired for epidemiological purposes. Other typing methods include antibiogram pattern, plasmid profile, DNA

fingerprinting, ribotyping and PCR-based analysis for genetic pleomorphism (Patricia, 2017).

2.8.4.6 Serological tests

Are of help in the diagnosis of hidden deep infections. Antistaphylolysin (antialphalysin) titers of more than two units per mL, especially when the titer is rising, may be of value in the diagnosis of deep seated infections, such as bone abscesses (Rachna and Anshul, 2009).

2.8.4.7 Antibiotic sensitivity tests

Is the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo. Ideal antibiotic therapy is based on determination of the etiological agent and its relevant antibiotic sensitivity. Empiric treatment is often started before laboratory microbiological reports are available when treatment should not be delayed due to the seriousness of the disease (Rachna and Anshul, 2009). Some antibiotics actually kill the bacteria (bactericidal), whereas others prevent the bacteria from multiplying (bacteriostatic) so that the host's immune system can overcome them. Antimicrobial susceptibility testing methods are divided into three types include:

Diffusion method: Stokes method and Kirby-Bauer method for agar.

Dilution method: Broth dilution and Agar dilution.

Diffusion gradient E-test method: This method is preferred over serial dilution method because of the ease with which quantitative results can be obtained. However, the method cannot be used when the antibiotic does not diffuse freely due to adsorption or incompatibility with the medium (Patricia, 2017).

2.8.5 Treatment

Benzyl penicillin is the most effective antibiotic, if the strain is sensitive but most clinical isolates of *S.aureus* are resistant to benzyl penicillin due to production of beta lactamase. Cloxacillin, oxacillin, flucloxacillin and methicillin are penicillinase resistant penicillins. *MRSA* (mecA gene positive) are also resistant to other penicillin and cephalosporin. Glycopeptides (vancomycin or teicoplanin) are the agents of choice in the treatment of systemic infection, but these agents are expensive and may be toxic. For mild superficial lesions, topical applications of drugs as bacitracin, chlorhexidine or mupirocin may be sufficient. The treatment of carriers is by local application of antibiotics such as bacitracin and antiseptics such as chlorhexidine (Rachna and Anshul, 2009).

2.9 Bee honey

Honey is a nutritional product produced by bees from nectar which they collect from plants as source of food. Honey bees go through complete metamorphosis (egg,larva, pupa, adult) and have a caste system. A colony typically consists of 20,000 to 90,000 members. In general honey bees are hairy and robust insects with brown and yellow bands. Adults have two large compound eyes, elbowed antennae and two pairs of transparent wings , they belong to kingdom Animalia, phylum Arthropods and class Insecta. Honey bees transform nectar into honey by a process of regurgitation and evaporation. They store it as a primary food source in wax honeycombs inside the beehive. The composition of honey usually depends on floral sources particularly the phytochemical components of the honey (Molan, 2001). The major components of honey are the sugars fructose (about 38.5%) and glucose (about 31.0%), other components include the enzymes produced by bees, sucrose between 1.0% and 12%, water 17.0% other sugars (such as maltose) 9.0%, ash 0.17% to 0.77% respectively according to the analysis of a typical honeys (Kamal *et al.*, 2002) and other

components. The enzymes commonly found in honeys are diastase (amylase), invertase (α-glucosidase), glucose oxidase, catalase and acid which contribute to their overall activity also bee defensin-1. Honeys also contain phenols ; the amount of which influences the antimicrobial activity of the honey (Aljadi and Yusoff, 2003). Other substances that have been identified in honeys are minerals such as K, Na, Ca, Mg, Fe, Zn, Cu, Ni and Co, with K . The composition of honey remains constant with storage at temperature of 20 ^{o}C or below hence temperature is of high importance in the storage of honey for the maintenance of potency when honey is to be used as a medicament. Individual honeys vary from their components due to the type of bees that produced them, the plants from where they are produced and the conditions of storage (Saif-ur-Rehman *et al.,* 2008).

Table 2.1 Honey composition (American Sugar Alliance, 2012): -

Honey…. Nutritional value per 100 g (3.5 Oz)	
Energy	1.27 kJ (304 kcal)
Carbohydrates	82.4 g
Sugars	82.12 g
Dietary fiber	0.20 g
Fat	0 g
Protein	0.30 g
Water	17.10 g
Riboflavin (vitamin B_2)	0.038 mg (3%)
Niacin (vitamin B_3)	0.121 mg (1%)
Pantothenic acid (vitamin B_5)	0.068 mg (1%)
Vitamin B_6	0.024 mg (2%)
Folate (vitamin B_9)	2 µg (1%)
Vitamin C	0.5 mg (1%)
Calcium	6 mg (1%)
Iron	0.42 mg (3%)
Magnesium	2 mg (1%)
Phosphorus	4 mg (1%)
Potassium	52 mg (1%)
Sodium	4 mg (0%)
Zinc	0.22 mg (2%)

2.9.1. Physico-chemical properties of Bee honey

PH of honey: it varies from honey to honey but generally they have an acidic pH; ranging between 3.2 and 4.5 and could be as low as 2.19 (Molan, 2006).

