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Antimicrobial and Antibiofilm Potentials of Methanolic Extract from Cultivated Tropical Hibiscus (*Hibiscus rosa sinensis* L.) Flower

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ABSTRACT

Using natural antimicrobials to treat microbial infections is one of the hopeful alternatives to combating antibiotic-resistant strains throughout the world. In this work, the antimicrobial and antibiofilm properties of methanolic extract derived from tropical *hibiscus* flower were investigated against a variety of microbial pathogens. Results showed that the extract contained considerable amounts of anthocyanins, polyphenols, and flavonoids. Furthermore, the extract contained high amounts of volatile compounds: Disulfide, di-tert-dodecyl (11.378%), Tridecanol, 2-ethyl-2-methyl- (11.940%), Didodecyl phthalate (13.701%), Eicosane (14.532%), and Octane, 2-methyl- (15.242%).

Interestingly, the growth, cell morphology, and biofilms of the tested pathogenic bacteria were highly affected by the extract. High sensitivity towards the extract was exhibited by the tested bacteria: *Staphylococcus aureus* (36±0.1 mm), *Micrococcus luteus* (45±0.5 mm), *Escherichia coli* (45±0.15 mm), and *Klebsiella pneumonia* (40±0.5 mm), with MIC values of 1, 0.5, 0.5 and 1 mg/mL, respectively. The extract inhibited the formation of biofilm in *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Klebsiella pneumonia* by 46.4, 47.6, 46.3 and 38.4% respectively. The strongest antifungal activity of the extract was against *Fusarium solani* (34.7%) and *Alternaria phragmospora* (33.8%) at 8 mg/mL. The findings of this study indicated that tropical *hibiscus* flower extract may be promising for the development and design of natural antimicrobial alternatives.

INTRODUCTION

Currently, due to the inappropriate antibiotic use, microbial pathogens are showing extremely high and frightening levels of antibiotic resistance. In fact, if adequate actions are not immediately taken, this antibiotic resistance crisis may soon force humanity to return to a pre-antibiotic period (O'Neill, 2016). Because of the slow progress in antibiotic discovery and development over the past few decades, the impact of antibiotic resistance on rates of mortality and morbidity has increased (Hutchings *et al.,* 2019).

Under unfavorable conditions, microorganisms can form biofilms, where the microbial cells attach to each other and embed themselves in a self-produced extracellular polymeric matrix that can adhere to both biotic and abiotic surfaces to resist and survive (Schulze *et al.*, 2021).

Bacterial biofilms are common in medical and industrial settings; they are undesirable and contribute to chronic infections because they can resist antibiotics, host defense, and other external stresses (Vestby *et al.,* 2020). Therefore, the search for new antibiotics from natural sources is a vital issue of modern medicine to overcome the negative effects of multidrug-resistant microbes on society, economy, and public health (Bakal *et al.*, 2017). The World Health Organization claims that plants are the best source for obtaining a wide range of antimicrobial drugs (WHO, 2002). The antimicrobial properties of plants are attributable to the wide variety of their secondary metabolites, particularly phenolic compounds, and flavonoids (AlSheikh *et al.,* 2020).

Tropical hibiscus (Scientific name: *Hibiscus rosa sinensis* L.) is a shrub belonging to the family Malvaceae and has red trumpet-shaped flowers (Bala *et al.*, 2022). It is native to China and is extensively cultivated as an ornamental plant in the tropical and subtropical regions (Ross, 2003). It is traditionally used for the treatment of flu and cough, bronchitis, stomach pain, dysentery and diarrhea,and also for regulation of menstruation and stimulation of blood circulation (Jadhav *et al.,* 2009)

Considering the above, the current study was designed to evaluate the antimicrobial and antibiofilm activities of a methanolic extract from tropical *hibiscus* flower against many microbial pathogens.

MATERIALS AND METHODS

1-Plant Material:

Fresh flowers of cultivated tropical hibiscus were collected from botanical garden of Aswan University, Egypt (Latitude 24º04 N, Longitude 32º57 E). Samples were transported to the laboratory and washed with tap water to eliminate dust. The extraction of the samples was carried out immediately.

2-Microbial Pathogens:

Four pathogenic bacteria and three pathogenic fungi were used in this study: the bacterial species were Gram-positive *Staphylococcus aureus (*ATCC 25923) and *Micrococcus luteus* (ATCC4698), and Gram-negative *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia* (ATCC4352). The fungal species were *Alternaria phragmospora (*LC475453), *Fusarium solani* (LC510255) and *Cochliobolus spicifer* (LC520251.1). Bacterial and fungal species were kindly taken from stock cultures of the Bacteriology and Mycology Laboratory, Botany Department, Faculty of Science, Aswan University, Egypt.

