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MICROBIOLOGY

Investigation of antibacterial and antifungal activity of *Saussurea costus* root extracts

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Abstract: Infectious diseases are a serious danger to public health, and plants may be a potential source of novel antimicrobial agents. In this 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 *Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Enterobacter cloacae, Enterococcus faecalis, Klebsiella pneumonia, Acinetobacter baumannii, Escherichia coli, and Candida 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 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*. *S. costus* root can be considered as a potential natural source of antimicrobial agents to fight pathogen microorganisms.

Key words: Antibacterial activity, antifungal activity, bacteria, extract, fungi, *Saussurea costus*.

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). In addition, the emergence of multidrug-resistant microorganisms has been reported 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 toward 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 (Abdallah et al. 2017, Nadda et al. 2020). 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 have been used since ancient times is *Saussurea costus (S. costus)*. *S. costus* is well known in Islamic medicine and 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 is currently inadequate research available in the literature regarding the antibacterial and antifungal property of *S. costus*. Hence there exists a gap in 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*.

MATERIALS AND METHODS

Plant material

S. costus root samples extraction process and the chemical analysis of the extracts was performed at the Research Laboratory Practice and Research Center of Tokat Gaziosmanpasa University. The antimicrobial activity assessment of the obtained *S. costus* root extracts were performed at the Microbiology Laboratory of Tokat Gaziosmanpaşa University Research and Application Hospital. The microbial species needed for this research were obtained from the microbial storage of the same hospital. In the current study, essential oil, aqueous, hexane-chloroform, and methanolic extracts were extracted from *S. costus* root.

Essential oil extraction

Essential oil of the plant was acquired by water distillation via Clevenger- type apparatus. 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.

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°C for further antimicrobial activity testing.

Methanolic and Hexane-Chloroform extractions

Methanolic extract was prepared from 100 g of the 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°C for further antimicrobial activity testing.

Sterility proofing of the extracts

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

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 the mobile phase was 0.8 ml/min, and the injection volume was 10 µl. Detection wavelength was set at 300/200 nm and the reference wavelength was 500/100 nm.

Identification of bacterial strains

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

Antimicrobial susceptibility screening

Disc diffusion test

Disc diffusion assay for antimicrobial susceptibility screening was conducted in accordance with the instructions provided by the Clinical Laboratory Standards Institute (CLSI 2016).

Preparation of impregnated discs

To prepare stock solutions, 200 mg of each extract was dissolved in one ml of their respective solvents, Dimethyl Sulfoxide (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.

Inoculums and inoculation procedure

The concentration of the inoculum was standardized to get a final concentration of approximately 1.5 x 108 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.

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 a positive control (Gentamycin and Ciprofloxacin), were applied. The essential oil was tested on a separate plate. Then, the plates were flipped and 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.

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, 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 (CLSI 2016).

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 0.5 McFarland diluted by a ratio of 1:100 (1.5 x 106 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).

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 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.

RESULTS AND DISCUSSION

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).

The results of the chemical analysis of all our tested extracts obtained by HPLC are shown in detail in Table I and Table II.

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 1 and Table III.

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.

Table I. 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.**

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 2 and Table III.

For *C. 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 3. The detailed results acquired in the assessment of the antimicrobial activity of *S. costus* by using disc diffusion assay are presented in Table III.

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 by 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.*

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

Compound	Percentage (%)		Compound	Percentage (%)	
	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

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

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. albicans* 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, and 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 IV, Figure 4 and 5.

Because of the rapid increase in the prevalence of resistant pathogenic microorganisms to antibiotics and the side effects of the current antimicrobial agents, attention