Osmolarity: high osmolarity because it is mainly a highly saturated mixture of monosaccharides with low water content, the molecules of which are mostly associated with the sugars. The resultant osmotic effect prevents microbial growth but the antimicrobial activity of honey is not exclusively due to the osmotic effect but other substances. These were observed when diluted honey inhibited microorganisms unlike concentrated sugar solution (French *et al.*, 2005).

Hydrogen peroxide : is produced in honey when glucose is broken down by glucose oxidase to produce gluconic acid and hydrogen peroxide.

$$\text{Glucose} + H_2O + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

The slow release of the hydrogen peroxide prevents toxicity to the tissues but acts as antiseptic without causing damage to the tissues when honey is applied to wound. The oxygen free radicals released by the hydrogen is oxidised by the iron present in honey which neutralises the effect on the tissue. The release of the hydrogen peroxide has been particularly linked to the antimicrobial activity of some honeys (Molan, 2006).

Phytochemical components: have major effect on the antibacterial properties of honeys which determines the potency of various honeys and they are dependent on the type of plants from which the bees derived the nectar. In manuka honey, it has been shown that the antimicrobial properties are not dependent on peroxide activity only but methylglyoxal (MGO) has been identified as one of the major active antimicrobial components responsible for the effectiveness against microorganisms (Atrott and Henle, 2009; Adams *et al.*, 2008).

Honey has antioxidant properties which are capable of neutralizing biologically destructive chemicals in the body. The antioxidant contents of honey diminish with time particularly from six months of storage not withstanding the temperature and storage containers (Jiménez *et al.*, 2006). Total phenolic content have been shown to influence the antioxidant activity of honeys although it is not solely responsible for it (Gheldof *et al.*, 2002).

Although there are numerous benefits of honey but sometimes contain microorganisms especially yeasts and spores of some organisms such as the *Bacillus species* (Cooper and Jenkins, 2009).

2.9.2 Medical importance of Bee honey

Honey was found to be many times more potent against coagulase-negative *staphylococci* , if bacterial inhibition were due to their osmolarity alone when compared with artificial honey or sugar solution even when diluted up to 7 to 15 fold honey still inhibited bacterial growth (Cooper *et al.*, 2002). Antimicrobial effects of honey have been demonstrated on common wound isolates such as *Pseudomonas* (Cooper *et al.*, 2002 b), *Staphylococcus* (French *et al.*, 2005), fungi (Koc *et al.*, 2009), *MRSA* (Cooper *et al.*, 2002), *VRE* (Cooper *et al*, 2002 a) and recently on biofilms invitro (Alandejani *et al.*, 2009).

2.9.3 Application of Bee honey in wound treatment

There are unique properties of honey that enhance wound healing potential which make honey an all encompassing remedy for wound treatment. These factors include the antibacterial activity which prevents mal-odour and inflammation, provides moist environment within the wound which aids debridement; and stimulation of wound healing processes. The use of sugars in the honey by bacteria instead of the amino acids in the dead tissues of the wounds further reduces the effect of honey on biofilm and QS unpleasant odour of the wounds (Molan, 2001). The overuse of antimicrobial agents is one of the major factors in the development and spread of drug resistance in

organisms, as well as in colonization and infection by drug-resistant organisms. The uses of honey in treatment of infected wounds and topical infections have been shown through several studies (Robson *et al.*, 2009).

CHAPTER THREE

3. Materials and Methods

3.1 Study Design

This is a descriptive case study.

3.2 Study Area

Soba teaching hospital in Khartoum State.

3.3 Study duration

The study was conducted during the period from June to August 2017.

3.4 Sample size

Fifty-seven isolates of *S.aureus* from wound swabs including *S.aureus* ATCC 29213 and *MRSA* ATCC 43300.

3.5 Study samples

S.aureus Isolates from Sudanese patients of different age groups and genders were suffering from wound infection attending Soba teaching hospital.

3.6 Data Collection

Fifty seven biofilm forming *S.aureus* isolated from human specimens (wound swab), were kindly donated by the hospital from the check list.

3.7 Sample collection

S.aureus isolated from wound swab (sterile cotton swab was used) were collected on nutrient agar slopes.

3.8 Ethical consideration:

Ethical approval for conducting the research was obtained from the College of Laboratory Medical Science. The participants were provided with information about the study and assured that all the obtained information will be kept highly confidential and will not be used for any other purpose than for this study.

3.9 Data analysis

The parameters were evaluated using correlation test. The level of significance was set at $p = 0.05$. All statistical analyses were performed using computerized SPSS version 21.

3.10 Methodology

3.10.1 Identification of clinical isolates

Specimens received were inoculated and incubated according to WHO SOPs.

S. aureus isolates were subcultured on blood, chocolate and Mac Conkey agar. Gram's stain was done; three drops of sterile normal saline were added in clean dry slides using sterilized wire loop. From pure culture of tested organism one colony was touched by the loop and mixed with normal saline spreaded evenly on an area of 15-20mm. Dried smears were fixed by heat, covered with crystal violet for 1 minute, washed with clean water, covered with lugol's iodine for 1 minute and rapid decolorization was done with acetone alcohol then washed, after that covered with neutral red for 2 minutes then washed. Dried smears examined microscopically using X100 and a drop of oil and appeared Gram positive cocci in clusters.

Catalase production was detected using 3ml hydrogen peroxide in which a wooden stick was immersed holding the tested organism and air bubbles was observed.

