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MEDICAL MICROBIOLOGY MASTER'S PROGRAM

INVESTIGATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *SAUSSUREA COSTUS* ROOT EXTRACTS

GASHA SALIH AHMED

Supervisor: Assoc. Prof. Dr. Umut Safiye ŞAY COŞKUN

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ETHICS CONTRACT

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The Student Who Prepared the Thesis

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ÖZET

SAUSSUREA COSTUS EKSTRAKTLARININ ANTIBAKTERIYEL VE ANTIFUNGAL ETKINLIKLERININ ARAŞTIRILMASI

Gasha S. Ahmed

Yüksek Lisans, Tokat Gaziosmanpaşa Üniversitesi Sağlık ve Fen Bilimleri Enstitüsü Tıbbi Mikrobiyoloji Anabilim Dalı

Tez Danışmanı: Assoc. Prof. Dr. Umut Safiye ŞAY COŞKUN

Bulaşıcı hastalıklar insan yaşamı için ciddi bir tehdit olmuştur ve tüm dünyada önde gelen morbidite ve mortalite nedeni olmaya devam etmektedir. Farmasötik üreticileri son on yılda birçok yeni ilaç üretmiş olsa da, mikroorganizmalar bu ilaçlara dirençli hale gelmekte ve bu nedenle ciddi bir küresel tıbbi kriz yaratmaktadır. Bu, bilim ınsanlarının dikkatini, potansiyel bir yeni ilaç kaynağı olarak bitki türevlerinin ve ekstraktlarının antimikrobiyal özellikleri üzerine araştırmalara çekmiştir. Bu çalışmada, *Saussurea costus (S. costus)* kökünün uçucu yağının, heksan-kloroform, metanolik ve sulu ekstraktlarının *S. aureus, P. aeruginosa, E. coli, S. epidermidis, E. cloacae, E. faecalis, K. pneumonia, A. baumannii, ve C. albicans'a* karşı antibakteriyel ve antifungal etkinlikleri araştırıldı. Bu amaçla disk difüzyon ve mikro dilüsyon duyarlılık testleri uygulandı. Ekstraktların fitokimyasal içeriklerini belirlemek için kimyasal analiz de yapıldı. Sonuçlarımız, *S. costus*'un uçucu yağı ve metanolik ekstraktının en yüksek antimikrobiyal aktivite sahib olduğunu, ardından heksan-kloroform ekstraktının, en düşük aktivite seviyesini gösteren sulu ekstraktın izlediğini gösterdi. Uçucu yağ, 3.12 µl/ml minimum inhibitör konsantrasyon (MIK) değeri ile *S. epidermidis* ve *C. albicans*'a karşı en yüksek aktiviteye sahip olduğu saptandı. Metanolik ekstraktın, 3.12 mg/ml MIK değeri ile *S. aureus*'a karşı en yüksek aktivite gösterdiği tespit edildi. Heksan-kloroform özütü ve sulu özütün ese, 6.25 mg/ml MIK değeriyle *S. aureus*'a karşı en yüksek aktiviteye sahip olduğu görüldü. Genel olarak, test edilen özütler, gram-pozitif bakterilere ve *C. albicans*'a karşı orta ile iyi antimikrobiyal aktiviteye sahipti, ancak test edilen gram-negatif bakterilerin çoğunda özütlere karşı duyarlılık tespit edilmedi. Bu nedenle, *S. costus* kökü, dirençli patojenlerin tedavisi için kullanılabilecek potansiyel bir doğal antimikrobiyal ilaç kaynağı olarak düşünülebilir.

Anahtar Kelimeler: *Saussurea costus; Antibakteriyel aktivite; Antifungal aktivite; Kök; Ekstrakt.*

ABSTRACT

INVESTIGATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *SAUSSUREA COSTUS* ROOT EXTRACTS

Gasha S. Ahmed

Master's program, Tokat Gaziosmanpaşa University, Institute of Health and Science, Department of Medical Microbiology

Thesis supervisor: Assoc. Prof. Dr. Umut Safiye ŞAY COŞKUN

Infectious diseases are a serious danger to public health, and they will continue to be one of the leading causes of mortality and morbidity around the globe, especially due to the ability of microorganisms to develop resistance to available antimicrobial agents. Plants may be a potential source for novel antimicrobial agents. In the current study, the antibacterial and antifungal activity of the essential oil, hexane-chloroform, methanolic, and aqueous extracts of *Saussurea costus (S. costus)* root were evaluated against *S. aureus, P. aeruginosa, S. epidermidis, E. cloacae, E. faecalis, K. pneumonia, A. baumannii, E. coli, and C. albicans*. For this evaluation, disc diffusion and micro-dilution susceptibility assays were performed. Chemical analysis was also performed to determine phytochemical constituents of the extracts. Our results showed that the essential oil and methanolic extract of *S. costus* root exhibited the highest antimicrobial activity, followed by hexane-chloroform extract, with aqueous extract showing the lowest activity. The highest activity with the lowest minimum inhibitory concentration (MIC) value was recorded as 3.12 µl/ml for the essential oil (against *S. epidermidis* and *C. albicans*), 3.12 mg/ml for the methanolic extract (against *S. aureus*), and 6.25 mg/ml for both hexane-chloroform and aqueous extracts (against *S. aureus*). In general, the tested extracts had moderate to good antimicrobial activity against the tested gram-positive bacteria and *C. albicans* as a representative fungal microorganism. However, most tested gram-negative bacteria had week or no susceptibility to the extracts. Hence, *S. costus* root can be considered as a potential natural source of antimicrobial agents to fight the global burden of antimicrobial resistant pathogens.

Keywords: *Saussurea costus*; Antibacterial activity; Antifungal activity; Root; Extract.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
COVID-19	Coronavirus disease-2019
CRAB	Carbapenem-resistant *Acinetobacter baumannii*
DMSO	Dimethyl sulfoxide
EIEC	Enteroinvasive *Escherichia coli*
EMB	Eosin methylene blue agar
EPEC	Enteropathogenic *Escherichia coli*
ESBLs	Extended-spectrum β-lactamases
ETEC	Enterotoxigenic *Escherichia coli*
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MHA	Muller Hinton agar
MHB	Muller Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
S. costus	*Saussurea costus*
SDA	Sabouraud Dextrose agar
STEC	Shiga-like toxin-producing *Escherichia coli*
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *enterococci*

1. INTRODUCTION

Throughout the ages, infectious diseases have been a serious danger to animal and human well-being, and they are a prominent cause of death around the world. Today, increased globalization and ongoing climate change have exacerbated the rapid spread of emerging infectious diseases. The field of infectious diseases is determined to encounter multiple obstacles in the next decades that will need a revolution in mankind's ability to quickly comprehend, develop, and discover novel diagnostic and therapeutic approaches for a vast range of human diseases (Mulat et al., 2019; Ahmad et al., 2009). In addition, the emergence of multidrug-resistant microorganisms has been reported from all over the world, which has created a serious global medical crisis (Nawab et al., 2011). Even though many new antimicrobial agents have been produced over the past decades, microbial resistance is continuously increasing towards different antimicrobial agents. In many cases, resistance emerges only a few years after the introduction of the medication (Njimoh et al., 2015).

Globally, the extracts of medicinal plants have been widely used for therapeutic purposes, and they have been used against many infections that are caused by microorganisms (Gautam et al., 2007). Plants act as a natural source of drugs and are considered to be an alternative to modern medicine, especially in developing countries for various reasons, including their lack of side effects or the presence of only mild ones, ease of availability, and low cost (Nadda et al., 2020; Abdallah et al., 2011). In order to discover and develop new antimicrobial agents, these traditional medicinal plants can be explored for their antimicrobial therapeutic potential.

One of the more important medicinal plants that has been used since ancient times is *Saussurea costus (S. costus), S. costus* is well known in Islamic medicine and has been used since the Islamic civilization era, it is also enlisted in ancient Chinese and Indian medicine (Deabes et al., 2021). The therapeutic value of *S. costus* lies in some active biochemical constituents that produce different physiological effects on the body. These biochemically active substances mainly include flavonoids, alkaloids, sesquiterpenes, phenolic compounds, tannins, carbohydrates, and glycosides (Abdelwahab et al., 2019). Many investigations have reported various therapeutic properties of *S. costus* root, such

as anti-parasitic, anti-inflammatory, wound healing, immuno-stimulatory, antioxidant, hepatoprotective, choleretic, antiulcerogenic, larvicidal, gastro-protective, cytotoxicity, cardiotonic, and anticancer activities (Al Otibi et al., 2020). However, there are currently inadequate research available in the literature regarding the antibacterial and antifungal property of *S. costus*. Hence there exist a gap of information in this area of research. More research is needed to evaluate *S. costus* antibacterial and antifungal activity.

The objective of this study is to evaluate the susceptibility of multiple pathogenic bacteria and fungi to various root extracts of *S. costus*.

2. LITERATURE REVIEW

2.1. Infectious diseases

Microbial infections have resulted in a large burden of illnesses, which are due to pathogenic bacteria, fungi, viruses, or parasites. Infectious diseases can be transferred, indirectly or directly, from an infected patient to a healthy one. Pathogenic microorganisms can destroy normal host cells in order to build a specialized niche that will enhances their survival in the host (Sofowora et al., 2013). Among common pathogenic microorganisms, bacteria are the most prevalent pathogens, which are responsible for many health defects and many opportunistic illnesses in immunocompromised individuals (Rathee et al., 2016). Similarly, fungal infections have become a major source of health problems, particularly in the hospital environment (Dean & Burchard, 1996).

Throughout the ages, infectious diseases have been a serious danger to animal and human well-being, and they are one of the leading causes of morbidity and death around the globe. Every year, 15% of all the deaths in the world are linked to infectious diseases (Eckhardt et al., 2020). The efficiency of improved antibiotics, vaccination, and sanitation programs in the 1960s led to a notion that infectious diseases would soon be eradicated. But instead, these diseases have remained major causes of suffering throughout the world. Moreover, microorganisms have adapted and evolved continuously; as a result, new infectious diseases have emerged (Hethcote, 2000). Today, the spread of new infectious diseases, worsened by globalization, has put a large portion of the population at risk of dangerous chronic or acute illnesses. Hence, the field of infectious diseases is determined to encounter multiple obstacles in the next decades that will need a revolution in mankind's ability to quickly comprehend, develop, and discover new diagnostic and curative therapies for a vast range of human conditions (Eckhardt et al., 2020; Hethcote, 2000).

2.2. Antimicrobial agents

Antimicrobials are defined as substances that control or eliminate the number of microorganisms via multiple methods, such as inhibition of cell wall synthesis, cell

membrane function inhibition, and inhibition of nucleic acid and protein synthesis (Kalayou et al., 2012). Figure 2.1 shows the main molecular targets of natural antimicrobial products. These substances have the ability to interact with the growth and reproduction of a vast range of microorganisms, such as bacteria, fungi, parasites, and viruses (Fane et al., 2017). These agents can have inhibitory or lethal effects even at low concentrations by suppressing or inhibiting the growth of microorganisms. Antimicrobials can be naturally produced by plants, molds, yeasts, and other microorganisms, or they may be formed by chemically modifying natural substances. In addition, there are entirely chemically synthesized agents that are chemically designed in the laboratory. Furthermore, some minerals such as silver, bromine, copper, etc., can have antimicrobial properties when present in high concentrations (Leisner, 2020).

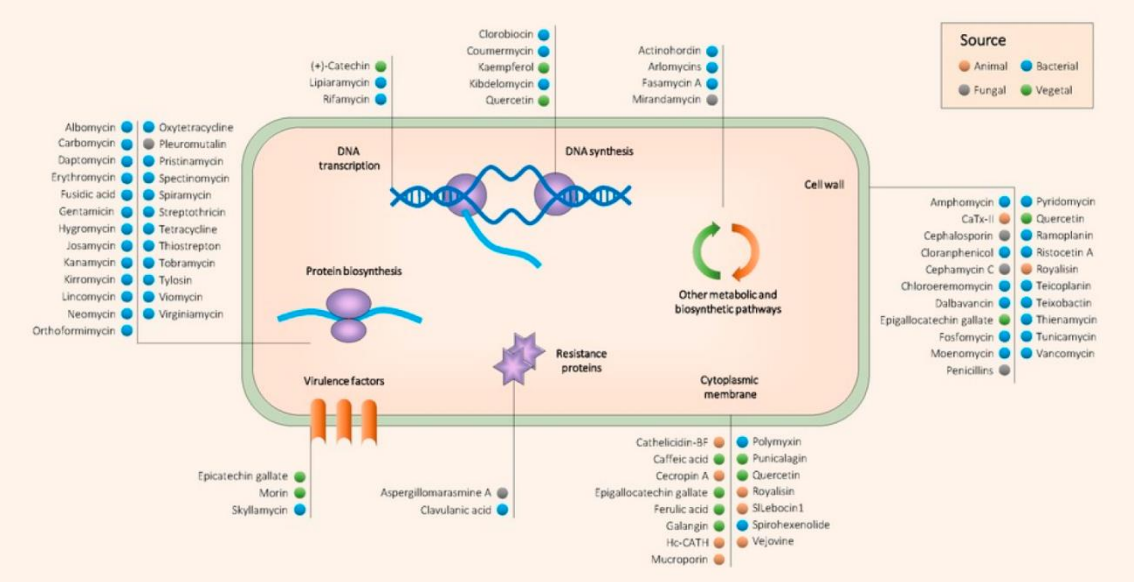

Figure 2.1. Natural antimicrobial products' main molecular targets (Álvarez-Martínez et al, 2020).

2.2.1. History of antimicrobial agents

When dated back, infectious diseases accounted for a huge proportion of human diseases as a whole. In the last half of the nineteenth century, microorganisms were first regarded as the causative agents of the infectious diseases that have been threatening

human life for millennia. Since then, scientists have begun to discover and observe effective antimicrobial agents against those pathogenic microorganisms. Salvarsan was reported as the world's first antimicrobial substance which was used as a treatment for syphilis, it was discovered in 1910 by Ehrlich (Saga and Yamaguchi, 2009). Following this, in 1928, Alexander Fleming's discovery of penicillin was considered a milestone. He noticed that a clear zone around a blue mold culture (Penicillium genus) was formed surrounded by *Staphylococcus aureus* colonies. Upon examination, he observed that the mold inhibited the growth of *Staphylococcus aureus*. Thus, he believed that a microorganism could produce agents that inhibit the growth of another microorganism. After that, this antibiotic was called penicillin, which would later become one of the world's most important antimicrobial drug. The discovery of penicillin led to the introduction of new antibiotics that significantly reduced the number of deaths from infectious diseases (Alharbi et al., 2014).

For the next twenty years, novel antimicrobial agents were introduced one after another, including streptomycin's discovery, which was the first discovered aminoglycoside antibiotic. Moreover, chloramphenicol, macrolides, tetracyclines, and glycopeptides (such as vancomycin) were isolated from soil bacteria and introduced into the pharmaceutical markets. In 1962, a synthetic antimicrobial substance called nalidixic acid, which is the first quinolone antibiotic, was discovered. Afterwards, studies and research have gained importance in improving each class of antimicrobial medications, leading to a broader antimicrobial spectrum. ß-lactams can be a good example (Lewis, 2013; Saga & Yamaguchi, 2009). The discovery of new antibiotic classes and the identification of microbial resistance are shown in Figure 2.2.