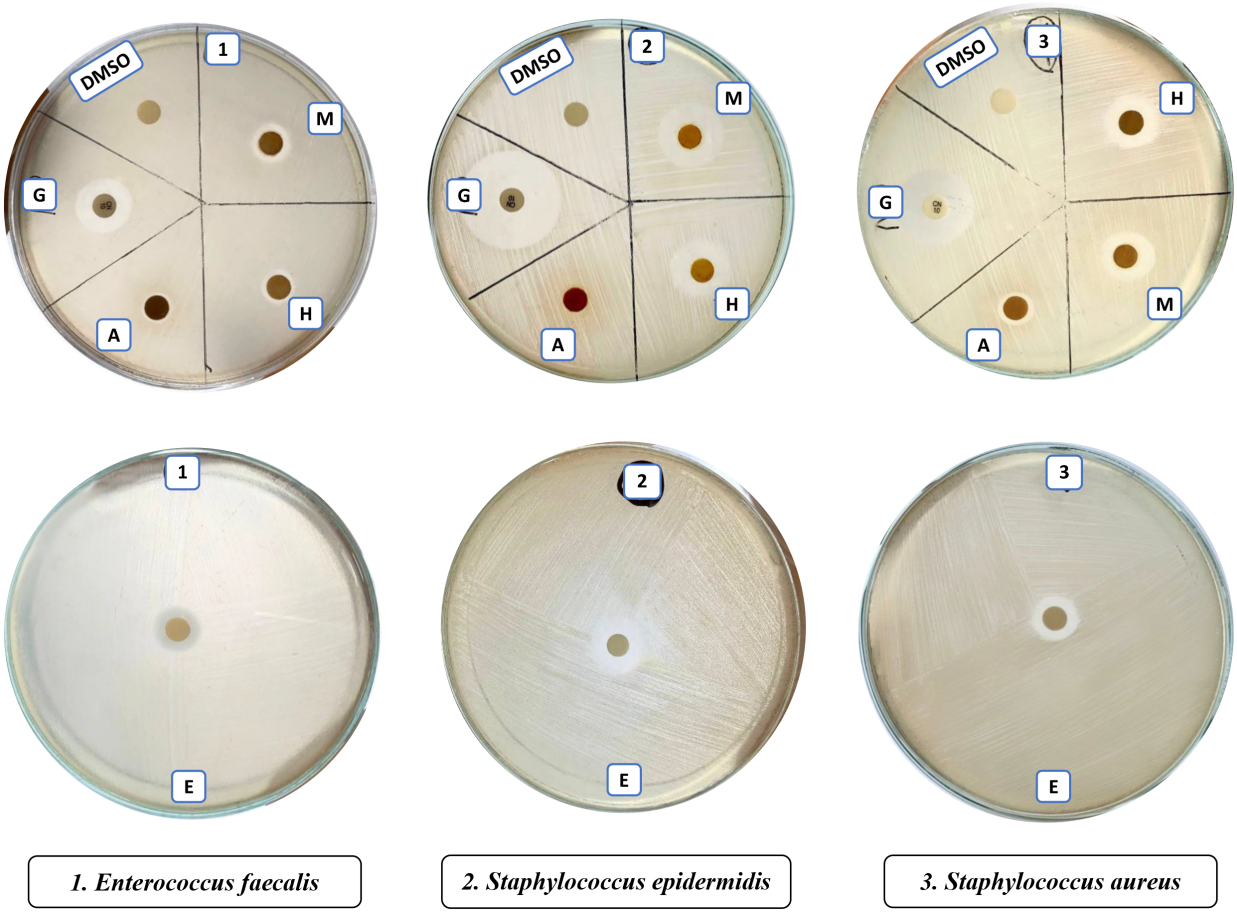

Figure 1. Inhibition zones showing antimicrobial activity of *S. costus* root against gram-positive bacteria. E) essential oil, A) aqueous, M) methanolic, and H) hexane- chloroform extracts, and G) gentamycin.

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). *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 phytochemicals (Abdallah et al. 2017). Phenolic compounds are one of the classes of phytochemical that are commonly present in plants and have both antifungal

Table III. The antimicrobial activity of different *S. costus* extracts according toinhibition 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* (200mg/ml)**	8.3 ± 1.5	-	11.6 ± 2.3	-	-	-	-	-	-
Methanolic extractof *S. costus* (200 mg/ml)	9.6 ± 0.6	13 ± 2.6	11 ± 2	9 ± 1.7	-	-	-	-	11.6 ± 1.5
Hexane/chloroformextract 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	-	-	-	-	-	-	-	-	-

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. (2021). Although the antimicrobial activity was low in the aqueous extract, any activity was likely due to Gallic acid and Catechin. The higher antimicrobial activity of methanolic extract may also stem from the presence of these compounds; Resveratrol, Gentisic acid, Quercetin, and t-cinnamic acid, in addition to those found in the aqueous extract.