Coagulase production was showed by emulsifying a colony of the tested organism in two drops of physiological saline making two thick suspensions then a drop of plasma was added to one of the suspensions and mixed gently by rotating. Clumping of the organism within 10 seconds was observed.

Deoxyribonuclease or DNase was detected by culturing the tested organism in a medium contain DNA, after overnight incubation at 37°C the medium was flooded with weak hydrochloric acid (1%HCL) solution. The acid made the un-

hydrolyzed DNA to precipitate and clear area surrounded the colonies indicating DNA hydrolysis by the enzyme produced.

Mannitol fermentation was confirmed by color change from red to yellow on MSA due to acidic pH.

Antibiotic sensitivity was done for penicillin discs, by seeding the agar surface by the tested bacterial inoculum that already compared with 0.5 McFarland standard to form a confluent growth after incubated at 37°C for 24 hours and read the zone of inhibition by mm.

Methicillin resistance was detected by oxacillin disc at 35°C and zone of inhibition was measured in mm by the ruler.

Each test was performed along with known positive and negative controls.

3.10.2 Preparation of nutrient agar and broth

Using a sensitive balance thirteen grams were added to 1 liter of distilled water for nutrient broth, twenty-eight grams added to 1 liter of distilled water for nutrient agar, boiled to dissolve the medium completely and sterilized in an autoclave at 15 Ibs pressure and 121ºC for 15 minutes. The sterilized medium, allowed to cool at 45 to 50°C in water bath. The freshly prepared and cooled medium was poured in sterilized small tubes for broth and glass petri dishes for agar under aseptic condition, on a horizontal surface to give a uniform depth of approximately 4 mm for agar. The agar medium allowed to cool to the room temperature. When the agar got solidified, the plates were dried for immediate use for 10–15 minutes at 60ºC by placing them in the upright position in the Hot air oven with the lids tilted. A representative sample of each batch of plates and tubes was examined for sterility by incubating at 37°C for 24 hours for quality control.

3.10.3 Bee honey types preparation

The honey was used *Acacia* honey from two different sources (Bees world AlHashim company, 2017) and (Bees kingdom company, 2017). This honey was

considered as a stock. Serial dilutions of the different honey types were prepared immediately prior to test using sterilized nutrient broth in small screw capped bottles under aseptic conditions. The prepared honey dilutions used were the following: 75% and 25 % (v/v). The 25% (v/v) is 25ml of stock honey was dissolved in 75ml of sterile distilled water**.** And the 75%(v/v) is 75ml of stock honey was dissolved in 25ml of sterile distilled water.

Diluted honey samples were incubated 30 minutes at 37^{o}C in a water bath with shaking intervals, incubation was carried out in the dark because both hydrogen peroxide and glucose oxidase are light sensitive.

For both honey types were collected using centrifugation, the bee hives in west Sudan and bees are feeding on *Acacia* flowers nectar, transported and stored in good conditions, and the physicochemical properties were determined at the National Research Center in the Khartoum State, these including pH, Refractive Index, Moisture (water content%), Free acidity(miliequivalent/kg), Total acidity (miliequivalent/kg), lactone (miliequivalent/kg), Reducing sugars % & Specific gravity. The Results were obtained in the table 3.2.

Type	**pH**	**Refractive index**	**Moisture**	**Free acidity**	**Total acidity**	**Lactone**	**Reducing sugars**	**Specific gravity**
***Acacia* honey AlHashim co.**	5.11	1.5030	13.4	37.00	41.50	8.50	64.38	1.4391
***Acacia* honey bees kingdom co.**	5.31	1.5036	13.2	34.50	39.50	7.00	62.29	1.4274
Reference values	3.4 to 6.1	–	Not more than 20%	Not more than50 meq/kg	Not more than50 meq/kg	–	Not less than 60%	Not less than 1.37

Table 3.2. Physicochemical properties of the Bee honey

3.10.4 Preparation of control antibiotic solutions

Penicillin 1000iu/5ml and (third generation cephalosporin) ceftriaxone 1mg/5ml was diluted immediately prior each susceptibility test using sterile distilled water in sterile 150×16mm test tube, in each time. it was diluted using the formula: RV/O. Honey bee types and the antibiotic solution were stored in the refrigerator at 8^{o}C.

3.10.5 Testing of antibacterial activity of Bee honey and antibiotics on biofilms

All equipment was thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms are sterilized by dry heat (hot air oven), or by steam (autoclave).

After nutrient agar plate was prepared, inoculation of *S.aureus* isolates, overnight incubation at 37 ^{o}C and appearance of typical colonies , the antimicrobial susceptibility testing was done directly by suspending the organisms in nutrient broth in sterile test tubes and compared to the density of a McFarland 0.5 turbidity standard, approximately corresponding to 1.5-2x10^{8} CFU/ml.

Inoculum or bacterial suspension was prepared by touching 2 colonies of similar appearance of purified tested organism, transferred to 3 ml nutrient broth in test tube, and agitated thoroughly.

Turbidity standard was prepared and read by spectrophotometer at 600nm and the reading was 0.1 and the suspension was calibrated to an OD of 0.128-0.175.