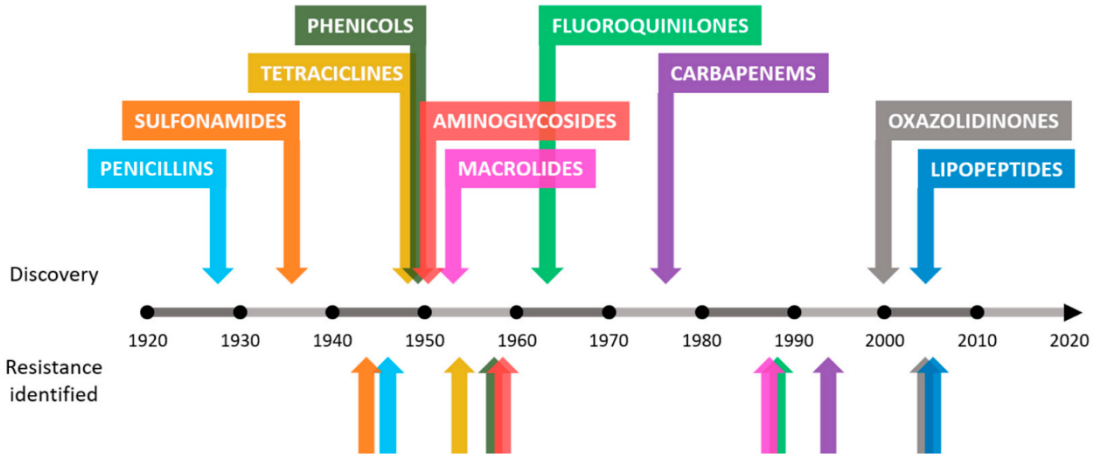

Figure 2.2. Discovery of new antibiotic classes and the identification of microbial resistance (Álvarez-Martínez et al, 2020).

2.3. Antimicrobial resistant microorganisms

The ability of bacteria to develop resistance to antimicrobial agents has become an unignorable problem in the therapy and control of diseases and has become a global problem (Greenwood et al., 2009). These drug resistant microorganisms have complicated the management of infections in many conditions, such as in cancer, AIDS, and immunocompromised patients, and particularly in the setting of hospital acquired infections (McGaw et al., 2001). From the discovery of antimicrobial agents for the management of infectious diseases, it has been reported that the human life expectancy has increased by approximately 10 years. The ability to treat previously fatal infectious diseases has contributed to this miraculous story of success in the prolongation of life **(**Alexander & Perfect, 1997). However, it soon became clear that it was highly improbable for pathogenic germs to surrender themselves unconditionally, as some bacteria quickly showed resistance to a variety of the already discovered effective medications (Cowan, 1999).

Various bacterial species are naturally resistant to certain antibiotic classes, either because their cell walls are impermeable to the drug or because they lack the necessary receptors. However, some other bacteria may acquire resistance genes at some point in their lives (Andersson, 2003). This is because antibiotic-resistant genes can be transferred

among different bacteria. Extra-chromosomal genes have been shown to be responsible for phenotypes of antimicrobial resistance, which may confer resistance to a whole class of antimicrobial. These antimicrobial resistance genes have been found to be linked to plasmids, which are large extra-chromosomal DNA that can be transferred. Other DNA mobile elements, such as integrons and transposons, can be found on plasmids. These mobile DNA elements transfer genetic materials responsible for the antimicrobial resistance characteristics, potentially leading to the rapid spread of drug resistance genes amongst different bacterial cells (Toutou et al., 2004). Furthermore, due to indiscriminate use of antimicrobial medications and microbial gene mutation, pathogenic microorganisms have gained resistance to many antimicrobial agents and have produced immense clinical issues in the management of infectious diseases (Davis, 1994). Resistance acquisition mechanisms and antimicrobial resistance mechanisms in bacteria are shown in Figure 2.3.

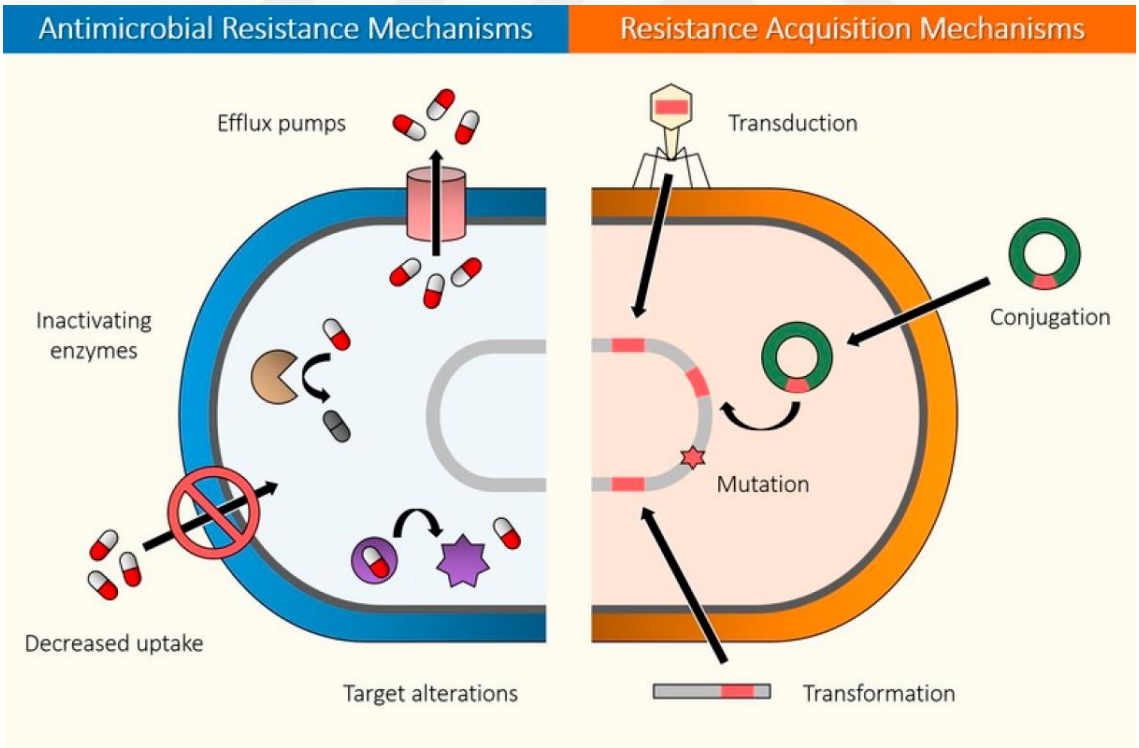

Figure 2.3. Resistance acquisition mechanisms and antimicrobial resistance mechanisms in bacteria (Álvarez-Martínez et al, 2020).

The most significant antimicrobial resistant pathogens amongst gram-positive bacteria are vancomycin-resistant *enterococci* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). And significant gram-negative resistance bacteria are extended-spectrum β-lactamases (ESBLs) in *Acinetobacter baumannii*, *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli* (Jones, 2001). Similarly, problems with drug resistance in fungi are now becoming a common issue in fungal infections **(**Alexander & Perfect, 1997). For example, invasive fungal diseases, which are mostly caused by some *Aspergillus sp. and Candida sp.,* are important causes of mortality and morbidity after hematopoietic organ transplantation (Schwartz & Patterson, 2018). Advances in prevention and treatment of invasive fungal infections post transplantation has has been made with good outcomes, however, the emergence of antifungal resistance amongst known fungal pathogens has complicated the situation (Perlin et al., 2017).

In recent times, the emergence of multidrug resistance in microorganisms has been reported from all over the world. Even though pharmaceutical manufacturers have produced many new antibiotics over the past decades, the ever-growing resistance to the available effective medications has become a major problem for medical society worldwide. The prevalence of antibiotic resistance is shown in Figure 2.4. Global warming, environmental degradation, humanity's invasions of new ecosystems, changes in economic patterns, and increased international travel will continue to provide opportunities for newly emerging and already existing infectious diseases that could be hard to treat (Hethcote, 2000). In addition, the overuse of antibiotics today can lead to the selection of antibiotic-resistant microbial strains in the future. Consequently, the effectiveness of many medications, which have been used frequently in the past as empiric treatments for infectious diseases, has decreased against nosocomial pathogens (Jones, 2001). It has been estimated that, over the last four decades, there has been an increase of about two-folds in microbial resistance to antimicrobials, in both the livestock and the medical sectors (Daboor & Haroon, 2012). The increase in microbial resistance has emerged to be a worldwide concern, hence highlighting the necessity to find novel antimicrobial medications that not only work on antimicrobial-resistant pathogens, but also eliminate persistent microorganisms and decrease the treatment length, as lengthy therapy is associated with toxicity and also produce poor compliance of patients (Mariita et al., 2010).

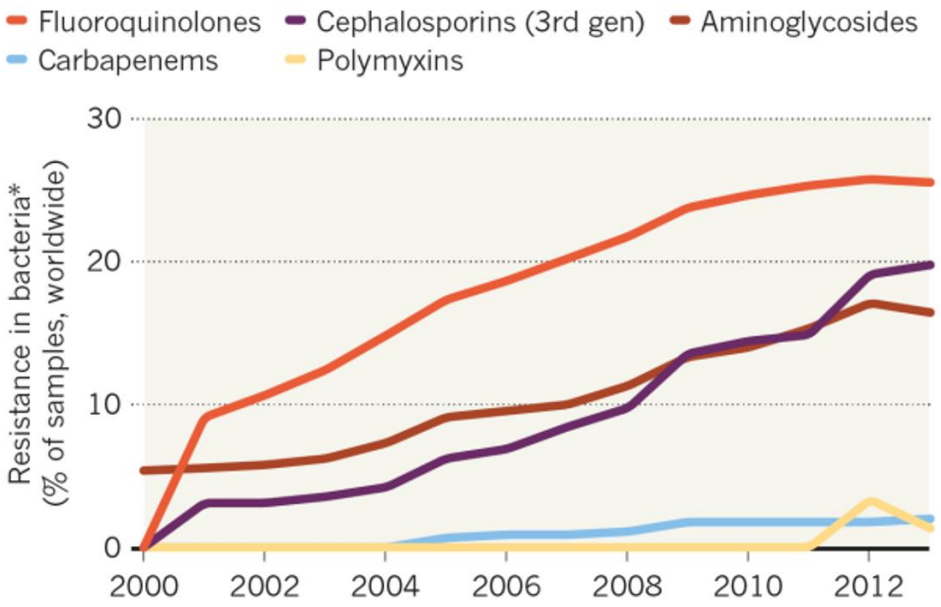

Figure 2.4. The prevalence of antibiotic resistance (Reardon, 2015).

2.3.1. Antimicrobial resistance and its socio-economic impact

Due to the significant reduction in mortality rates achieved through antimicrobial therapies, millions of human lives have been saved. However, the efficiency of most antimicrobials is being threatened by increasing resistance in microorganisms. The misuse and over-use of medications may cause the selection of antimicrobial-resistant bacteria and fungi, which has made infectious diseases more difficult to treat and has raised the mortality and morbidity of previously treatable infections **(**Paphitou, 2013; Byarugaba, 2004). The current global leading causes of death and prediction for antimicrobial resistance related deaths by 2050 are shown in Figure 2.5. Additionally, there is evidence proving that genes of antibiotic resistance can be horizontally transferred among different strains of bacteria, which their control is nearly impossible (Leisner, 2020). Furthermore, producing even very deadly bactericidal agents is no longer an applicable choice, financially and clinically. As the powerful medications that are toxic to bacterial cells are often toxic to humans as well and the adverse side-effects might be quite worse than the recovery.

Infections by antimicrobial resistant microorganisms are more inclined to require more toxic and expensive drugs, increase the risk of death, and prolong hospitalization

(Graf & Martin, 2000). Hence, as multidrug resistant microorganisms (Superbugs) become more prevalent, additional costs of therapy will become a bigger threat to the national, regional, and local health care systems (DiazGranados et al., 2008). The pharmaceutical industries are struggling to keep up with antimicrobial resistance microorganisms, and bringing news and more effective medications to markets has become difficult (Graf & Martin, 2000). This has increased the need to search for a new, cheaper, and safer antimicrobials as an alternative to the older medications, in order to give a proper and effective drug to patients.

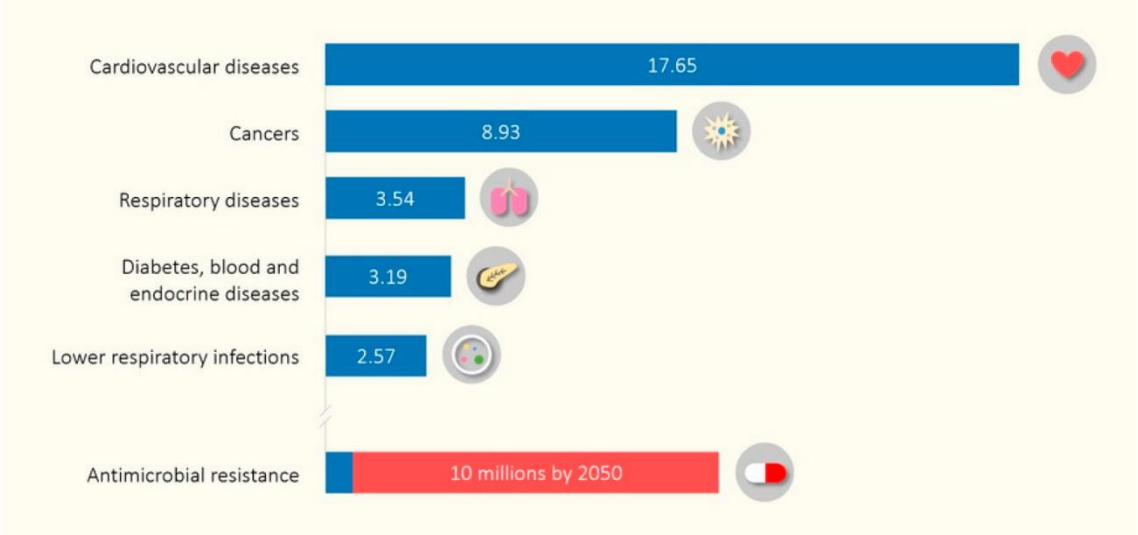

Figure 2.5. Current global leading causes of death and prediction for antimicrobial resistance related deaths by 2050 (Álvarez-Martínez et al, 2020).

2.4. Medicinal plants

A medicinal plant is a plant that can be utilized for medical purposes, as one or multiple parts contain substances that have therapeutic potential or are precursors for the synthesis of effective medications (Sofowora et al., 2013). Plants have existed before mankind; innately, they are the first source utilized for their medicinal benefits. Historically, the etiological agents that caused diseases were mostly unknown to human beings; therefore, the process of trial and error of medicinal plants usage increased, eventually leading to the introduction of effective medicine from plants. The subsequent information from this trial and error formed the foundation of recent medicine (Petrovska,

2012). In the past, plants have been a precious source of natural products for staying healthy, many of them are based on their uses in traditional herbal medicine. In many regions of the world, herbal medicines are still considered as the first choice of treatment, because traditional medications readily fit with the socio-cultural life of those populations (Musa *et al*., 2011). In most part of the third world, medicinal plants have been observed to be utilized as a normative basis for maintaining health (Sofowora et al., 2013). Furthermore, medicinal herbs are used by nearly 80 percent of the world, because herbal medications are cheap, readily available, and most importantly, has the potential to introduce new templates for new medicines (Shazadi et al., 2010). Among the recognized plant species (estimated 250,000–500,000), only a small part has been studied pharmacologically and chemically for the presence of antimicrobial molecules. Currently, it is estimated that more than half of all new clinical medications in many countries are either natural products or their derivatives (Hosseinzadeh et al., 2015).