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. (2014) reported that the contents of dehydrocostus lactone and costunolide in the *S. costus* roots growing at a high altitude were 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. Minhas et al. found that flavonoids have the best correlation with the antibacterial potential of *S. costus* extracts against *P. aeruginosa* (Minhas et al. 2017). This agrees with Goswami & Chatterjee (2014) and Xie et al. (2015), who revealed that flavonoids can impart antibacterial activity by preventing nucleic acid synthesis and cytoplasmic membrane function. Negi et al. (2014) observed that samples with higher levels of dehydrocostus lactone and costunolide had greater antibacterial activity. Dehydrocostus lactone and costunolide were also found in our study in significant proportions. Additionally, antimicrobial activities may also stem from the presence of substantial amounts of sesquiterpenes such as Elemene, Caryophyllenes, and Curcumene found in both hexane extract and essential oil. The essential oil also contained important antimicrobial compounds such as Thymol phenol and Humulene.

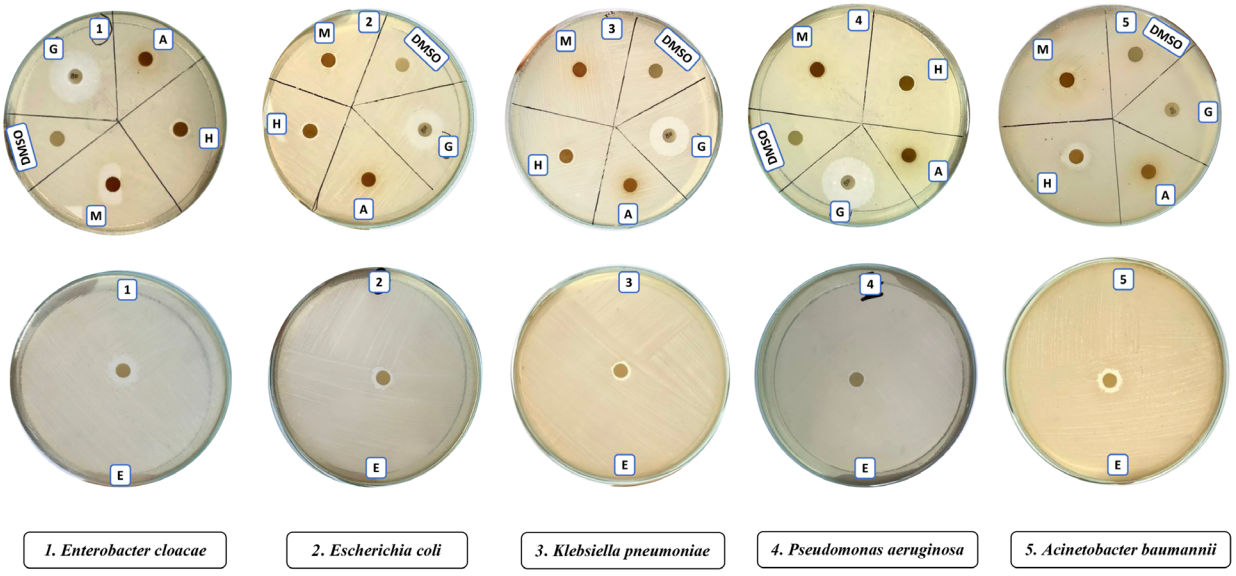

Figure 2. Inhibition zones showing antimicrobial activity of *S. costus* root against gram-negative bacteria. E) essential oil, A) aqueous, M) methanolic, and H) hexane- chloroform extracts, and G) gentamycin.