50µl of each 57 biofilm forming *S.aureus* were cultivated in sterile U shape 96 well microtitre plates and mixed with 50µl of concentrations of *Acacia* honey from two sources (25% and 75%) and antibiotics (penicillin and ceftriaxone) performed in duplicates. Each microtiter plate included 12 wells without inoculum (negative control), 12 inoculated wells without added honey (positive

control) and at least 12 wells for each 25% and 75% (v/v) diluted honey solution already prepared with nutrient broth incubated for 24 hours at 37 ^{o}C, effects on biofilm were monitored by spectrophotometer to give OD (at 620nm), biomass (by staining with crystal violet).

Planktonic bacteria from each microtiter dish was removed by briskly shaking the dish out over the waste tray. Plates were washed using normal saline with vigorous shake and poured over the waste tray, repeated three times in between each microtiter dish was inverted and vigorously taped with on paper towels to remove any excess liquid. the plates were allowed to air-dry.

100 µl of 0.1% crystal violet solution was added to each well. left for 10min at room temperature.

Each microtiter dish was poured out over the waste tray to remove the crystal violet solution. Dishes were washed successively for three times by normal saline (This step removed any crystal violet that is not specifically staining the adherent bacteria).

Each microtiter dish was inverted and vigorously taped with on paper towels and gauze to remove any excess liquid. Allow the plates to air-dry.

(At this stage, the staining is stable and the dried plates may be stored at room temperature for at least several weeks).

100 µl of 30% acetic acid was added to each stained well (the appropriate solvent).

The dye was allowed to solubilize by covering the plates and incubating 10 to 15 min at room temperature.

Briefly mixed contents of each well by pipetting, and 100 µl of the crystal violet/acetic acid solution was transferred from each well to a separate well in an optically clear sterile flat-bottom 96-well plate. The OD of each of these samples was measured at a wavelength of 620 nm.

Reading of optical density: Using turbidimetry to measure the light blocked as decrease in the light transmitted through the solution dependent on size and concertation of the bacterial particles and uses a spectrophotometer for measurement by adjusting wavelength (Anna and Donald, 2010).

3.10.6 Disposal of cultures

All cultures to be discarded were sterilized even if they were apparently negative. Cultured plates and microtiter plates were collected in autoclavable plastic bags. Disposable containers for discard, such as plastic petri dishes, kept separated from cultures in non-disposable containers such as test tubes, glass bottle, and glass petri dishes to be sterilized and reused.

CHAPTER FOUR

4.Results

4.1 Susceptibility of clinical isolates to penicillin

Out of 57 *S. aureus* 37 (64.9%) isolates were sensitive to penicillin, 4 (7%) were intermediate to penicillin and 16 (28.1%) were resistant, as shown in figure 4.3.

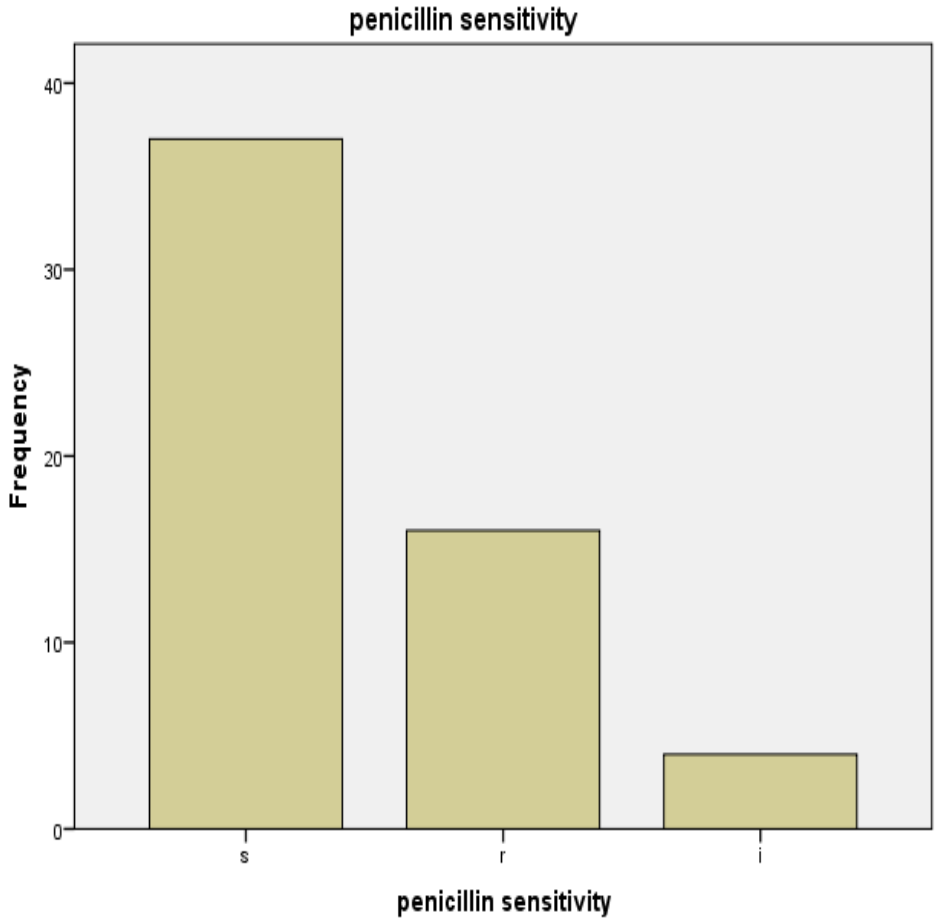

Figure 4.3 Frequency of penicillin sensitivity of *S. aureus* isolates; sensitive, intermediate and resistant

4.2 Susceptibility of clinical isolates to oxacillin antibiotic

There were 51 (89.5%) *MSSA* and 6 (10.5%) *MRSA*, as shown in figure 4.