2.4.1. Phytochemicals in medicinal plants

Medicinal plants are the natural source of compounds that may be used to treat many conditions. Phytochemicals are necessary components of plant metabolism, and they are also called secondary metabolites (Rates, 2001). There are approximately 350.000 different species of plants currently growing in the world, and it is predicted that a single species contains more than 5000 distinct chemical substances. Plants might have evolved these substances as a self-defense against pathogens and pests, and aid plants to become established in their environment. Plants contain a wide range of these chemicals that have significant effect on human health (Raskin *et al.,* 2002). About one hundred thousand likely secondary metabolites have been identified and categorized, many of which have been applied as active components in modern medicine. Chemicals such as phenolic compounds, flavonoids, tannins, alkaloids, glycosides, berberine, terpenoids, and quinine, in which they are all produced by plants, and are commonly used to treat human diseases (Rates, 2001).

2.4.2. Drug derivative from medicinal plants

Since the dawn of civilization, society have developed a huge interest in drugs derived from plants (Shazadi et al., 2010). Medications derived from plants have been a part of traditional health care for ages in most regions of the world. They have also been

used for the management of various human conditions over the centuries (Oluma et al., 2004). Currently, over 120 active ingredients are vastly used in modern medicine that are extracted from higher plants, and 80% of these active substances show a positive correlation between their modern clinical application and their traditional use (Fabricant & Farnsworth, 2001). Modern microbiological techniques demonstrate that plant products play a significant role in the health care system and drug production, because higher plants often possess significant potency against pathogenic fungi and bacteria. Secondary metabolites of plants comprise a significant bioactive resource for researching beneficial medications. Secondary metabolites are used by plants to safeguard themselves from viruses, fungi, and bacteria; thus, they can be used in nearly the same way for medical purposes to manage infectious diseases (Wink, 2008).

Bioactive components extracted from roots, leaves, flowers, and other parts of plants have shown antimicrobial activity against many bacteria and fungi. These plant antimicrobial substances inhibit microbial growth via various mechanisms, and they might have an important clinical value in the management of resistant microbial strains (Shankar et al., 2010). A crucial property of plant extracts and their constituents is the hydrophobicity, that allows them to split the cell membrane lipids of bacteria, thus disrupting the structure of the cell and increasing their permeability (Sikkema *et al.*, 1994). In addition, extracts acquired from multiple plants have been shown to have many important biological activities. Furthermore, the active ingredients of many synthetic medications, are structural analogs of substances isolated from plants. Thus, herbal drugs have been used to manage a vast spectrum of conditions, and their antimicrobial characteristics would make them a potential source of potent medications (Srivastava et al., 2005).

In previous decades, many microorganisms have shown increasing resistance against currently available antimicrobials; and they have created a problem in the management of infectious diseases (Cunha, 2001). This has drawn the attention of pharmaceutical manufacturers and scientific societies toward research on the antimicrobial properties of plant derivatives and extracts in order to use them as a source of novel antimicrobial agents, and as an alternative to the existing drugs for the treatment of infectious diseases (Romulo 2018). Herbal medications have some advantages over synthetic medications such as; better patient tolerance, relatively less expensive (Mulat et

al., 2019), and adverse side effects associated with synthetic drugs are usually less in herbal medications (Cunha, 2001). In addition, the use of medicinal herbs has always played an important role in disease control and prevention, such as heart conditions, diabetes, stomach problems, intestinal disorders, and many cancers (Mohanta et al., 2003). Other activities such as analgesic, antiallergic, antihypertensive, antioxidant, anti-inflammatory, antiulcer, antifungal, antiviral, antibacterial, and anti-malarial activities have also been widely described (Mulat et al., 2019; Sofowora et al., 2013). Therefore, there is a growing interest in medicinal plants as a powerful source to fight diseases.

2.5. *Saussurea costus*

One of the more important medicinal plants that are used in many medicines all over the world is *Saussurea costus* (*S. costus*), which is also referred to in the literature as *Saussurea lappa, Aucklandia lappa,* and *Aucklandia costus* (Wei et al., 2014). The plant is commonly referred to as Mu Xiang in China, Kuth in India, and Udi Hindi in Turkey (Nadda et al., 2020). In Arab countries, it is known as Al-Kost Al-Hindi, Al-Kust, and Al-Qust (Saif-Al-Islam, 2020). *S. costus* is a potential herb that is a member of the Asteraceae family (Kamalpreet et al., 2019). The Asteraceae family is one of the major flowering plants globally, that contains nearly 1000 genera and 30,000 species (Butola & Samant, 2010). *S. costus* is considered the most commercially feasible species amongst all other species of the *Saussurea* genus, and it is also well known for its therapeutic uses in various indigenous medicinal systems (Nadda et al., 2020). Over the years, in both traditional and conventional medication systems, *S. costus* has been found to offer a variety of theraputic potentials (Pandey et al., 2017).

2.5.1. Geographical distribution

S. costus is indigenous to India, China, and Pakistan. It grows primarily in the Himalayan area at an altitude of 2500–3500 meters (Gwari et al., 2013). In addition, *S. costus* has a wide distribution throughout Asia, various parts of Europe, and North America It typically grows in moist habitats, and also grows in arctic regions and alpine habitats (Semwal et al., 2020; Kamalpreetet al., 2019).

2.5.2. Morphological description of *S. costus*

S. costus is a tall, perennial plant that reaches a height of 1 to 2 meters; its size varies based on the altitude at which it grows (Semwal et al., 2020). The stem of the plant is stout, upright, and fibrous, meanwhile the root is long and stout, measuring about 60 centimeters in length, and has a strong, distinctive odor. The fresh roots are grey to light yellow in color; while the dried ones are brown or dull-red, generally ridged and wrinkled, and tastes slightly bitter (Madhavi et al., 2012; Kamalpreet et al., 2019). Figure 2.6 shows the dried root of *S. costus*. Leaves are membranous, lobate, stalked, and irregularly toothed; the basal leaves are large in size, and have long and lobately winged stalks, while the upper leaves are small. Flowers are stalkless and bluish-purple to black in color; they appear in clusters of 2-5 and are borne either axially or terminally on the leaves (Madhavi et al., 2012). And the fruit of *S. costus* is cupped, compressed, curved, and hairy (Pandey et al., 2017). Seeds of *S. costus* maintain their viability for more than a year. Naturally, the seeds shed in autumn, lie under the snow in winter and begin to sprout in spring (Madhavi et al., 2012).

Figure 2.6. The dried root of *S. costus.*

2.5.3. Chemical constituents of *S. costus*

A variety of biologically active chemical ingredients have been isolated from the root of *S. costus*. *S. costus extracts,* such as hexane, methanolic, and petroleum extracts, have been examined by some researchers for their phytochemical compositions (Madhuri

et al., 2012). In the chemical assessment of *S. costus* root, resins, reducing sugars, alkaloids, and tannins were found (Pandey et al., 2017). The odorous components isolated from the root were: salt of valeric acid, tannin, manganese (Madhavi et al., 2012). Additionally, other chemicals like anthraquinones, flavonoids, terpenes, and lignans were also found in the extract (Wei et al., 2014). Various terpenes isolated from *S. costus* oil include; Costunolide, Dihydrocostunolides, Dihydrocostus lactone, α-cycloCostunolide, 12-methoxy dihydro costus lactone, β-hydroxyDehydrocostus lactone, β-cycloCostunolide, Lappadilactones, Betulinic acid methyl ester, Betulinic acid, Cynaropicrin, Reynosin, Alantolactone, Isoalantolactone, Sassureamine A-E, Iso-dihydrocostunolide, 1β-hydroxy arbusculin A, Arbusculin B, Sesquiterpenoides, 22-dihydro stigmasterol and Shikokiols. (Zahara et al., 2014; Nadda et al., 2020). Meanwhile, in other investigations, the quantity of all of these chemicals may be vastly different. Differences in the chemical composition of *S. costus* extract could be attributable to a variety of causes related to phenophases, ecotype, chemotype, and differences in environmental factors, including relative humidity, photoperiod, irradiance, and temperature, may result in varying chemical constituents. In addition, genetic background may influence the chemical properties of secondary metabolites in plants (Zahara et al., 2014).

2.5.4. Traditional use of *S. costus*

S. costus has been known for over 2500 years and has been widely used based on many historical medical systems, including Unani, Ayurveda, and Siddha (Zahara et al., 2014). The therapeutic effects of *S. costus* are also described in traditional Tibetan and Chinese systems of medicine (Nadda et al., 2020). Furthermore, *S. costus* is well documented in Islamic medicine, which is mentioned in the Holy Al-hadith said by Prophet Muhammad (PBH) and used by traditional practitioners from the era of Islamic civilization (Saif-Al-Islam, 2020).

In the southern part of India, the roots have been used for asthma, toothache, dysentery, rheumatism, and skin diseases (Nadda et al., 2020). The root is mostly used in Ayurvedic medicine to treat leukoderma, ringworm, arthritis, stomachaches, and itching (Ashwinia et al., 2019). *S. costus* was used for anthelmintic, aphrodisiac, carminative, brain-stimulating, kidney diseases, and liver diseases in the Unani medicinal system

(Madhuri et al., 2012). This plant is an important medicine for erysipelas, gout disease, and promotes spermatogenesis. Furthermore, the root and its powder have been used traditionally for a variety of health issues such as: gastritis, diarrhea, ulcers, allergies, asthma, inflammation, cough and throat infections, paralysis, epilepsy, scalp scabies, typhoid, joint pain, and blood-related disorders (Zahara et al., 2014; Rana et al., 2014).

In ancient times, people applied root paste of *S. costus* on areas of inflammation; also, the oil derived from the dried roots was used on joints to ease discomfort (Zahara et al., 2014). Seeds of *Carum carvi* and floral parts of *S. costus* were grounded and mixed with desighee (butter) in equal portions and mildly heated to make a thick paste. This paste was applied twice a day to the external parts of the ears to cure earaches for 2 to 3 days. Furthermore, *S. costus* roots were grounded with water to make a paste, which was applied to the skin to treat blisters and boils; it was also used to treat leprosy for 7 to 8 days. Furthermore, the powdered roots are traditionally used as insecticides and are sprinkled over crops. It is sometimes used as a hair shampoo to eliminate lice and blacken grey hair. *S. costus was* also used for ethnoveterinary purposes as it was used to cure heart diseases in cattle (Zahara et al., 2014).

2.5.5. Safety and toxicity study of *S. costus*

People all over the globe use *S. costus* to treat a wide range of health issues. Hence, the assessment of safety and toxicity of *S. costus* parts and their components are of significant concern, because there is little information available regarding their side effect and safety profile. Generally, *S. costus* root might be safe to consume when taken correctly. However, it may contain aristolochic acid as a contaminant, which may have carcinogenic effects and nephrotoxic effects ((Nadda et al., 2020; Mujammami, 2020). An allergic reaction may be induced by *S. costus* in people who are allergic to the plant and its compositions, such as the sesquiterpene lactones; contact dermatitis has been observed in those exposed to sesquiterpene lactones. In addition, the safety of *S. costus* in breast-feeding and pregnant women has not been proven yet (Mujammami, 2020).

As an aspect of the toxicity assessment, Garg and colleagues investigated the toxicity of *S. costus* extract. A four different solvent mixture were used to create an extract, which were chloroform, water, petroleum ether, and ethanol. The extract of *S. costus* were administered to healthy adult albino mice of body weight 20–25g; the

mortality percentages were observed to be 0%, 0%, 33.33%, 66.66%, 100%, and 100%, respectively. At up to a dose of 250 mg/kg body weight, the extracts did not cause any toxic symptoms of mortality in mice (Garg et al., 2016). In an acute toxicity evaluation, eight mice were given 1 mL of *S. costus* water extract for seven days, and no toxicity effect was observed (Sen & Chakraborty, 2020).

2.5.6. Pharmacology and clinical properties of *S. Costus*

From *S. costus* extracts, a diverse range of biologically active constituents have been isolated. However, the most important active constituents responsible for various pharmacological activities are flavonoids and sesquiterpenes (Hassan & Masoodi, 2020). *S. costus* is a medicinally valuable plant, many active constituents extracted from *S. costus* are reported to possess therapeutic benefits (Zahara et al., 2014). Thus, it has been tested for a variety of pharmacological properties. Various activities of *S. costus* have been shown such as; angiogenesis, antiarthritic, anti-convulsant, anticancer, anti-inflammatory, hepatoprotective activities, etc., by using various appropriate in-vivo and in-vitro models (Madhuri et al., 2012; Zahara et al., 2014).

2.5.6.1. Anticancer effect

S. costus root has been reported to possess anti-cancer effects against a variety of cancers, such as ovarian cancer, lung cancer, colon cancer, and some other forms of carcinoma (Semwal et al., 2020). The hexane extracts of *S. costus* root apoptosis in androgen-insensitive human prostate cells (Kim et al., 2008). Also, the extracts stopped the migration of prostate cancer cells (Kim et al., 2012). In their study, Tian et al. showed that the anticancer property of *S. costus* extracts is mediated by autophagy and apoptosis regulation in cells of prostate cancer (Tian et al., 2017). In human oral cancer cells, the root of *S. costus* have been found to inhibit propagation of cancerous cell (Moon et al., 2013). Ko and associates demonstrated that an ethanolic extract of *S. costus* root have effect on stomach cancerous cells and has healing properties either alone or in combination with modern chemical medications (Ko et al., 2004). Furthermore, according to Choi et al., *S. costus* and its derivatives costunolide, inhibit breast cancer growth and metastasis in mice. Hence, they stated that *S. costus* might be a potential anticancer drug (Choi et al., 2013).

2.5.6.2. Anti-inflammatory properties

The various bioactive substances obtained from *S. costus* have an anti-inflammatory effect in humans and animals (Kamalpreet et al., 2019). Various compounds present in *S. costus* have the potential to assist pharmaceutical industries in the development of medicines to treat skin inflammation, such as costunolide, costic acid, and alantolactone (Lim et al., 2015). Damre et al. evaluated the anti-inflammatory effect of sesquiterpene lactone isolated from *S. costus* root*;* they used a cotton pellet granuloma assay in rats, and it was noticed to be effective in inflammation (Damre et al., 2003). Gokhale et al. assessed the role of ethanolic extracts of *S. costus* root in chronic and acute inflammation. The findings of that study showed that the extract has significant anti-inflammatory activity in animal models at various doses (Gokhale et al., 2002). Moreover, it was found that methanolic extract of *S. costus* inhibits approximately half of the inflammation-inducing factors (Lee et al 1995).