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 (Abdallah et al. 2017, Al Otibi et al. 2020). 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. (2017) 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. 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

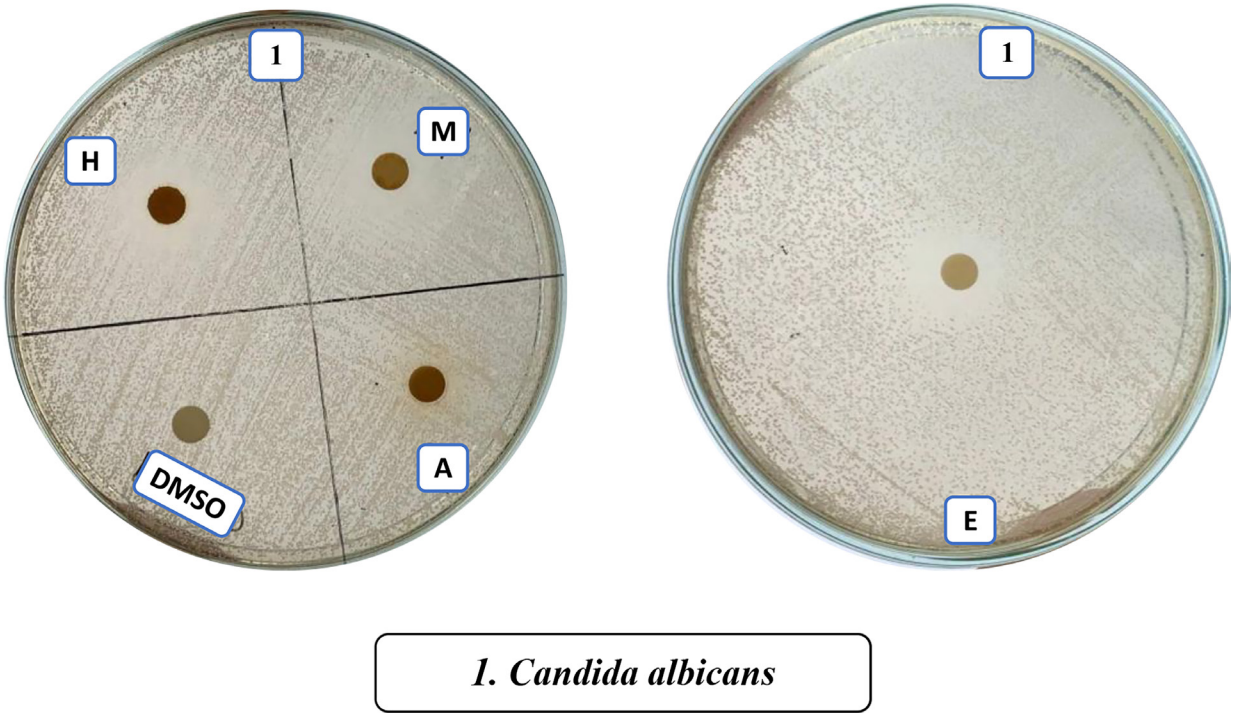

Figure 3. Inhibition zones showing antimicrobial activity of *S. costus* root against C. albicans. E) essential oil, A) aqueous, M) methanolic, and H) hexane-chloroform extracts.

root. Some studies report the susceptibility of both gram-positive and negative bacteria to *S. costus* root extracts. Minhas et al. (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 the 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. (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 et al. (2014) 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).

On the other hand, other studies reported higher susceptibility of gram- positive bacteria. In an investigation by Abdullah 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

Table IV. 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)	Essentialoil (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

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 et al. (2021) 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