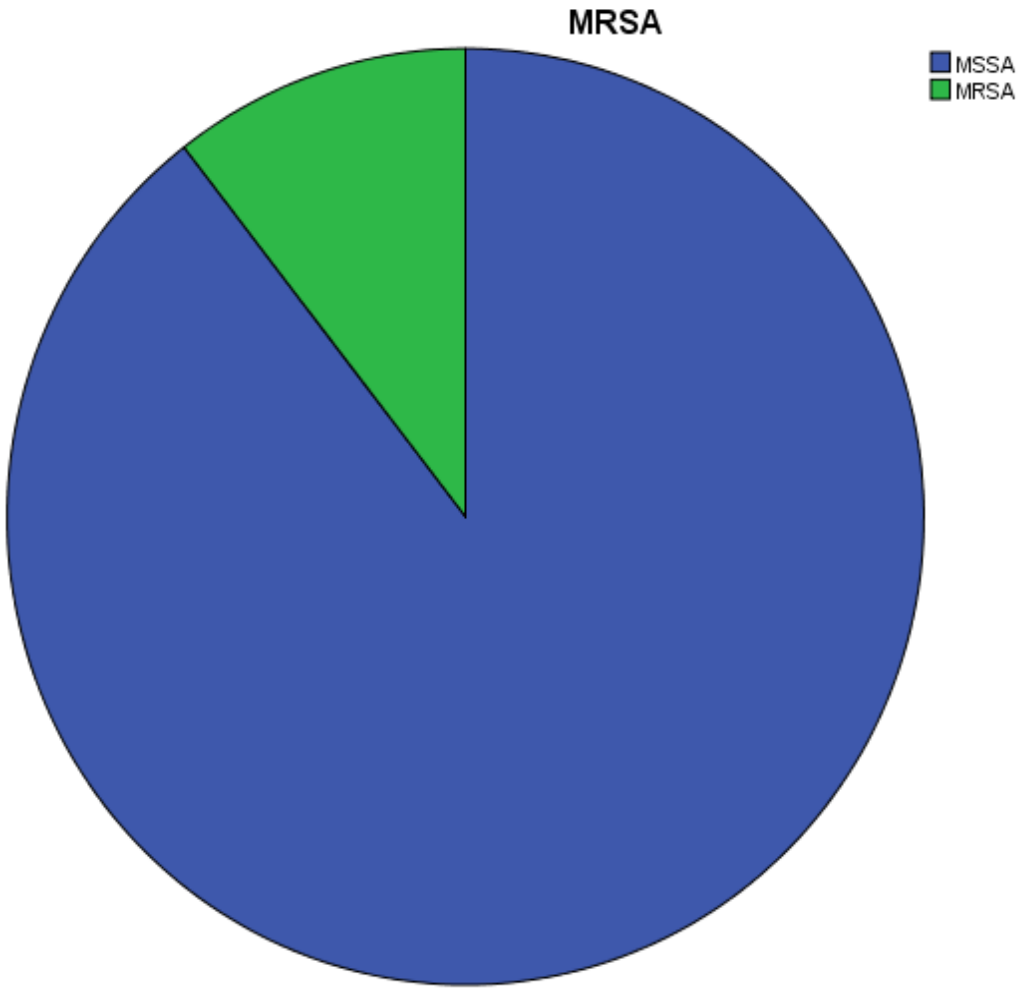

Figure 4.4 frequency of MRSA among *S. aureus* isolates

4.3 Susceptibility of clinical isolates biofilms to honey and antibiotics

Using microdilution method the MIC for *Acacia* honey is 25% diluted honey which is the lowest concentration that affected and inhibited the biofilm formation compared to untreated controls, but the higher concentration 75% diluted honey had a lower effect on the inhibition of biofilm formation. For antibiotics, ceftriaxone inhibited the biofilm formation unlike penicillin which enhanced its formation as shown in the tables 3,4,5,6,7 and 8. *MSSA* and *MRSA* can form biofilms with no difference invitro.

Antimicrobials in biofilm biomass	*p*. value*
ceftriaxone	0.097
Benzyl penicillin	0.658
Hashim honey 25%	0.000
Bees world honey 25%	0.000
Hashim honey 75%	0.075
Bees world honey 75%	0.401

Table 4.3 The effect of Antimicrobials on biofilm formation

* Results show difference between the wells containing bacterial biofilms with antimicrobials & the wells containing bacterial biofilms alone.

Parameter	Mean ± SD
biofilm biomass	0.16 ± 0.17
Hashim 25%	0.14 ± 0.17
Bees world 25%	0.13 ±0.05
Hashim honey75%	0.12 ± 0.03
Bees world honey 75%	0. 13 ±0.04
penicillin	0.65±0.47
ceftriaxone	0.10±0.01

Table 4.4 shows mean ± standard deviation for the bacterial biofilm biomass, inhibition of *S. aureus* biofilm by *Acacia* bee honey and antibiotics.