2.5.6.3. Hepatoprotective

S. costus has been investigated in several scientific studies for its hepatoprotective effectivity (Nadda et al., 2020). In a study by Chen et al., dehydrocostus lactone and costunolide, isolated from *S. costus,* showed an inhibitory effect on human hepatocellular carcinoma (Chen et al., 1994). In a study, the anti-hepatotoxic activity of *S. costus* extract was investigated by Yaeesh et al.; based on their findings, they concluded that an aqueous-methanolic extract of *S. costus* root has hepatoprotective properties; it kept the levels of liver enzymes in the blood from rising in a dose-dependent manner, and it considerably slowed the progression of hepatic damage in mice (Yaeesh et al., 2010).

2.5.6.4. Gastro-protective and anti-ulcer effects

S. costus extracts have anti-ulcer activity and significantly reduce the risk of gastric ulceration (Madhavi et al., 2012). In a study by Yoshikawa et al., sesquiterpene isolated from *S. costus* root had an anti-ulcer activity in rats and inhibited the formation of stress-induced ulcer (Yoshikawa et al., 1993). Dehydrocostus lactone and costunolide were also reported to have gastroprotective effects in rats with gastric mucosal lesions induced by acidified ethanol (Matsuda et al., 2000).

2.5.6.5. Respiratory inflammation and asthma

Based on the traditional utilization of *S. costus*, its roots are useful for the treatment of throat infections, colds, and coughs. The root of *S. costus* has been studied for its ability to treat asthma and chronic bronchitis. Lee et al. investigated the respiratory effects of sesquiterpene lactones isolated from *S. costus* root against antigen-induced degranulation in rats and in an ovalbumin-induced allergic asthma murine model. The study's findings reveled that sesquiterpene lactones reduced mucus production and lung inflammation, and limited Th2 cytokines expression and secretion in the lung tissues and bronchoalveolar lavage fluid. Thus, they concluded that these compounds have the potential to be anti-allergic medications; the most potent phytochemical was dehydrocostuslactone, followed by costunolide and alantolactone (Lee et al., 2018).

2.5.6.6. Antidiarrheal activity

Traditionally, *S. costus* has been used to treat dysentery and abdominal pain. Today, many people in the third world depend on herbal medications to cure diarrhea. In Wistar rats, a methanol extract of *S. costus* roots was examined for antidiarrheal efficacy. It was found that the administration of 100mg, 300mg, and 500 mg/kg body weight doses showed diarrheal inhibition of 26.33%, 32.28%, and 66.77%, respectively. At the same time, it was observed that, at a dose of 5 mg/kg body weight, the standard drug loperamide reduced diarrheal stool by 68.02%. This study revealed that *S. costus* at a dose of 500 mg/kg body weight has almost the same effect as loperamide in reducing diarrheal stool. Thus, this finding clearly demonstrates that the methanol extract of *S. costus* possess a substantial antidiarrheal activity (Negi et al., 2013).

2.5.6.7. Antioxidant activity

S. costus is known to have antioxidant activities along with multiple other bioactivities. Plant-derived antioxidants remain an important resource to combat various health issues (Singh et al., 2018). Several phytochemical compounds of *S. costus* exhibit antioxidant properties, such as polyphenols and flavonoids, chlorogenic acid, palmitic acid, stigmasterol, myrcene, and tannin (Shing et al., 2002). The antioxidant activities of various solvent extracts of *S. costus* roots were investigated by Singh et al. Different solvent extracts were prepared, such as methanol, dichloromethane, and hexane extract.

According to their findings, among all the extracts tested, the methanolic extract of the root contained the maximum number of phytochemicals, and also had the highest flavonoid and phenolic content. Thus, methanolic extracts of the roots had the highest antioxidant activity (Singh et al., 2018). In another study, the antioxidant potential of *S. costus* extract was evaluated by Lee et al. They claimed that the extracts had strong antioxidant properties (Lee & Kang, 2020).

2.5.7. Antimicrobial activity of *S. Costus*

S. costus, or combined with other herbs, has the potential to be applied clinically in the management of pathogenic bacteria, fungi, and viruses. Various phytoconstituents of *S. costus* have been shown to have antimicrobial potential; this potential of *S. costus* has been reported in traditional and conventional medication systems over the years.

2.5.7.1. Antiparasitic activity

In a study, the anti-nematode efficacy of *S. costus* roots was evaluated by Akhtar et al. in naturally infected children. Their research showed that *S. costus* roots have significant antinematode activity. Oral dosages that are most effective were 50 and 40 mg/kg body weight, and no serious adverse effects were observed (Akhtar et al., 1991). In another study, Lirussi and associates assessed the effects of extracts obtained from seventeen plants used in traditional Chinese medicine against *Trypanosoma cruzi* (*T. cruzi*). These extracts were tested on Wistar rats with the epimastigote stage of *T. cruzi*. This stage is more sensitive than the others, and considering that, this stage is appropriate for identifying active compounds against the parasite. Among the seventeen tested plants, *S. costus* was one of the plants that showed the greatest activity against *T. cruzi,* with a 100% inhibition rate. In addition, *S. costus* did not show any cytotoxic effects. This result allowed them to suggest that *S. costus* could be a source of new molecules that are medically effective against *T. cruzi* (Lirussi et al., 2004).

2.5.7.2. Antiviral activity

S. costus root extract was studied by Chen et al. for its potential against the hepatitis B virus in hepatoma Hep3B cells of humans. Active constituents isolated from *S. costus,* including dehydrocostus lactone and costunolide, suppressed hepatitis B surface antigen expression. The suppression was also found in the HepA2 cell line. Thus, compounds

found in *S. costus* root extract were confirmed to have significant anti-hepatitis B activity, and the extract has the potential to be utilized as potent anti-hepatitis B virus drugs in the future (Chen et al., 1995). Saif-Al-Islam, in his recent review, studied the most important pharmacological and medicinal properties of *S. costus*, and based on some important activities of this plant, he concluded that *S. costus* may help in the management of COVID-19 (Saif-Al-Islam, 2020).

2.5.7.3. Antibacterial and antifungal activity

The antimicrobial activity of *S. costus* root extract was examined by Khalid et al. against multiple bacterial species. The gram-positive bacteria were *Bacillus subtalis, Staphylococcus aureus*, and *Entereococcus faecalis; the* gram-negative bacteria were *Salmonella typhi* and *Pseudomonas aeruginosa*. Based on the findings of this study, extracts of *S. costus* showed a strong antimicrobial activity against the tested bacteria. Therefore, they stated that extracts may serve as a foundation for the discovery of novel antimicrobial agents to overcome the issue of increasing resistance to common medications (Khalid et al., 2011).

In a study by Chang and associates, the antimicrobial activity of ethanolic extract obtained from *S. costus* root was investigated on six foodborne pathogenic bacteria; *Bacillus subtilis, Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Vibrio parahaemolyticus,* and *Salmonella choleraesuis*. Also, the antibacterial properties of *S. costus* roots were compared to those of synthetic antibiotics like tetracycline and ampicillin. The ethanol extracts of *S. costus* were fractionated using different solvents such as *n*-hexane, chloroform, and *n*-butanol. The ethanol extract and *n*-hexane fraction showed strong activities against *Vibrio parahaemolyticus* and *Bacillus cereus* compared to ampicillin. Therefore, based on their data, they stated that *S. costus* has the potential to be effective as an antimicrobial agent against food-borne pathogens, and in the food safety industry, it is likely to be used as an alternative to chemical preservatives (Chang et al., 2011).

In another study by Negi et al., the antibacterial activity of methanolic extract of *S. costus* was tested against S*taphylococcus aureus, Escherichia coli, Enterococcus faecalis, and Citrobacter freundii*. Their results showed that the extract has concentration-

dependent antibacterial activity. They also observed that samples with higher levels of dehydrocostus lactone costunolide had greater antibacterial activity (Negi et al., 2014).

Al Otibi et al. investigated the antifungal property of *S. costus* root extract against multiple phytopathogenic fungi. The tested fungi were *Helminthosporium sativum, Fusarium solani*, *Macrophomina phaseoloni*, *F. moniliforme,* and *F. oxysporum.* Various solvent extracts of the *S. costus* root were used, such as water, ethanol, methanol, dichloromethane, and ethyl acetate. Methanol, dichloromethane, and ethanol extracts showed strong antifungal activity against all tested fungi. Their findings indicated that the roots of *S. costus* are a great source of bioactive constituents that have the ability to affect the stability and integrity of the fungal cell membrane structure, eventually resulting in the death of fungal cells. Therefore, *S. costus* root can be used in controlling phytopathogens that damage vegetables and fruits during the period of harvest and postharvest. They may thus function as cheaper, more environmentally friendly, and safer substitutes to synthetic fungicides that are harmful and toxic (Al Otibi et al., 2020).

S. costus stem has been explored for its antibacterial activity against five strains of *Helicobacter pylori*. It is one of the important pathogenic bacteria that can cause multiple conditions, such as gastritis, gastric carcinoma, and dyspepsia, as well as autoimmune diseases and endocrine diseases. The findings showed that *S. costus* extracts strongly inhibited all of the tested strains (Li et al., 2005).

Yu et al. investigated the effect of an ethanolic extract of *S. costus* root on the acid production and growth of *Streptococcus mutans (S. mutans)*, and also the synthesis and adherence of water-insoluble glucans. *S. mutans* is an important and well-known cariogenic bacterium. Their findings revealed that an ethanol extract of *S. costus* inhibited the production of acid and growth of *S. mutans* significantly and also significantly reduced bacterial attachment ability in a dose-dependent manner. This outcome demonstrated that *S. costus* remarkably inhibited *S. mutans's* cariogenic activity (Yu et al., 2017).

The mentioned studies suggest that *S. costus* extracts may have the potential to be used against some pathogenic bacterial and fungal microorganisms. Thus, it may have a role in the defense action against multidrug-resistant pathogens in human, and it might be an alternative to antimicrobials in the treatment of some infections. Hence, further quality

studies are required to determine the effectiveness of the plant against various microbial species.

2.6. Tested microorganisms

2.6.1. *Escherichia coli (E. coli)*

E. coli is the most important and most common member of *Escherichia genus*, which is an Enterobacteriaceae family member. *E. coli* is a gram-negative, facultative anaerobic, rod-shaped, lactose fermenting, oxidase-negative, non-spore-forming, catalase-positive, motile bacterium which prefers to live in warm-blooded organisms in the lower intestine. The majority of E. *coli* strains are not harmful and are naturally found in the healthy human microbial flora. However, some strains can lead to infection in both immunocompromised and healthy people (Gomes et al., 2016) When the intestine is injured, these bacteria enter the peritoneum and can become opportunistic pathogens, or when the bacteria change their original place, such as in the case of urinary tract infection (UTI). Pathogenic strains have a various pathogenicity island coding for specific toxins and adhesions that make them capable of resulting in disease. The pathogenic *E. coli* are classified into five main pathotypes: Shiga-like toxin-producing *E. coli* (STEC) causes severe foodborne illness, hemorrhagic diarrhea, and hemolytic uremic syndrome. Enterotoxigenic *E. coli* (ETEC) is linked to watery diarrhea. Uropathogenic *E. coli* (UPEC) which cause the majority of the UTIs. Enteroinvasive *E. coli* (EIEC) causes dysentery by mechanically destroying the intestinal epithelium. And, Enteropathogenic (EPEC) causes diarrhea in children under the age of two years (Bekal et al., 2003; Kumar, 2015). *E. coli* is responsible for more than 80% of all community-acquired UTIs and also many hospital-acquired infections. In addition, *E. coli* is the most common gram-negative rod isolated from patients with sepsis. Furthermore, it is a prominent cause of peritonitis, meningitis, and abscesses (Murray et al., 2017). To treat severe diarrheal cases, cotrimoxazole or doxycycline can be administered. Nitrofurantoin, cotrimoxazole, or fluoroquinolones including ciprofloxacin are used to treat UTIs. However, different defense mechanisms have evolved in *E. coli* to combat the action of many antibiotics. In many instances, one strain can possess antibiotic resistance genes for multiple classes of

antibiotics and transfer the gene to other bacteria via horizontal gene transfer, which complicates the treatment; such as in some strains that produce class A extended spectrum β-lactamases, which is the most prevalent mode of resistance in *E. coli* towards β-lactam agents (Galindo-Méndez, 2020).

2.6.2. *Pseudomonas aeruginosa (P. aeruginosa)*

P. aeruginosa is a non-fermenter, gram-negative, rod-shaped, motile bacterium. It occurs as single bacterium, in pairs, or in short chains occasionally. Although described as obligate aerobes, they have the ability to grow anaerobically by using nitrate or arginine as an alternate electron acceptor (Zimmermann et al., 1991). *P. aeruginosa* produce sweet or grape-like odor, some strains hemolyze blood. They are catalase and oxidase positive, and grow well at 37°C-42°C (Procop et al., 2020). They are not found in the normal human microbiome; in the environment, they can be found in soil, vegetation, water, and decaying organic matter. Unfortunately, they can be found throughout the hospital. Because of its simple growth requirements and nutritional versatility, it has a wide environmental distribution. As they are capable of using many organic substances as sources of nitrogen and carbon, some strains can even grow in distilled water by using trace nutrients (Harvey, 2007).

P. aeruginosa is an opportunistic microorganism, which can cause serious infections to previously damaged tissues including burns, or in immunosuppressed patients such as HIV/AIDS and cancer. It can cause a variety of diseases such as; ecthyma gangrenosum, hot tub folliculitis, malignant external otitis, osteomyelitis, and it also causes pneumonia in patients suffering from cystic fibrosis. *P. aeruginosa* possesses a variety of virulence factors, including; biofilms, mucoid exopolysaccharide capsules, adhesins, and various enzymes and toxins. In addition, it has a type III secretion system, which is especially effective at toxin injection into host cells. Furthermore, lipopolysaccharide, which exists in various immunotypes and is responsible for many of the endotoxic properties of the organism. These virulence factors keep them alive and make them resistant to the majority of commonly used antibiotics (Gillespie & Bamford, 2003; Kumar, 2015). *P. aeruginosa* is one of the most resistant microorganisms observed in medical laboratories. It is intrinsically resistant to many antibiotics due to its low permeability of its outer membrane, various multidrug efflux pumps, and various

enzymes such as β-lactamases, aminoglycoside-modifying enzymes. And also, it can develop resistance to new antibiotics by mutations and/or horizontal transfer of resistance genes. Some strains, such as those that produce metallo-beta-lactamase, are reported to be resistant to all antibiotics except colistin (Murray et al., 2017).