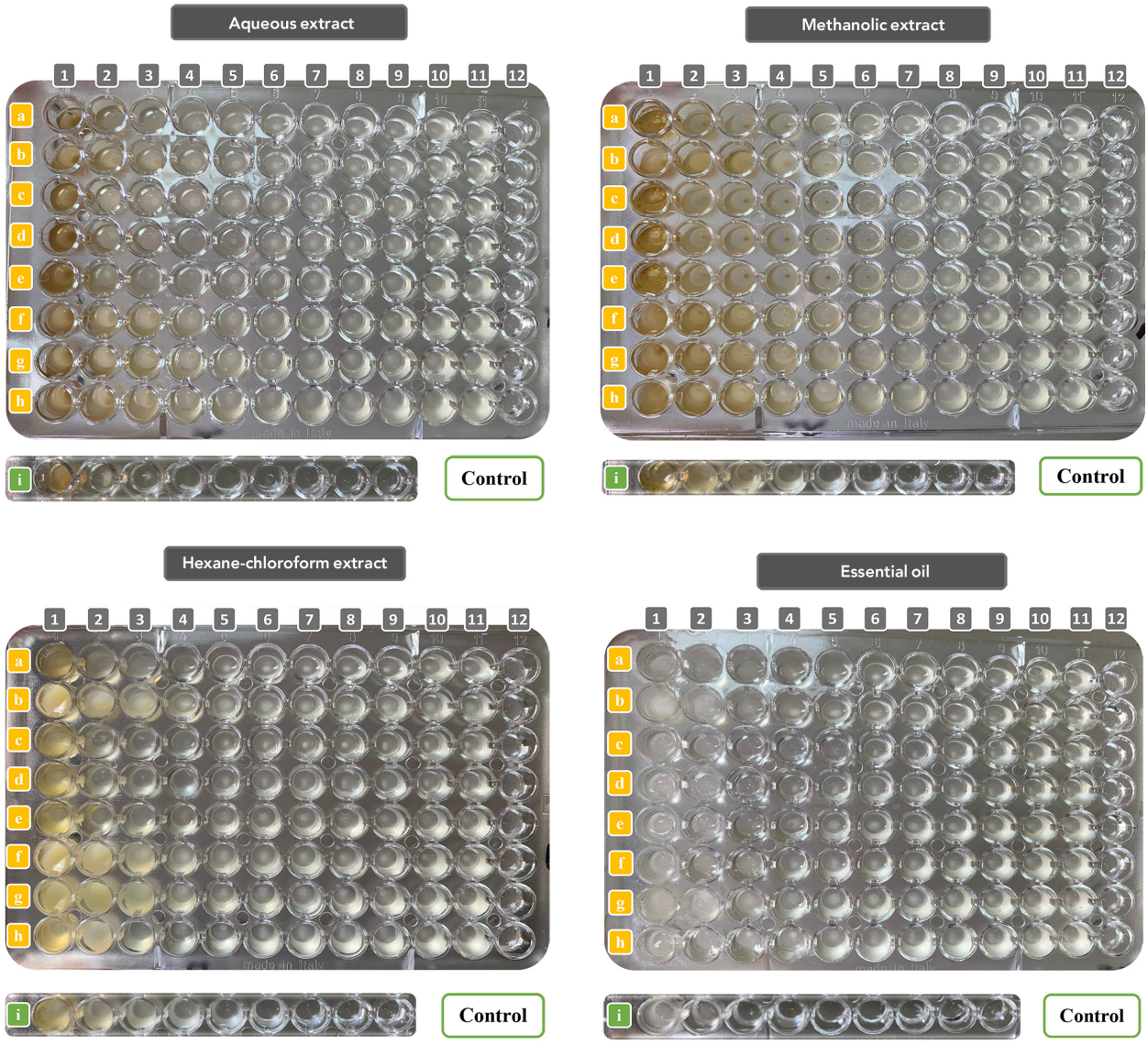

Figure 4. Microdilution assay to determine MIC of *S. costus* extracts against gram-positive and gram-negative bacteria. a) *E. faecalis*; b) *E. coli*; c) *S. epidermidis*; d) *S. aureus*; e) *E. cloacae*; f) *K. pneumoniae*; g) *P. aeruginosa*; h) *A. baumannii*. i) 50 mg/ml; 2) 25 mg/ml; 3) 12.5 mg/ml; 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).