CHAPTER FIVE

5.1 Discussion

Chronic wounds are costly and difficult to treat, and bacterial biofilms are important contributors to delay in healing. Honey is a promising alternative treatment for these wounds and studies have indicated that is able to prevent bacterial biofilms and eliminate established biofilm invitro (Alandejani,2009). In this laboratory study aiming to investigate the effect of honey on bacterial biofilms isolated from wound swabs, *MSSA* and *MRSA* can both form biofilms and the low concentration of *Acacia* honey 25% was found to affect the formation of *S. aureus* biofilm (MIC) and reduction of biofilm biomass was more than the higher concentration 75% (v/v). The dilution may have enhanced hydrogen peroxide which is produced in honey when glucose is broken down by glucose oxidase to produce gluconic acid and hydrogen peroxide. The release of the hydrogen peroxide has been particularly linked to the antimicrobial activity of some honeys (Molan, 2006). Activity of honey maybe due to low pH, hydrogen peroxide, phenolics, bee-drived enzyme glucose oxidase and methylglyoxal (MGO) and unknown components which will proportionally increase the antibacterial activity (Zmantar *et al.,* 2010).

Claire *et al*., (2015) revealed that *MSSA* clinical strain tested exhibited high levels of biofilm formation in vitro even after short-duration (4 h) incubation. Another study showed that the *MRSA* and *MSSA* can form biofilms with variable masses, Manuka honey was effective at preventing biofilm formation by *S. aureus* at 8% (w/v) concentration compared to untreated controls unlike other types of honey like Medihoney was effective at 16% (w/v) and Clover honey was much less active to affect the biofilm at 32% (w/v). Medihoney was found to prevent the formation of *P. aeruginosa* biofilm, as well as inhibiting and disrupting established biofilms. A lower concentration of Medihoney was

required to prevent the formation of biofilms (Cooper *et al.,* 2014). The effect of 40% (w/v) Manuka honey on the 30 hour - biofilm of a strong biofilm forming *P.aeruginosa* isolated from wound in a continuous culture wound model showed evidence of an inhibitory effect (okhiria, 2009).

Here the isolated biofilms showed susceptibility to ceftriaxone antibiotic, but penicillin showed reduced susceptibility and made isolates more prolific biofilm-formers than the progenitor strains. There were 37 (64.9%) isolates sensitive to penicillin, 4 (7%) intermediate and 16 (28.1%) resistant in agar diffusion. Frequently using of penicillin and self-medication, availability as tablets and injection increased the resistance rates, penicillin is a natural, narrow spectrum antibiotic and ceftriaxone is also can inhibit the cell wall (Andrews, 2004). That disagree with study said the biofilm formation ability of the strain was evaluated in the presence of 12 antibiotics from different families including β-lactams. None induced biofilm formation, with an inverse relation between basal biofilm production and antibiotic-driven biofilm inducibility (Kaplan *et al*., 2012). A few studies have examined the effect of honey on *S.aureus* biofilms showing it to be active, with different antibiofilm activities which are not consistent among these studies due to difference in antibacterial components in the honey that varies according to the floral and geographical source of nectar, storage time and conditions and other possible treatments that may have occurred (Alandejani,2009; Adams *et al*., 2008).

5.2 Conclusion

This study concluded that the concentration of *Acacia* bee honey that affected *S.aureus* forming biofilm invitro was 25%. The other 75% bee honey concentration didn't affect biofilm formation significantly. Also, *MSSA* and *MRSA* can form biofilms invitro.

Interestingly the isolates showed susceptibility to ceftriaxone antibiotic, but for benzyl penicillin the isolates showed reduced susceptibility and made the isolates more prolific biofilm-formers than the progenitor strains.

5.3 Recommendation

Determination of MIC and MBC of honeys on cultures in the microtitre and the continuous culture assays relative to time will provide more information about the effect of honey on biofilms. But other techniques can determine the active components responsible for the antimicrobial activity for example chromatography to extract honey active ingredients. Further advanced techniques of biofilm formation detection are recommended such as colony biofilm assay, kadouri drip-fed biofilm, the air-liquid interface (ALI) assay, assaying esterase activity in biofilms, vitality of biofilm assessed by epifluorescent microscopy & examination of biofilm by scanning electron microscopy.

Furtrher studies are needed to link the QS inhibitory effect of bee honey invitro to the effect of honey in wound using molecular techniques to investigate the effect of bee honey on biofilm with confirmatory in vivo trials to evaluate the antimicrobial activity of bee honey.