2.6.3. *Enterococcus faecalis (E. faecalis).*

E. faecalis is gram-positive, catalase-negative, facultatively anaerobic bacteria that belong to a group of organisms known as lactic acid bacteria. They are usually oval in shape and arranged in pairs or short chains. *E. faecalis* is commensal bacteria and part of the normal enteric microbiota (Fisher & Phillips, 2009). These bacteria can survive in harsh environments and have adapted to a variety of ecological niches. They can proliferate in the presence of high levels of bile salts, high PH levels, sodium chloride, and in high temperatures. Thus, only a few environmental factors prevent the growth of enterococci (Sirichoat et al., 2020**;** Micallef et al., 2013). They are opportunistic pathogens; life-threatening disease caused by some strains has become a serious issue in immunocompromised people and has become one of the most common causes of nosocomial infections, with a high mortality rate. The ability of *E. faecalis* to adhere to tissues, to form biofilms, and antibiotic resistance all contribute to their virulence. Several factors that influence adherence and biofilm formation have been identified, including surface proteins, pili, membrane glycolipids, and gelatinase. (Fisher & Phillips, 2009). *E. faecalis* infections include UTI, endocarditis, neonatal sepsis, hepatobiliary infections, bacteremia, and surgical wound infections. Respiratory tract infections (e.g., pneumonia, sinusitis, and otitis) and central nervous system infections (e.g., meningitis) have been described, but rarely occur (Riede et al., 2022).

Antibiotic resistance is common among enterococci. Traditionally, treatment for systemic enterococcal infections consists of a cell-wall active antibiotic (e.g., vancomycin or ampicillin) combined with an aminoglycoside. However, several cell-wall active antimicrobial agents (e.g., nafcillin, oxacillin, and cephalosporins) have no activity against some strains of enterococci. In addition, enterococci have developed and shown resistance to some of the commonly used cell-wall active antibiotics (e.g., vancomycin), such as in VRE. (Arias et al., 2010; Sirichoat et al., 2020**).**

2.6.4. *Staphylococcus aureus (S. aureus)*

S. aureus is a facultative anaerobic and gram-positive bacterium belonging to Staphylococcaceae family. *S. aureus* is a non-motile, catalase-positive, coagulase-positive, non-spore forming bacterium that forms grape-like clusters with large yellow colonies. It is a normal microbial flora on human skin and mucosal surfaces. However, it can cause infection by direct colonization, toxin generation, or both if it over proliferates or the skin is injured (Nizet & Bradley, 2011). It possesses various virulence factors, such as structural components that enhance adhesion to host tissues and avoid phagocytosis, such as capsules, biofilms, protein A, teichoic acid, and variety of toxins such as Panton-valentine leukocidin toxin, exfoliative toxins, hemolysin, toxic shock syndrome toxin 1, and enterotoxin, hydrolytic enzymes such as coagulase, hyaluronidase, fibrinolysin, lipases, and nucleases. *S. aureus* is the causative agent for a wide range of health defects in humans, which include diseases caused by toxin (scalded skin syndrome, food poisoning, and toxic shock syndrome), pyogenic diseases (wound infections, cellulitis, impetigo, folliculitis, carbuncles, and furuncles), and also causes bacteremia, endocarditis, pneumonia, and meningitis (Harvey, 2007; Kumar, 2015). The overuse of antimicrobials against *S. aureus* has led to the emergence of resistance strains. For example, methicillin-resistant *S. aureus*, healthcare and community-acquired infections with methicillin resistant *S. aureus* are caused significant problems worldwide. It has become the leading cause of community-acquired soft-tissue and skin infections (Kumar, 2015). wound sepsis is still a major cause of mortality in burnt patients due to colonization with methicillin-resistant *S. aureus* on the wound surface, which can lead to serious clinical complications and systemic infections. This strain is resistant to all ß-lactam antibiotics as well as several other antibiotic classes including the aminoglycosides, clindamycin, erythromycin, and quinolones (Naqvi et al., 2007; Giedraitienė et al., 2011). Those strains of *S. aureus* that have intermediately developed resistance to vancomycin are called vancomycin intermediate *Staphylococcus aureus* (VISA), and those that have completely developed resistance are called vancomycin resistant *Staphylococcus aureus* (VRSA) (Gardete & Tomasz, 2014). Ultimately, *S. aureus* infections are treated by testing these bacteria against a variety of antibiotics and then selecting the most effective antibiotic among them. The quest for novel antibiotics and vaccines to combat *S. aureus* infections is ongoing, but antibiotic overuse contributes to the rapid resistance.

2.6.5. *Staphylococcus epidermidis (S. epidermidis)*

S. epidermidis is a clustering non-spore-forming, gram-positive coccus, catalase-positive, non-motile, coagulase-negative, and facultatively anaerobic bacteria (Namvar et al., 2014). *S. epidermidis* is considered a normal skin microbial flora that maintains a benign relationship with its host and avoids being colonized by other microbial pathogens, such as *S. aureus* Otto, 2009). However, it is also a leading cause of healthcare-acquired infections and it is frequently found as a contaminant in blood culture specimens that have been improperly collected. Instrumentation procedures such as medical implantation and catheterization, as well as immunosuppressive medications, are some examples of the risk factors for hospital-acquired *S. epidermidis* infection (Mahon et al., 2018). Nosocomial infections caused by *S. epidermidis* are endocarditis involving prosthetic cardiac valves, bacteremia linked with indwelling vascular catheters, other infections associated with cerebrospinal fluid shunts, postsurgical ocular infections, prosthetic joints, bacteremia in newborns under intensive care, and vascular grafts (Tille, 2015). The ability of *S. epidermidis* to develop biofilm is a critical virulence factor. This allows the bacterium to attach to abiotic and biotic surfaces and to resist antibiotics and the host immune system (Gomes et al., 2014).

2.6.6. *Klebsiella pneumoniae (K. pneumoniae)*

K. pneumonia is a non-motile, rod-shaped, lactose-fermenting, gram-negative, non-spore forming, facultative anaerobic bacterium, which is a member of the family Enterobacteriaceae. It can be found in the environment, in soil and surface waters, as well as on medical devices (Paczosa & Mecsas, 2016). It can also normally colonize the gastrointestinal tract of humans. *K. pneumoniae* is an important opportunistic pathogen, which is considered one of the common causes of nosocomial pneumonia, and the bacteria is responsible for 3% to 8% of all healthcare-associated bacterial infections (Haeggman et al., 2004). A subset of hypervirulent serotypes of *K. pneumoniae* with increased production of polysaccharide capsule can infect healthy people and can cause potentially fatal community-acquired infections, including necrotizing fasciitis, meningitis, endophthalmitis, pyogenic liver abscess, and pneumonia that usually involves necrotic alveolar space destruction, formation of cavities, and production of blood-tinged

sputum. This bacterium also causes urinary tract, wound, and soft-tissue infections, and bacteremia/sepsis (Li et al., 2014; Riede et al., 2022).

For their survival and evasion of the immune system during infection, *K. pneumoniae* uses a number of virulence factors, such as polysaccharide capsules, lipopolysaccharides, outer membrane proteins, pili, and determinants for iron acquisition and utilization of nitrogen sources (Li et al., 2014). Additionally, *K. pneumoniae* produces different enzymes that degrade the antibiotic structure, making it a multidrug-resistant bacterium. Some strains produce beta-lactamases, which confers resistance to amoxicillin and ampicillin. Furthermore, there are strains that produce extended-spectrum beta-lactamases (ESBL), which confers resistance to cephalosporins, fluoroquinolones, and aminoglycosides. Carbapenems are often used to treat these strains. Meanwhile, there are some strains that can also produce carbapenemases, which confers resistance even to carbapenems. In this case, *K. pneumoniae* can be treated with colistin and tigecycline (Nordmann et al., 2009; Haeggman et al., 2004). Carbapenem resistance has been reported to associated with the up-regulation in efflux pumps, increased in ESBL enzymes production, and the outer membrane alteration (Ashurst & Dawson, 2018), and it is resistant to other antimicrobial agents due to the acquisition of plasmids containing multiple resistance genes. Thus, the management of patients with *K. pneumoniae infections* is a major clinical challenge (Murray et al., 2017).

2.6.7. *Acinetobacter baumannii (A. baumannii)*

A. baumannii are plump gram-negative coccobacilli, strictly aerobic, oxidase-negative, catalase positive, non-motile, non-fastidious, glucose-oxidizing bacteria (lee et al., 2017). *A. baumannii* is saprophytic, recovering in nature (soil, water, and foodstuffs) and in the hospital environment. They can thrive on moist surfaces such as humidifiers, mechanical ventilation, and catheters, as well as on dry surfaces such as human skin. These bacteria are also found in the normal oropharyngeal flora of a small portion of healthy individuals, and they can multiply rapidly during hospitalization (McConnell et al 2013; Villegas & Hartstein, 2003). These organisms can cause community-acquired infections, although the great majority of infections are healthcare-associated infections (Tille, 2015). They come in second after *P. aeruginosa* in the frequency of isolation of all non-fermenters in the medical microbiology laboratories responsible for nosocomial

infections. *A. baumannii* is an opportunistic pathogen that mainly affects patients with compromised immune systems and coexisting conditions. It causes several infections, such as hospital and community acquired pneumonia, endocarditis, bacteremia, soft tissue and skin infections, meningitis, and urinary tract infections (McConnell et al., 2013). By genomic and phenotypic analysis, various virulence factors have been identified including capsular polysaccharides, phospholipases, lipopolysaccharides, outer membrane porins, proteases, biofilm formation, iron-chelating systems, and protein secretion systems (lee et al., 2017; Lin and Lan, 2014).

A. baumannii isolates are frequently resistant to a vast range of antimicrobials, including penicillin, fluoroquinolones, and first and second generation of cephalosporins. *A. baumannii* is susceptible to a variety of aminoglycosides and β-lactams, as well as β-lactamase inhibitor combinations (ampicillin-sulbactam and piperacillin-tazobactam). However, carbapenemases have been reported lately among *A. baumannii*, which exhibits resistance to carbapenems. These isolates are known as carbapenem-resistant *A. baumannii* (CRAB), and they are often only susceptible to tigecycline and colistin. When a CRAB is isolated, patients are often forced to be isolated in mobile isolation chamber units (Mahon et al., 2018; Tille, 2015). Several resistance mechanisms of *A. baumannii* are known, including multidrug efflux pumps, target modifications, permeability defects, and enzymatic degradation of antimicrobial agents (Gordon & Wareham, 2010; Kim et al., 2012).

2.6.8. *Enterobacter cloacae (E. cloacae)*

E. cloacae is a gram-negative, rod-shaped bacterium belonging to the Enterobacteriaceae family of Enterobacterales order. *E. cloacae* is a motile, non-spore forming, oxidase-negative, urease-positive, and facultatively anaerobic bacteria. It can be found in both aquatic and terrestrial habitats (water, soil, sewage, and food). *E. cloacae* is found in both human and animal intestinal tracts as a commensal microbiota (Davin-Regli & Pagès, 2015). In the last ten years, they have gained clinical value as an opportunistic pathogen and are considered one of the most common Enterobacter species that cause infection in the hospital environment, especially in those patients who are admitted to the intensive care unit and those using mechanical ventilation system (Annavajhala et al, 2019). It can cause a variety of illnesses, such as endocarditis,

osteomyelitis, skin and soft tissue infections, septic arthritis, lower respiratory tract infections, UTIs, and bacteremia. Its ability to cause infections may be related to its capability to form biofilms and to secrete a number of cytotoxins such as enterotoxins and pore-forming toxins. Infections caused by this bacterium can be managed with aminoglycosides, fluoroquinolones, carbapenems, and polymyxins. (Mezzatesta et al., 2012; Potron et al., 2013).

2.6.9. *Candida albicans (C. albicans)*

C. albicans is an opportunistic fungi that lives as a relatively harmless commensal in nearly 70% of humans' gastrointestinal tracts, mouths, and genitourinary tracts. It can cause health defects in people with compromised immune systems, or even in healthy individuals. Candidiasis is caused by *C. albicans*. In less severe cases, *C. albicans* may cause thrush, which is a mucosal infection characterized by the presence of white spots in the infected membranes. It primarily affect gastrointestinal epithelial cells and oropharyngeal or vaginal mucosa. However, in more severe cases, *C. albicans* can cause potentially fatal systemic infections in critically ill patients, with about a 30% mortality rate (Kabir et al., 2012). Systemic candidiasis is common in people with compromised immune systems, including transplant recipients, HIV-infected patients, low-birth-weight infants, and individuals undergoing chemotherapy (Pfaller & Diekema, 2007).

C. albicans pathogenicity is determined by two key factors. One is related to the host's immune status, and the other is linked to the pathogen's virulence factors. Broadly, the microbial factors that play a role in pathogenicity are: adherence to the host cell, hydrolytic enzymes secretion, phenotypic switching, dimorphic phenotype, and the formation of biofilm (Kabir et al., 2012; McCullough et al., 1996). Adhesion is regarded as a crucial step in the colonization and establishment of *C. albicans* infections. *C. albicans* is an adaptable pathogen that can stick to a wide range of surfaces, including endothelial cells, epithelial cells, extracellular matrix, and implanted inert materials in the host's body (Pendrak & Klotz, 1995). It has a wide range of secreted hydrolytic enzymes, including secreted aspartyl proteinases (SAPs), which can breakdown a variety of significant host factors (Schaller et al., 2005). Phospholipases A, B, and C are also secreted by *C. albicans* and are related to the host cell's damage, penetration, and adherence *(*Ghannoum, 2000). Another important characteristic of *C. albicans* is its

potential to produce biofilm on both abiotic and biotic surfaces (Ganguly & Mitchell, 2011). Moreover, *C. albicans* phenotypic switching is regarded as an important virulence determinant; for this pathogen, a variety of colony types like wrinkled, smooth, rough, irregular, stippled, and star have been identified. *C. albicans* can grow as a unicellular budding yeast or a filamentous form of hyphae or pseudo-hyphae (Kabir et al., 2012; Murray et al., 2017). This plasticity in morphological or phenotypic states can play a significant role in its pathogenicity. Candidiasis treatment varies according to the infection severity and its location. Oral thrush can be managed by an oral nystatin suspension. Azole or topical antifungals are used to treat skin and vaginal infections. Severe infections are usually managed via azole antifungals, amphotericin, or echinocandins like Micafungin (Pappas et al., 2016; Riede et al., 2022).

3. MATERIALS AND METHODS

3.1 Research setting

S. costus root samples were obtained from a local herbal shop from Tokat, Turkey. The extraction process of the plant was conducted at the Chemistry department, Gaziosmanpaşa university and the chemical analysis of the extracts was performed at the Research Laboratory Practice and Research Center. The antimicrobial activity assessment of the obtained *S. costus* root extracts were performed at the Microbiology Laboratory, Tokat Gaziosmanpaşa University Hospital. The microbial species needed for this research were obtained from the microbial storage of the same hospital.

3.2. Used materials

In the extraction of *S. costus* root extract and performing the antimicrobial activity assessment, the following materials and instruments were used:

3.2.1. Equipment:

- Sterile cotton swabs (CITOSWAB, China)
- Hot plate with stirrer (IKA, China)
- Autoclave (Nuve, Turkey)
- Incubator (Muve, Turkey)
- Vortex mixer (ISOLAB Laborgeräte GmbH, Germany)
- Refrigerator (Vestel, Turkey)
- Clevenger device
- Lauda Alpha RA 8 (Lauda, Germany)
- Heidolph Rotatory (Heidolph, Germany)
- Forceps
- Oven (Elektromag, Turkey)
- Loops
- Sterallized test tube (4a medical, Turkey)
- McFarland densitometer (DENSICHEK)
- Micropipette (Transferpette, Germany)
- Agilent 1260 High Performance Liquid Chromatography (Agilent, USA)
- Sterallized syringe (BERIKA, Turkey)

- Ruler
- Sterile hand gloves
- Parafilm tape
- Glassware (Petri dish, test-tubes, beakers, flasks, round bottle)
- 96-well microplates (LP ITALIANA, Italy)
- Precision Balance (DIKOMSAN ELEKTRONIK, Turkey)

3.2.2. Chemicals and Reagents:

- Tween 80 (Sigma Aldrich, Germany)
- Dimethyl sulfoxide (DMSO) (CARLO ERBA, France)
- Methanol (CH_3OH) (Merck KGaA, Germany)
- Hexane (C_6H_{14}) (Merck KGaA, Germany)
- Chloroform ($CHCl_3$) (Merck KGaA, Germany)
- Sterile saline solution.