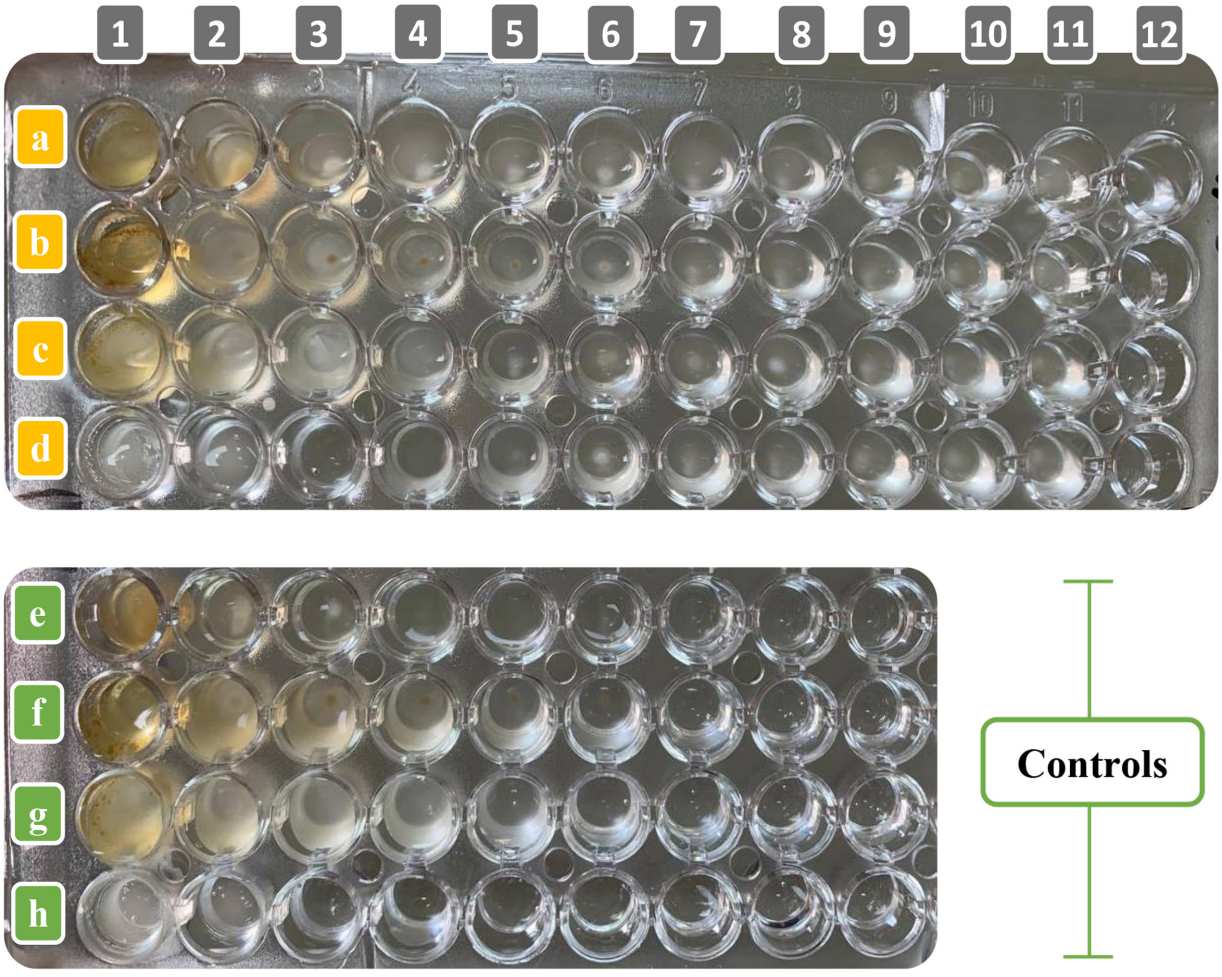

Figure 5. Microdilution assay to determine MIC of *S. costus* extracts against *C. albicans*. a) Aqueous extract; b) Methanolic extract; c) Hexane-chloroform extract; d) Essential oil; e) Aqueous extract control; f) Methanolic extract control; g) Hexane- chloroform extract control; h) Essential oil control. i) 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).

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 susceptible to many extracts (Silhavy et al. 2010). 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. 2020). In a research by 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). This contradiction in the results of different studies may be attributed to multiple reasons, such as the use of different solvents/ extraction methods, different microbial strains, different concentrations of the extract, and different geographical sources for the *S. costus*.

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. (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. subtilis*. Hence, they concluded that in the treatment of some infections, *S. costus* extracts can be used instead of antibiotics.

CONCLUSION

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. 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. 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. *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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