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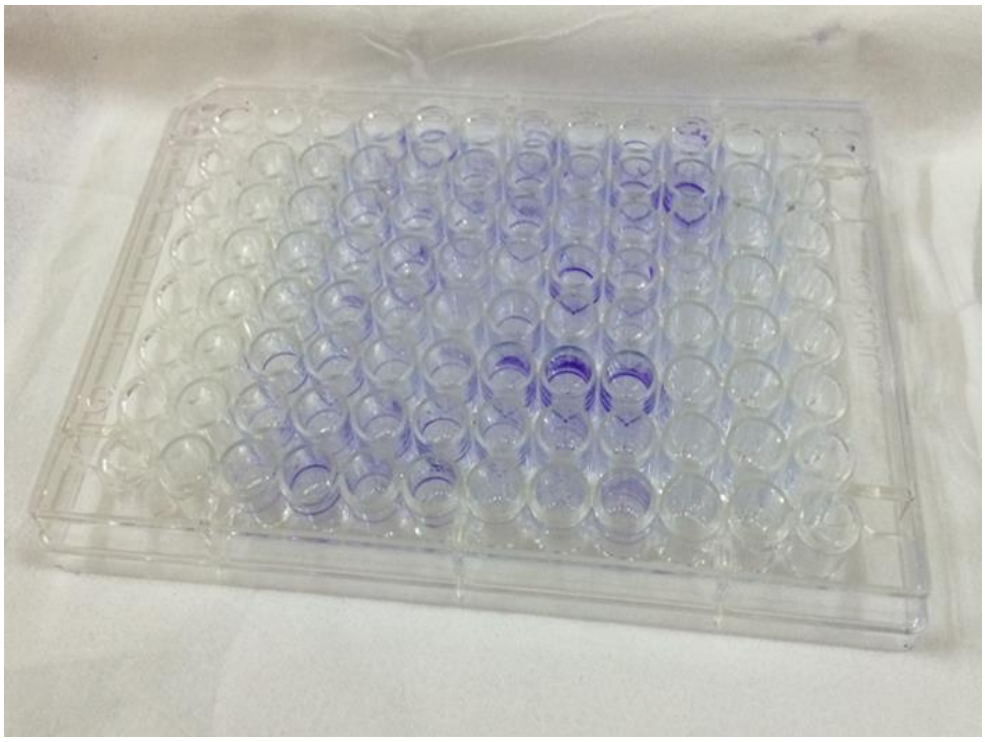
Appendices

Appendix-I

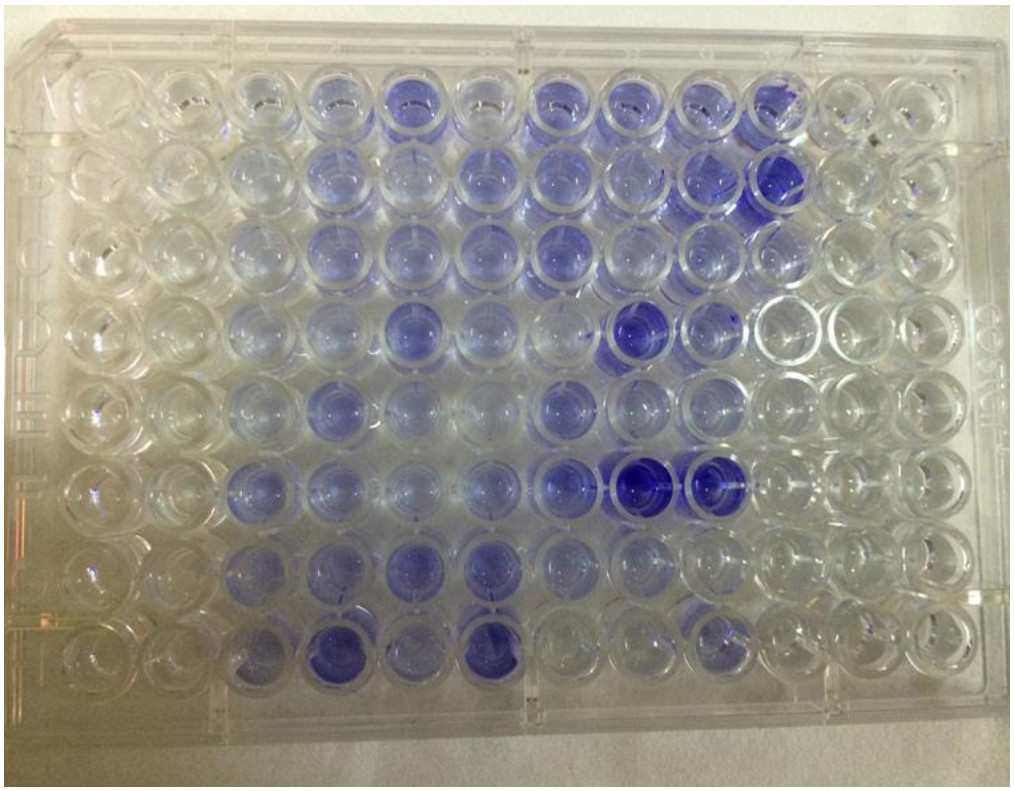
Color plate 1. Addition of diluted honey to microtiter plates

Appendix-II



Color plate 2. The biofilm adhering to the wells

Appendix-III



Color plate 3. The biofilm biomass after staining and addition of acetic acid

Appendix-IV

Materials

A. Equipment

1- Autoclave
2- Hot air oven
3- Incubator
4- Refrigerator
5- Sensitive balance
6- Light microscope with oil immersion lens
7- Wire loops with handle
8- Bunsen burner
9- Rack
10- Antibiotic applicator
11- Water bath
12- Spectrophotometer
13- Ruler
14- Marker
15- Automatic pipette (1000, 100 µl and multichannel pipette)
16- Colorimeter
17- Sterile Microtiter plates (U and flat shape)
18- Distiller for distilled water