3.2.3. Bacteriological media

- Sabouraud Dextrose agar (SDA) (HIMEDIA, India)
- Eosin methylene blue agar (EMB) (HIMEDIA, India)
- Muller Hinton agar (MHA) (HIMEDIA, India)
- Blood agar (HIMEDIA, India)
- Cation adjusted Muller Hinton broth II (MHB) (Becton, Dickinson, USA)

3.2.3. Other materials:

- *S. costus* root
- Sterellized distilled water
- Antibiotics discs (Gentamycin 10 mcg & Ciprofloxacin 5 mcg) (Bioanalyse, Turkey)
- Blank discs (Bioanalyse, Turkey)
- Ethanol

3.3. Plant extraction

In the current study, essential oil, aqueous, hexane-chloroform, and methanolic extracts were extracted from *S. costus* root.

3.3.1. Essential oil extraction

Essential oil of the plant was acquired by water distillation via Clevenger-type apparatus, which is shown in Figure 3.1. An amount of 200 g of *S. costus* root powder was placed in Clevenger's bowl then 600 ml of water was added and the heating process began. The oil was collected in the accumulation chamber of the device, which was operated for six hours, and was transferred to a sterile brown bottle. In total, approximately one ml of essential oil was obtained from the 200 g of *S. costus* root at the end of the distillation process. Then, the obtained essential oil was stored at +4 °C in a refrigerator to be used later in chemical analysis and antimicrobial activity screening.

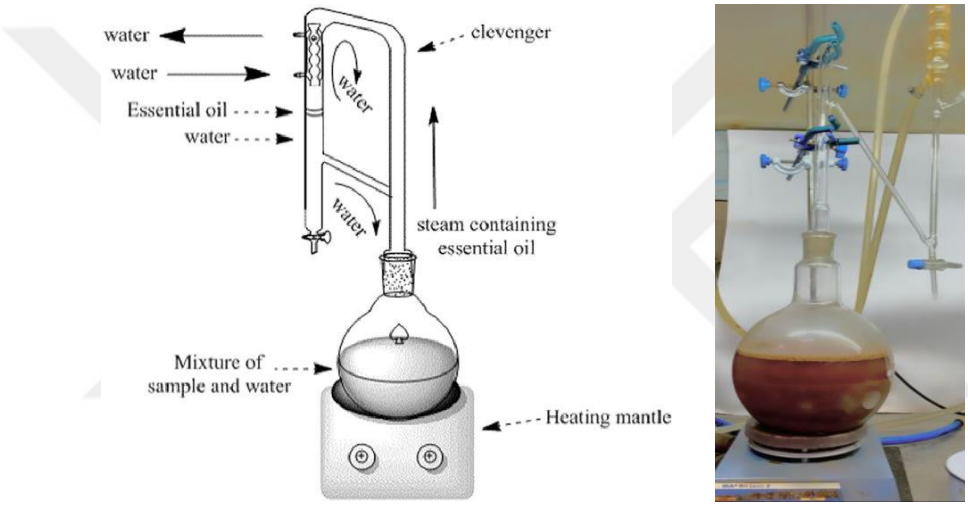

Figure 3.1. Clevenger device

3.3.2. Aqueous extraction

In order to obtain crude aqueous extract, 200 g of dried root of *S. costus* was mixed with 600 ml of sterile distilled water, and was heated and stirred for nearly four hours. After the extract was cooled, it was filtered via Whatman No. 1 filter paper and vacuum filtration. Then the resulting filtrate was concentrated via Lauda Alpha RA 8 with a Heidolph Rotatory evaporator to acquire the crude aqueous extract. The resulting extracts were stored at 4 ^{o}C for further antimicrobial activity testing. Figure 3.2 shows Lauda Alpha RA 8 with a Heidolph Rotatory evaporator.

3.3.3. Methanolic and Hexane-Chloroform extractions

Methanolic extract was prepared from 100 g of dried powdered root of *S. costus* soaked with 250 ml of methanol, it was placed on a stirrer and left for 72 hours. Then the content was homogenized and filtered through Whatman No. 1 filter paper. After that, the filtrate was transferred into a round bottom flask and was concentrated using Lauda Alpha RA 8 with a Heidolph Rotatory evaporator to remove the solvent and obtain a dark-gum like crude methanol extract. The same procedure was followed for the preparation of Hexane-Chloroform extract by dissolving 100 g of dried root powder with 200 ml of hexane and 50 ml of chloroform. The extracts were stored at 4 ^{o}C for further antimicrobial activity testing.

Figure 3.2. Lauda Alpha RA 8 with a Heidolph Rotatory evaporator to remove the solvent from extracts.

3.3.4 Sterility proofing of the extracts

The obtained *S. costus* extracts were sterility proofed by transferring two ml of the extract into 10 ml of MHB and incubating it for 24 hours at 37 C^{0}. After the period of incubation, the absence of bacterial turbidity or clearness of the broth were observed and 100 µl was transferred onto a clear blood agar plate to ensure the sterility of the extracts.

3.4. Chemical analysis of the extracts

Chemical analysis of the extracts was performed by High Performance Liquid Chromatography (HPLC) on Agilent 1260 Infinity (Agilent, USA) which was equipped with an ACE GENERİX 5 C18 column (4.6 mm × 250 mm) coupled with a diode array detector (DAD). The temperature of the column was set at 30 °C. Separation was achieved via a gradient elution with (A) 0.1% Phosphoric acid (H3PO4) in water (83%), and (B) 100% Acetonitrile (17%). The flow rate of mobile phase was 0.8 ml/min, and the injection volume was 10 µl. Detection wavelength was set at 300/200 nm and reference wavelength was 500/100 nm.

3.5. Identification of bacterial strains

The test microorganisms for this study were *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Enterococcus cloacae* (ATCC 23355), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), and *Candida albicans* (ATCC 90028). All clinical isolates were obtained from the Tokat Gaziosmanpaşa University Hospital, Microbiology Laboratory, which were stocked and stored at -80 °C. The microbial strains were revived using blood agar, EMB agar, and SDA.

3.6. Antimicrobial susceptibility screening

3.6.1 Disc diffusion test

Disc diffusion essay for antimicrobial susceptibility screening was conducted in accordance to the instructions provided by the Clinical Laboratory Standards Institute, (CLSI) (Jorgensen & Turnidge, 2015; Jorgensen et al., 2007).

3.6.1.1. Preparation of impregnated discs

To prepare stock solutions, 200 mg of each extract was dissolved in one ml of their respective solvents, DMSO and sterile distilled water for organic and aqueous extracts, respectively, to produce a final concentration of 200 mg/ml. For essential oil, 200 µl was dissolved in one ml of DMSO (200 µl/ml). Sterilized six mm blank discs were then impregnated with 20 µl of the prepared extract stock solutions. In this study, negative

controls were DMSO-loaded discs, and the positive controls were standard antibiotic discs, which contained Gentamycin (10 mcg) and Ciprofloxacin (5 mcg) for all bacterial strains.

3.6.1.2. Inoculums and inoculation procedure

The concentration of the inoculum was standardized to get a final concentration of approximately 1.5 x 10^8 CFU/ml. A few colonies from an agar culture plate were suspended in three ml of sterile physiological saline and vortexed thoroughly to prepare a homogeneous microbial suspension. The turbidity of the inoculum was equal to the 0.5 McFarland standard. Then, a sterile cotton swab was put into the microbial suspension, and after that, it was streaked over the whole surface of MHA agar plates. Separate plates were used for each type of microorganism.

3.6.1.3. Application of impregnated discs

The prepared plant extract impregnated discs were applied to the inoculated MHA by using sterile forceps. After adding the discs, to achieve proper contact with the agar surface, the discs were gently pressed. Each test plate had a maximum of five discs on it to avoid inhibition zones overlapping, they were set at about similar distances from one another. Three discs with different extracts, a negative control (DMSO), and the last one was a positive control (Gentamycin and Ciprofloxacin), were applied. The essential oil was tested on a separate plate. Then, the plates were flipped and were incubated at 37°C for 24 hours (48 hours for fungi). For the antimicrobial activity evaluation, the diameters of the inhibition zones surrounding the control and treated discs were measured. To make sure the results were accurate, each experiment was done three times, and their average was calculated.

3.6.2. Minimum Inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC)

MIC was determined as the maximum dilution of the extracts that inhibited microbial growth but did not kill the microorganism (no visible microbial growth as compared to the control tube), and MBC/MFC was determined as the concentration that killed the microbial species. By following the CLSI (Jorgensen et al., 2007; Jorgensen & Turnidge, 2015), the antibacterial activity of *S. costus* extracts against the test microbial

isolates was investigated in-vitro to determine MIC and MBC/MFC values using a broth microdilution susceptibility assay for quantitative measurement.

3.6.2.1. Preparation of extract dilutions and inoculation procedure

One gram of plant aqueous extract was dissolved in 10 ml of sterile distilled water to prepare a stock solution with a 100 mg/ml concentration. Similarly, 1 g of methanolic and hexane-chloroform extracts were each dissolved in 10 ml of DMSO, resulting in an initial concentration of 100 mg/ml of plant extract. For essential oil, 100 µl was dissolved in 1 ml of DMSO. All tests were carried out in MHB supplemented with 2% Tween-80 detergent (with a final concentration of 0.5%) to enhance the extracts' solubility. The active concentration of the extract was determined using 96-well microplates with the microdilution method. An amount of 100 µl of the prepared MHB medium was dripped into every well of the microplates. Then, 100 µl of *S. costus* extract (100 mg/ml or µl/ml) was transferred to the first well, and 9 dilutions of the extract were prepared by two-fold serial dilution at every turn to prepare 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, and finally 0.2 mg/ml concentrations (The unit is µl/ml for essential oil). Subsequently, all the wells were inoculated with an equal amount of microbial suspension of a 0.5 McFarland diluted by a ratio of 1:100 (1.5×10^6 CFU/ml) in MHB broth, except for the negative control well. For each serial dilution line, one growth control well containing MHB and microorganisms, one solvent control well containing MHB, DMSO, and microorganisms, and one sterility control well containing only MHB were left. All tests were performed two times to ensure dependability. After inoculation, the microplates were incubated for 24 h at 37°C (48 hours for fungi).

3.6.2.2. Determination of MIC and MBC values

To assess the presence of microbial growth, the turbidity of the wells and the presence of white pellets were observed on the day after. To ensure the presence or absence of microbial growth, 10 µl of each well was inoculated on blood agar by drop plate method and incubated for 24 hours at 37 ºC (48 hours for fungi). Following incubation, the blood agar plates were observed to confirm the absence of microbial growth. The MIC value was determined using the lowest concentration that showed no observable growth, and the determination of MBC values was done using the lowest concentration that showed no microorganism.

4. RESULTS

4.1. Chemical analysis

The results for chemical analysis of all our tested extracts obtained by HPLC are shown in detail in Table 4.1 and 4.2.

Table 4.1. Chemical composition analysis of aqueous and methanolic *S. costus* root extracts.

Compound	**Concentration (mg/g)**	
	Aqueous extract (2.47)	**Methanolic extract (2.96)**
Ascorbic acid	0.22	0.15
Alizarin	ND	ND
Gallic Acid	1.6	1.5
Protocatechuic acid	ND	0.03
Catechin	0.18	0.15
Hydroxybenzoic acid	ND	ND
Vanillic acid	0.05	ND
Gentisic acid	0.06	0.16
p-Coumaric acid	ND	0.03
Rutin	ND	0.01
Ferulic acid	ND	0.02
Flavones	ND	ND
Naringin	ND	ND
o-Coumaric acid	ND	0.06
Neohesperidin	ND	ND
Coumarin	ND	0.04
Resveratrol	0.03	0.36
Quercetin	0.04	0.13
t-Cinnamic acid	ND	0.22
Hesperidin	0.18	ND

*** ND** = not detected.

Table 4.2. Chemical composition analysis of hexane-chloroform extract and essential oil of *S. costus*.

Compound	Precentage (%)		Compound	Precentage (%)	
	H.C.E.	E.O.		H.C.E.	E.O.
1,8,11,14-Heptadecatetraene	20.98	40.87	Hexadecanoic acid, 14-methyl-, methyl ester	0.09	ND
1,8,11,14-Heptadecatetraene-isomer	0.80	ND	Humulene	0.26	2.43
1,8,11-Heptadecatriene	2.08	1.78	Lignoceric acid methyl ester	0.14	ND
15-Tetracosenoic acid, methyl ester	0.34	ND	Linoleic acid, methyl ester	21.64	ND
1-Pentadecene	0.36	ND	Linolenic acid, methyl ester	11.43	ND
22-Tricosenoic acid	0.17	ND	Linolenin, 1-mono-	0.06	ND
24-Norursa-3,12-diene	4.18	ND	Methyl 9.cis.,11.trans.t,13.trans.-octadecatrienoate	0.17	ND
2-Isopropyl-4α,8-dimethyl-1,2,3,4,4α,5,6,7-octahydronaphthalene	0.14	0.58	Myristic acid, methyl ester	0.17	ND
3-Ethyl-3-hydroxyandrostan-17-one	3.30	ND	Oleic acid, methyl ester	0.71	ND
4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene	0.14	ND	Palmitic acid, methyl ester	6.51	ND
4,5-di-epi-aristolochene	ND	0.45	Palmitoleic acid, methyl ester	0.34	ND
4-terpineol	ND	0.55	Pentadecanoic acid, methyl ester	0.11	ND
8-Cedrene-13-ol	0.16	ND	Reynosin	0.21	ND
Arachidic acid methyl ester	0.20	ND	Stearic acid, methyl ester	0.92	ND
Behenic acid, methyl ester	0.06	ND	Thymol	ND	2.92
Bohlmann k2631	0.26	2.13	trans-α-Bisabolene	0.13	ND
Caryophyllene	1.72	7.23	α-Atlantone	0.28	ND
Caryophyllene oxide	1.01	1.79	α-Cedrene	0.07	0.59
cis-13-Eicosenoic acid	0.11	ND	α-Curcumene	0.71	2.86
cis-α-Bergamotene	0.27	1.46	α-Ionone	0.12	4.58
cis-γ-Bisabolene	0.20	ND	α-Selinene	0.63	1.85
Costal	0.19	ND	β-Cyclocostunolide	0.30	1.50
Costol-isomer	0.12	ND	β-Elemene	1.47	9.31
Dehydrosaussurea lactone	0.38	ND	β-Eudesmol	0.16	ND
Dihydrodehydrocostus lactone	1.92	0.71	β-Selinene	0.96	ND
Dihydrodehydrocostus lactone-isomer	8.56	5.69	β-Sesquiphellandrene	0.13	ND
Dihydropseudoionone	0.12	1.93	γ-Curcumene	0.46	1.35
Dihydro-α-ionone	0.20	2.66	γ-Linolenic acid, methyl ester	0.50	ND
Elemol	0.10	ND	δ-Guaiene	0.57	ND

Estragole	ND	0.93	Not identified	2.66	3.86

*** ND** = not detected; **H.C.E.** = Hexane-chloroform extract; **E.O.** = Essential oil.

4.2. Antimicrobial activity of *S. costus*

The antimicrobial activity of essential oil, hexane-chloroform, methanolic, and aqueous extracts of *S. costus* root were tested against *P. aeruginosa, E. coli, S. aureus, S. epidermidis, E. cloaca, E. faecalis, K. pneumonia, A. baumannii, and C. albicans* using disc diffusion method and micro-dilution susceptibility assays. Additionally, the antimicrobial activity of the root was compared to other synthetic antibiotics, including Gentamycin (10 mcg) and Ciprofloxacin (5 mcg).