B. Glassware

1- Petri dishes
2- Flasks with different sizes
3- Measuring cylinder
4- Beakers
5- Sterile containers

6- Test tubes (large and small)

C. Disposable materials

1- Disposable syringes

2- Universal containers

3- Swabs

4- Tips

Appendix- V

A. Reagents:

1- Crystal violet stain

Crystal violet …….…….... 20g

Ammonium oxalate ……..... 9g

Absolute ethanol or methanol ……..… 95ml

Distilled water ……. 1 liter

Procedure:

- Weight the crystal violet on a piece of clean paper, transferred to a one-liter brown bottle pre marked.
- Add the absolute ethanol or methanol and mix until the dye is completely dissolved.
- Weight the ammonium oxalate and dissolve in in about 200ml of distilled water, add the stain and pour distilled water to 1 liter and mix well.
- Label the bottle and store it at room temperature. The stain is stable for several months.

2- Lugol's iodine solution

Potassium iodine solution …….……. 20g

Iodine ……. 10g

Distilled water ……. 1 liter

Procedure:

- Weight the potassium iodine, transfer to one-liter brown bottle pre marked.
- Add about quarter of the volume of water and mix until the potassium iodine solution is completely dissolved.

- Weight the iodine, add to potassium iodine solution, mix until the iodine is dissolved.
- Add distilled water to 1 liter, mix well, label the bottle and mark as toxic, store in a dark place.

3- Turbidity standard equivalent to 0.5McFarland (barium sulphate)

Concentrated sulphuric acid …….……1ml

Dihydrate barium chloride ($BaCl_2.H_2O$)…..…..0.5g

Distilled water …… 150ml

Procedure:

- Prepare 1%(v/v) solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of distilled water, mix.
- Prepare 1%(w/v) solution of barium chloride by dissolving 0.5g of dihydrate barium chloride in 50ml of distilled water, add 0.6ml of barium chloride to 99.4ml of sulphuric acid solution and mix well.

B. Preparation of media:

1- Blood agar base

Blood agar base is recommended as base to which blood maybe added for use in the isolation and cultivation of fastidious pathogenic microorganisms.

Ingredients for g/l:

Beef heart infusion (beef extract) …….……..5000

Tryptose ……. 10

Sodium chloride …….. 5

pH……..… 7.3

Directions:

Suspend 40g in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cool to 50°C and aseptically add 5% v/v sterile defibrinated blood, mix well and pour into sterile Petri dishes.

2- DNase agar medium

Ingredients for g/l:

Tryptose ……..…… 20

Deoxyribonucleic acid …….. 2

Sodium chloride …… 5

Agar ……...…….. 12

pH…….. 7.2

Directions:

Suspend 3.9g in 1 liter of distilled water, bring to boil to dissolve completely. Sterilize by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cool to 50°C and pour into sterile Petri dishes. Dry the surface of the medium before inoculation.

3- Mac Conkey agar medium

Is a differential medium to distinguish between bacteria by neutral red indicator which changes colour when acid is produced following fermentation of lactose sugar.

Ingredients for g/l:

Peptic digest of animal tissue …… 17

Protease peptone ……..…… 3

Lactose …….. 10

Bile salts …….. 1.5

Sodium chloride …… 5

Neutral red ……0.3

Agar …….. 15

pH at 25°C …… 7.2

Directions:

Suspend 51.53g in 1000ml distilled water, heat to boiling to dissolve the medium completely. Sterile by autoclaving at 15 lbs pressure at 121°C for 15 minutes, mix and pour into sterile Petri dishes.

4- Muller Hinton agar medium

Is used for testing susceptibility of common and rapidly growing bacteria using antimicrobial disc, it is manufactured to contain low level of thymine, thymidine, calcium and magnesium.

Ingredients for g/l:

Casein acid hydrolysate ……..…… 17

Beef heart infusion ……..…… 2

Starch soluble …….. 1.5

Agar …… 17

pH at 25°C ……. 7.3

Directions:

Suspend 38g in 1000ml distilled water, heat to boiling to dissolve the medium completely. Sterile by autoclaving at 15 lbs pressure at 121°C for 15 minutes, mix and pour into sterile Petri dishes.

5- Nutrient agar medium

Is used for cultivation of less fastidious organisms, can be enriched with blood or other biological fluids.

Ingredients for g/l:

Peptone …….. 10

Beef extract …… 10

Sodium chloride ……..…..... 5

Yeast extract ……. 1.5

Agar…… 15

pH at 25°C ……. 7.3

Directions:

Suspend 28g in 1000ml distilled water, heat to boiling to dissolve the medium completely. Sterile by autoclaving at 15 lbs at 121°C for 15 minutes, mix and pour into sterile Petri dishes.

6- Nutrient broth medium

Is used for cultivation of less fastidious organisms and sterility testing, can be enriched with blood or other biological fluids for agar.

Ingredients for g/l:

Peptone ……..….. 5

Beef extract …… 1.5

Sodium chloride …….…..... 5

Yeast extract ……. 1.5

pH at 25°C ……. 7.4

Directions:

Suspend 13g in 1000ml distilled water, heat to boiling to dissolve the medium completely mix and pour into tubes or flasks. Sterile by autoclaving at 15 lbs at 121°C for 15 minutes.