4.2.1. Disc diffusion

The findings revealed that *S. costus* root extracts, among gram-positive bacteria, exhibited the highest antibacterial activity against *S. epidermidis* with an average inhibition zone of 13 mm, 15.3 mm, and 12.5 mm inhibition zone for methanolic, hexane-chloroform, and essential oil extracts, respectively, as for aqueous extract, there was no discernible inhibition zone. For *S. aureus,* all extracts showed moderate to good activity with 13.3 mm, 12 mm, 11.6 mm, and 11 mm inhibition zones for hexane-chloroform, essential oil, aqueous, and methanolic extracts, respectively. Also, *E. faecalis* showed a moderate susceptibility to the essential oil by showing 11.5 mm inhibition zone, and weak susceptibility to the methanol, hexane-chloroform, and aqueous extracts with 9.6 mm, 8.6 mm, and 8.3 mm inhibition zones, respectively. Inhibition zones showing the antimicrobial activity of *S. costus* root against gram-positive bacteria are shown in Figure 4.1.

For gram-negative bacteria, all strains were resistant to the aqueous extract. However, for the other extracts, *E. cloacae* showed 11 mm inhibition zone for essential oil, 9 mm for methanolic extract, and 7.6 mm for hexane-chloroform extract. *E. coli* was resistant to all the extracts except for essential oil, which only showed an inhibition zone of 8 mm. Only Hexane-chloroform extract and essential oil exhibited weak activity against *K. pneumonia* and *A. baumannii,* with 7 mm and 7.5 mm inhibition zones for *K. pneumonia,* and 8.3 mm and 8 mm inhibition zones for *A. baumannii*, respectively. *P. aeruginosa* was observed to be completely resistant to all the tested extracts. Inhibition

zones showing the antimicrobial activity of *S. costus* root against gram-negative bacteria are shown in Figure 4.2.

For *Candida albicans*, all extracts except aqueous extracts showed good antifungal activity. The highest inhibition zone (14.6 mm) was recorded for hexane-chloroform extract, followed by essential oil (14 mm) and methanolic extract (11.6 mm). Inhibition zones showing the antimicrobial activity of *S. costus* root against *C. albicans* are shown in Figure 4.3. The detailed results acquired in the assessment of the antimicrobial activity of *S. costus* by using disc diffusion assay are presented in Table 4.3.

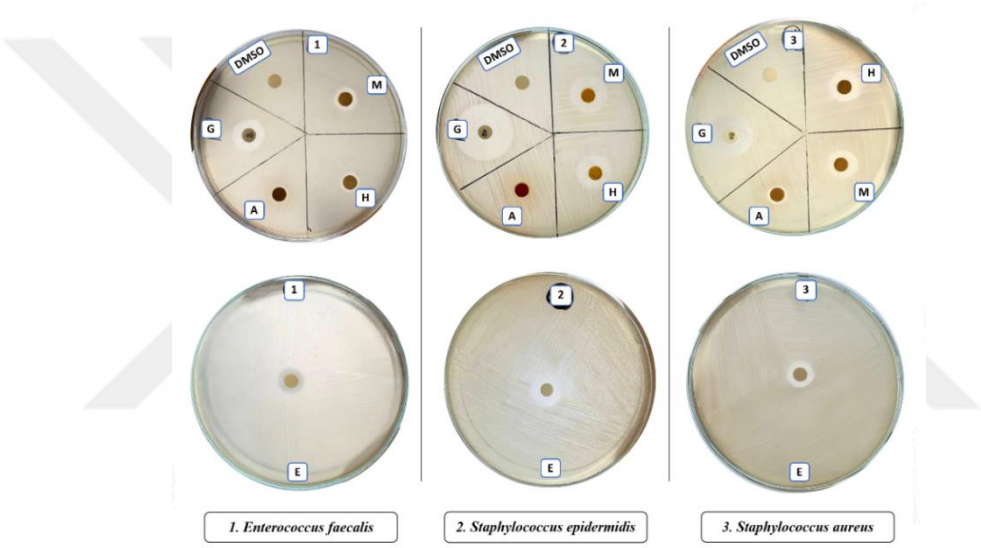

Figure 4.1. Inhibition zones showing antimicrobial activity of *S. costus* root against gram-positive bacteria. **E**) essential oil, **A**) aqueous, **M**) methanolic, and **H**) hexane-chloroform extracts, and **G**) gentamycin.

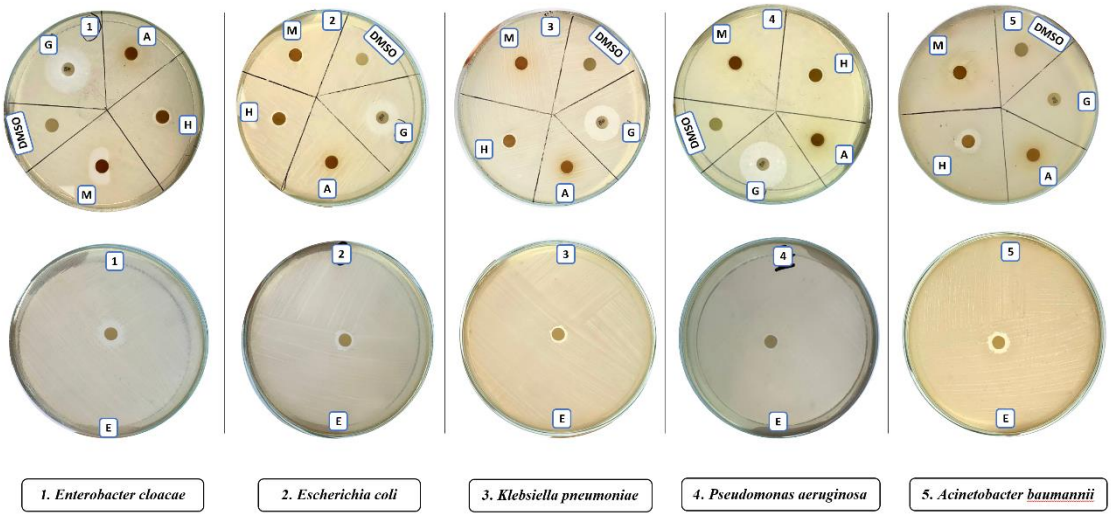

Figure 4.2. Inhibition zones showing antimicrobial activity of *S. costus* root against gram-negative bacteria. **E**) essential oil, **A**) aqueous, **M**) methanolic, and **H**) hexane-chloroform extracts, and **G**) gentamycin.

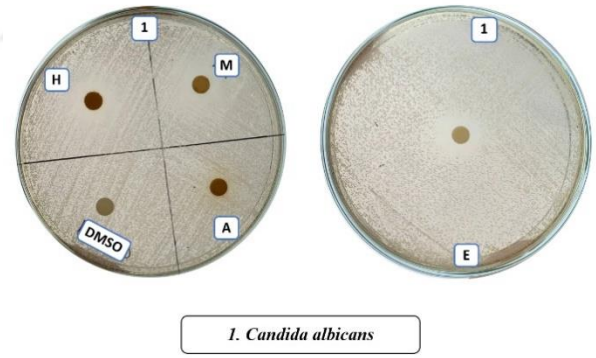

Figure 4.3. Inhibition zones showing antimicrobial activity of *S. costus* root against *C. albicans*. **E**) essential oil, **A**) aqueous, **M**) methanolic, and **H**) hexane-chloroform extracts.

Table 4.3. The antimicrobial activity of different *S. costus* extracts according to inhibition zones.

Tested substance	Mean inhibition zones (mm)								
	Gram-positive bacteria			Gram-negative bacteria					Fungi
	E. faecalis	*S. epidermidis*	*S. aureus*	*E. cloacae*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. baumannii*	*C. albicans*
Aqueous extract of *S. costus* (200 mg/ml)	8.3 ± 1.5	-	11.6 ± 2.3	-	-	-	-	-	-
Methanolic extract of *S. costus* (200 mg/ml)	9.6 ± 0.6	13 ± 2.6	11 ± 2	9 ± 1.7	-	-	-	-	11.6 ± 1.5
Hexane/chloroform extract of *S. costus* (200 mg/ml)	8.6 ± 0.6	15.3 ± 1.5	13.3 ± 0.6	7.6 ± 1.5	-	7 ± 1	-	8.3 ± 1.5	14.6 ± 1.5
Essential oil of *S. costus* (200 µl/ml)	11.5	12.5	12	11	8	7.5	-	8	14
Ciprofloxacin (5 mcg)	22.5	32.5	26	33	36.5	22	22.5	-	-
Gentamicin (10 mcg)	14	24	18	21	15	15	23	-	-
DMSO	-	-	-	-	-	-	-	-	-

4.2.2. MIC and MBC/MFC

The aqueous extract exhibited the highest activity with the lowest MIC (6.25 mg/ml) against *S. aureus*, followed by *S. epidermidis* and *E. faecalis* with MICs of 12.5 mg/ml and 25 mg/ml, respectively. The aqueous extract did not show a bacteriostatic effect on other tested bacterial species. *C. albicans*' growth was significantly reduced with

increasing the aqueous extract concentration; however, no MIC was achieved at any tested concentration.

The lowest MIC value (3.12 mg/ml) for methanolic extract was recorded for *S. aureus*. At a concentration of (6.25 mg/ml), the extract inhibited the growth of *S. epidermidis*. Moreover, *E. faecalis* showed no visible growth at 50 mg/ml, hence it was recorded as the MIC. For *E. cloacae* the MIC was recorded at 12.5 mg/ml. The growth of *E. coli, P. aeruginosa, K. pneumonia, and A. baumannii* was not inhibited at any tested concentration. MIC value for *C. albican*s was recorded at 6.25 mg/ml.

Hexane-chloroform extract revealed the highest antimicrobial activity against *S. aureus* (MIC value of 6.25 mg/ml), followed by *S. epidermidis* (12.5 mg/ml), *E. faecalis* (25 mg/ml), *E. cloacae* and *A. baumannii* (50 mg/ml). However, hexane-chloroform did not show any observable antimicrobial activity against *P. aeruginosa* and *K. pneumonia*. For *C. albicans* the MIC was 12.5 mg/ml.

The essential oil worked well against *S. epidermidis, C. albicans, S. aureus, and E. cloacae* with MIC values of 3.12, 3.12, 6.25, 12.5 µl/ml, respectively. No visible growth of *E. faecalis, A. baumannii*, and *K. pneumonia* was seen at 50 µl/ml concentration, hence it was recorded as the MIC for these bacteria. The growth of *E. coli* and *P. aeruginosa* were not affected by any of the tested concentrations.

The MBC/MFC values were recorded at a concentration higher than the MIC value at which no microorganism was present. Further detail regarding the MIC and MBC/MFC values are shown in Table 4.4, Figure 4.4 and 4.5.

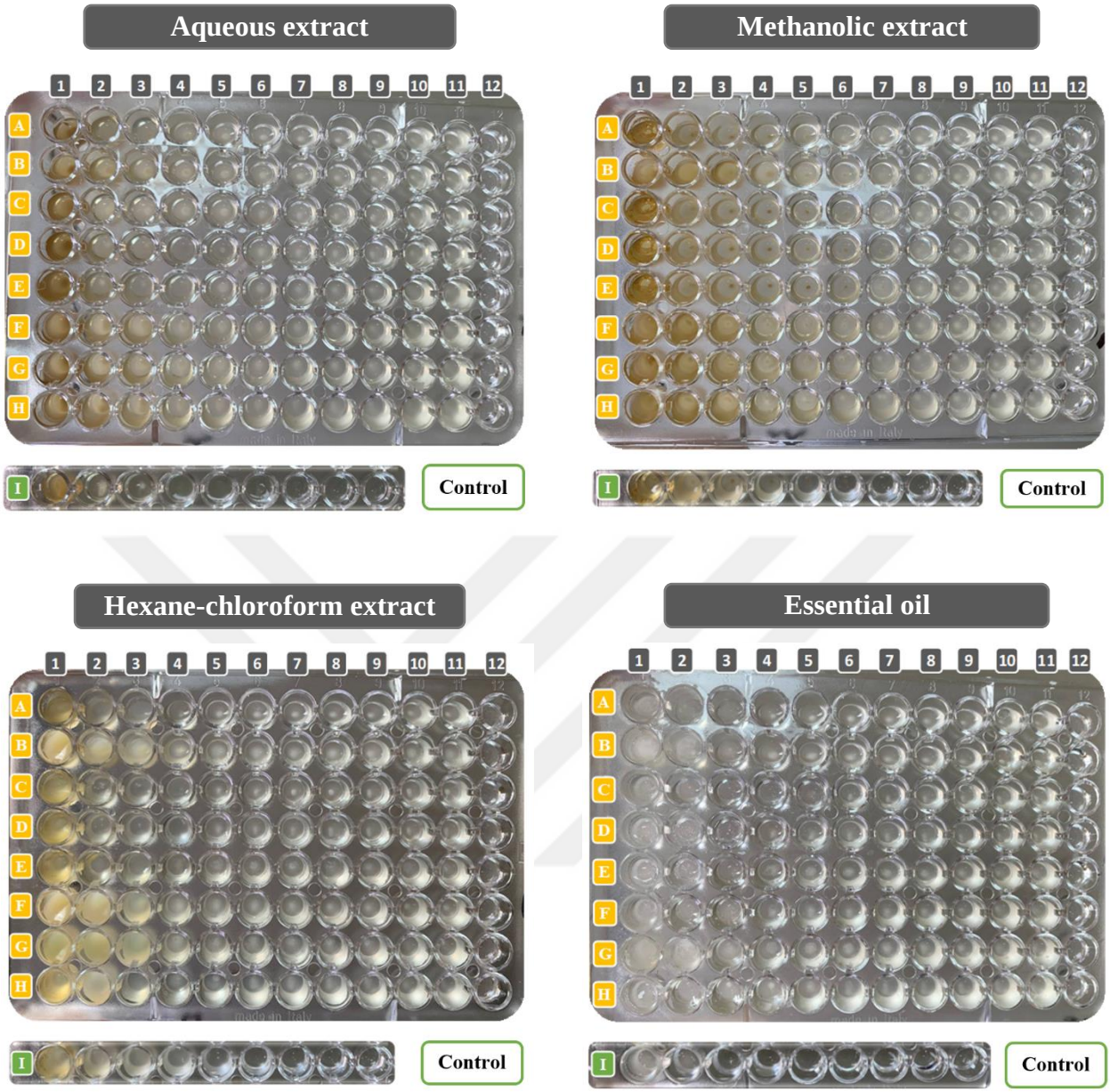

Figure 4.4. Microdilution assay to determine MIC of *S. costus* extracts against gram-positive and gram-negative bacteria. **A**) *E. faecalis*; **B**) *E. coli*; **C**) *S. epidermidis*; **D**) *S. aureus*; **E**) *E. cloacae*; **F**) *K. pneumoniae*; **G**) *P. aeruginosa*; **H**) *A. baumannii*. **1**) 50 mg/ml; **2**) 25 mg/ml; **3**) 12.5 mg/ml; **4**) 6.25 mg/ml; **5**) 3.12 mg/ml; **6**) 1.56 mg/ml; **7**) 0.78 mg/ml; **8**) 0.39 mg/ml; **9**) 0.2 mg/ml; **10**) Growth control (CAMHB + Bacteria); **11**) Solvent control (CAMHB + DMSO + Bacteria); **12**) Sterile control (CAMHB only). (The unit for essential oil concentration is µl/ml instead of mg/ml).

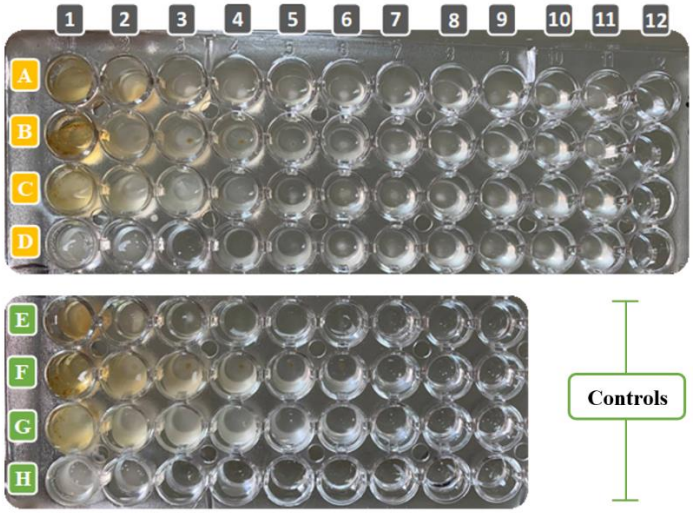

Figure 4.5. Microdilution assay to determine MIC of *S. costus* extracts against *C. albicans*. **A**) Aqueous extract; **B**) Methanolic extract; **C**) Hexane-chloroform extract; **D**) Essential oil; **E**) Aqueous extract control; **F**) Methanolic extract control; **G**) Hexane-chloroform extract control; **H**) Essential oil control. **1**) 50 mg/ml; **2**) 25 mg/ml; **3**) 12.5 mg/ml; **4**) 6.25 mg/ml; **5**) 3.12 mg/ml; **6**) 1.56 mg/ml; **7**) 0.78 mg/ml; **8**) 0.39 mg/ml; **9**) 0.2 mg/ml; **10**) Growth control (CAMHB + Bacteria); **11**) Solvent control (CAMHB + DMSO + Bacteria); **12**) Sterile control (CAMHB only). (The unit for essential oil concentration is µl/ml instead of mg/ml).

Table 4.4. The MIC and MBC/MFC for different extracts of *S. costus*.

Tested extracts				Aqueous extract (mg/ml)	Methanolic extract (mg/ml)	Hexane/chloroform extract (mg/ml)	Essential oil (µl/ml)
MIC and MBC/MFC values	Gram-positive bacteria	*E. faecalis*	MIC	25	50	25	50
			MBC	50	>50	50	>50
		S. epidermidis	MIC	12.5	6.25	12.5	3.12
			MBC	25	12.5	25	6.25
		S. aureus	MIC	6.25	3.12	6.25	6.25
			MBC	12.5	6.25	12.5	12.5
	Gram-negative bacteria	*E. cloacae*	MIC	12.5	12.5	50	12.5
			MBC	25	25	>50	25
		E. coli	MIC	-	-	-	-
			MBC	-	-	-	-
		K. pneumoniae	MIC	-	-	-	50
			MBC	-	-	-	>50
		P. aeruginosa	MIC	-	-	-	-
			MBC	-	-	-	-
		A. baumannii	MIC	-	-	50	50
			MBC	-	-	>50	>50
	Fungi	*C. albicans*	MIC	-	6.25	12.5	3.12
			MFC	-	12.5	25	6.25

5. DISCUSSION

Due to the significant reduction in mortality rates achieved through antimicrobial therapies, millions of human lives have been saved. However, the efficiency of most antimicrobials is being threatened by increasing resistance in microorganisms. The misuse and over-use of medications may cause the selection of antimicrobial-resistant bacteria and fungi, which has made infectious diseases more difficult to treat and has raised the mortality and morbidity of previously treatable infections **(**Paphitou, 2013; Byarugaba, 2004).

Infections by antimicrobial resistant microorganisms are more inclined to require more toxic and expensive drugs, increase the risk of death, and prolong hospitalization (Graf & Martin, 2000). Hence, as multidrug resistant microorganisms (Superbugs) become more prevalent, additional costs of therapy will become a bigger threat to the national, regional, and local health care systems (DiazGranados et al., 2008).

Because of the rapid increase in the prevalence of resistant pathogenic microorganisms to antibiotics and the side effects of the current antimicrobial agents, attention has been drawn to the study of biologically active substances derived from plants to treat those pathogens (Essawi & Srour, 2000). Hence, evaluating the antimicrobial activity of plant extracts has been seen as a way to achieve this goal, as many naturally occurring substances of plants have been observed to have some level of antimicrobial activity (Kumar et al., 2006). The findings of many investigations suggest that medicinal plants can be a potential source of novel antimicrobial agents, even against various antibiotic-resistant pathogens (Kone et al 2004). In the current study, the use of *S. costus* root extracts in treating selected bacterial and fungal pathogens were evaluated. Furthermore, phytochemical analysis of the extracts was also performed. Quality studies regarding the antimicrobial activity of *S. costus* root is scarce, and the available studies are highly controversial and have only tested the extract's efficacy on a few microbial species.

S. costus is one of the more important medicinal plants that is used in many local medicines all over the world, which is also referred to in the literature as *Saussurea lappa*, *Aucklandia lappa,* and *Aucklandia costus* (Wei et al., 2014). *S. costus* is indigenous to India, China, and Pakistan and grows primarily in the Himalayan region (Gwari et al., 2013).

Previous studies regarding the phytochemical analysis of *S. costus* root extracts have shown that its extracts contain a wide range of phytochemicals, including flavonoids, glycosides, alkaloids, resins, carbohydrates, steroids, saponins, phenolic compounds and many more (Abdelwahab et al., 2019). Hence, the diverse biological activities of the plant root, including antifungal and antibacterial activities, may be due to the presence of this vast diversity of photochemicals (Abdallah et al., 2017). Phenolic compounds are one of the classes of phytochemical that are commonly present in plants and have both antifungal and antibacterial activities. In our study, more phenolic compounds with higher concentrations were found in methanolic extract compared to aqueous extract. Similar findings were observed by Deabes et al. (Deabes et al., 2021).

Among the reported studies in the literature, the extract contents and concentrations obtained from the *S. costus* root varied, this may be due to a variety of environmental factors. Negi et al. (Negi et al., 2014) reported that the contents of dehydrocostus lactone and costunolide in the *S. costus* roots growing at a high altitude was significantly higher than in lower altitude samples. This variation in bioactive compounds may affect the therapeutic value of this medicinal plant. The available studies report different results regarding the phytochemicals that are responsible for the antimicrobial property of *S. costus*. Acylated flavone glycosides were detected in the roots of *S. costus,* and these glycosides are known to be the reason for the antifungal activity of this plant (Negi et al., 2013). Minhas et al. (Minhas, 2017) found that flavonoids have the best correlation with the antibacterial potential of *S. costus* extracts against *P. aeruginosa*. This agrees with Goswami et al. and Xie et al., who revealed that flavonoids can impart antibacterial activity by preventing nucleic acid synthesis and cytoplasmic membrane function (Goswami & Chatterjee, 2014; Xie et al., 2015). Negi et al. observed that samples with higher levels of dehydrocostus lactone and costunolide had greater antibacterial activity (Negi et al., 2014).

The findings of our study indicated that *S. costus* root has moderate to good antimicrobial activity against gram-positive bacteria and *C. albicans* as a representative fungal microorganism, and this activity increases proportionally to the increase in extract concentration.

There is a contradiction in the literature regarding the antimicrobial activity of *S. costus* root against some pathogenic microorganisms. This may be related to the solvents

used in the extraction methods and the existence of different chemical constituents, which are influenced by the extracting solvent's polarity (Al Otibi et al., 2019; Abdallah et al., 2017). Therefore, the susceptibility of microorganisms may depend on the bioactive constituents extracted by the solvents, because microorganisms respond differently to various phytochemicals. Mohamed et al. tested the inhibitory activity of various solvent extracts of *S. costus* roots against various bacteria. Methanolic extract had the highest antimicrobial activity against all of the pathogens tested, while water extracts showed the least (Mohamed et al., 2017). Similar findings were observed in our study as essential oil, methanolic and hexane-chloroform extracts showed high antimicrobial activity, but water extract possessed the lowest antimicrobial activity in both disc diffusion and microdilution assays.

It is worthy to note that among the few conducted studies previously carried out on the antimicrobial property of *S. costus* root extracts, solely the disc diffusion method has been used by most of them in order to determine its antimicrobial efficacy, which may not be an uptodate method especially for organic extracts. However, in the current study, both disc diffusion and microdilution assays were used to provide a more accurate result.

According to the previously published research on *S. costus* root, there is controversy regarding the susceptibility of gram-positive and negative bacteria to the extracts of the plant root. Some studies report the susceptibility of both gram-positive and negative bacteria to *S. costus* root extracts. Minhas et al. (Minhas, 2017) tested the antibacterial activity of various *S. costus* extracts (ethanolic, aqueous ethyl, petroleum ether, acetate, and methanolic extracts) against *P. aeruginosa*, *E. coli* and *S. aureus*. Antibacterial activity against *S. aureus* was found to be greatest in ethanolic extract. The antibacterial activity against *P. aeruginosa* was highest in aqueous extract. While all the tested extracts showed almost the same significant activity against *E. coli*. In their study, Hasson et al (Hasson et al., 2013) also reported that *S. costus* exhibits a significant level of antibacterial activity against a number of pathogenic gram-negative and gram-positive bacteria, such as *P. aeruginosa, E. coli, K. pneumonia,* and *S. aureus.* Meanwhile, a study by Negi even reported that *E. coli* (gram-negative) had a higher susceptibility to the *S. costus* extract (highest zone of inhibition with lowest MIC) when compared to the gram-positive *S. aureus* (highest MIC) (Negi et al., 2014).

On the other hand, other studies reported higher susceptibility of gram-positive bacteria. In an investigation by Abdullah et al. (Abdallah et al., 2017), the antimicrobial screening of ethanolic and methanolic extracts of *S. costus* root was performed against several pathogenic bacteria. The findings revealed that the extracts were more effective against gram-positive bacteria; *B. cereus* had the highest susceptibility to the extracts, followed by *S. epidermidis, S. saprophyticus*, and *S. aureus*. However, the extracts had weak or no activity against gram-negative bacteria; *P. vulgaris, P. aeruginosa*, and *E. coli* showed only weak susceptibility. While *K. pneumonia* and *S. flexneri* showed no susceptibility at all to the extract. This is in agreement with the findings of (Mohamed et al., 2017), which showed that methanolic extract of *S. costus* root considerably inhibited the growth of *B. subtilis* and *S. aureus* (gram-positive), but it had no effect on *P. aeruginosa* and *E. coli* (gram-negative). In addition, the results of a study by Deabes and colleagues showed that *S. costus* extract had antibacterial effect against tested gram-positive strains (*B. cereus, S. sciuri,* and *S. aureus*) (Deabes et al., 2021). Our study supported the last-mentioned studies, as all the tested extracts exhibited higher antimicrobial activity against gram-positive bacteria and fungi, but only had weak or no activity against gram-negative bacteria.

The cell wall composition of these bacteria may explain the differences in microbial susceptibility to extracts. Gram-negative bacteria have an outer membrane that acts as a barrier to various chemicals, making them less susceptible to various extracts (Kalayou et al., 2012). This explains why gram-negative bacteria were not inhibited by the *S. costus* extract. However, the gram-positive bacteria possess a thicker covering of peptidoglycan sheet that is highly permeable to a wide range of chemicals, making them more susceptable to many extracts (Nester et al., 2004). This could be the reason why the *S. costus* extracts were effective against gram-positive bacteria in our experiment.

The roots of *S. costus* are a great source of active constituents that can affect the integrity and stability of the fungal cell membrane structure, eventually resulting in the death of fungal cells (Al Otibi et al., 2019). In a research by Abdallah et al. (Abdallah et al., 2017), methanol and ethanol extracts of *S. costus* were used to treat *C. albicans* and *A. niger. C. albicans* was resistant to the extracts, whereas the growth of *A. niger* was inhibited at a MIC of 50mg/ml. This contradicts our results and also the findings of

Mohamed et al. who reported a significant antifungal activity of *S. cotsus* extract against *C. albicans* **(**Mohamed et al., 2017).

S. costus is a rich source of valuable components that can be used to develop new therapeutic agents and can even substitute antibiotics in the treatment of certain pathogens. Khalid et al. **(**Khalid et al., 2011**)** stated that the methanolic extracts of the *S. costus* root can be preferred over cefuroxime and gentamycin in treating *S. aureus* infections and metronidazole in treating infections caused by *B. subtalis*. Hence, they concluded that in the treatment of some infections, *S. costus* extracts can be used instead of antibiotics.

6. CONCLUSIONS

Our study aimed to investigate the antimicrobial activity of different types of *S. costus* root extracts against multiple gram-positive and gram-negative bacteria, and also a candidate fungus. We also aimed to analyze the phytochemical content of each extract. Based on our results and the evaluation of the literature, the following conclusions were reached:

1- Regarding the phytochemical content of the plant root, various results have been obtained in the literature, which can be attributed to different geographical and environmental factors. This has resulted in variations in the reported antimicrobial activity among the available studies in the literature, as different compounds produce different effects.

2- In our study, the highest variation of phytochemicals was found in hexane-chloroform extract, followed by essential oil and methanolic extract. The aqueous extract contained the least variation and concentration of these compounds.

3- Our results indicated that *S. costus* root extracts have moderate to good antimicrobial activity against gram-positive bacteria and *C. albicans* as a representative fungal microorganism, and this activity increases proportionally to the increase in extract concentration. Meanwhile, weak or no activity was observed against gram-negative bacteria.

4- *S. costus* root can be considered as a potential natural source of antimicrobial agents to fight the global burden of antimicrobial resistant pathogens. We suggest further studies on more and different microbial species, especially antimicrobial resistant strains, in order to determine the full potency of *S. costus* root.

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