3-Preparation of Methanolic Extract from Flowers:

The extract was prepared using the method of Muangrat *et al.,* (2017). Fresh flowers (100 g) were homogenized with the same volume (w/v) of methanol and then filtered. Methanol was evaporated by using a rotary evaporator to get fraction with methanol and the obtained extract was stored at 4ºC for further study.

4-Phytochemical Screening of The Extract:

4.1 Estimation of Anthocyanins Content:

The content of anthocyanins was determined according to the procedure described by Pa´sko *et al.* (2009). The absorbance of the extract was measured at wavelengths of 530 and 657 nm. The total anthocyanin (µmol) per gram of fresh flowers was calculated using the following equation:

$$\text{Anthocyanin (mg /g FW)} = \frac{\text{Abs } 530 - 0.33 \times \text{Abs } 657}{M} \times \frac{V}{\text{g}}$$

where: A = absorbance at 530 and 657 nm, V = volume of extract (mL), g= weight of samples (gm) and M = fresh mass of fresh flowers (gm)

4.2 Estimation of Polyphenols Content:

Polyphenols were spectrophotometrically quantified following the method of Ainsworth and Gillespie (2007). One milliliter of the extract was mixed with 1 mL of diluted Folin–Ciocalteu reagent with distilled water (1:10). The mixture was vortexed for 3 min, and then 1 mL of sodium carbonate solution (10 %, w/v) was added. The mixture was left at the room temperature for 1 h, and then the absorbance was measured at 700 nm. Total polyphenols content of the extract was calculated as mg gallic acid equivalents per gram of fresh weight using the equation of gallic acid standard curve: $y = 0.0001x - 0.004$, ($R^2 = 0.7728$), where y is absorbance at 700 nm and x is gallic acid concentration (mg/mL).

4.3 Estimation of Flavonoids Content:

Aluminum chloride assay according to the procedure of Da Silva *et al.* (2015) was used to assess the content of total flavonoids in the extract. Briefly, the reaction mixture contained 1 mL of the extract, 300 µL of sodium nitrite (5%, w/v), 300 µL of aluminum chloride (10%, w/v) and 2 mL of sodium hydroxide (1M) was left at room temperature for 12 min to settle. Absorbance was read at 510 nm. Total flavonoids concentration was calculated using quercetin standard curve equation: $y = 0.0011x + 0.8962$, ($R^2 = 0.7185$), where y is absorbance at 510 nm and x is quercetin concentration (mg/mL).

4.4 Estimation of Volatile Compounds by GC-MS:

TR-5MS GC column (Thermo Scientific) was used to separate the compounds present in the extract. The extract was diluted in hexane in a ratio of 1:10, and then 1 µL of the diluted extract was loaded into the column. The temperature of the column gradually increased from 60 to 240 ^oC. The compounds were propelled by helium (1 mL/min). Unknown compounds in the extract were identified by comparing their mass spectra and retention times with those of the reference compounds in the NIST library (Rassem et al. 2018)

5-In Vitro Antibacterial Activity:

The agar-well diffusion method as described by Salamon *et al.* (2019) was followed to evaluate the antibacterial activity of the extract. Onto the surface of Mueller Hinton agar plates, 100 µL of bacterial inoculum (10^7 CFU/mL) of each tested pathogenic bacterium was uniformly spread. Plates were left at room temperature for 20 min to absorb. Wells (6 mm) were made in the agar, and then filled with 100 µL of the extract, 100 µL of methanol as negative control and 100 µL of ampicillin (1 mg/mL) as positive control. Plates were incubated for 24 h at 37 ^oC. The growth inhibition zones that indicate the antibacterial effect were observed and their diameters were measured in millimeters. Three replicates were made for each tested bacterium.

5.1 Minimum Inhibitory Concentration (MIC):

Broth dilution assay as described by Meenu Krishnan *et al.* (2018) was used to determine the MIC values of the extract. The extract was diluted two-fold with Mueller Hinton broth to obtain the final concentrations of 4, 2, 1, 0.5 and 0.25 mg/mL. In test tubes, 5 mL of each concentration was inoculated with 10 µL bacterial suspension (10^7 CFU/mL). Mueller Hinton broth and Mueller Hinton broth supplemented with ampicillin (1 mg/mL) were served as negative and positive control respectively. Tubes were incubated for 24 h at 37 ^oC. To each tested bacterium, three replicates of each concentration were done. The lowest concentration of extract that inhibited visible growth was considered as MIC.

6-Effect of Extract on Morphological Shape of Bacteria:

Staphylococcus aureus and *Escherichia coli* were chosen as representatives for Gram-positive spherical-shaped and Gram-negative rod-shaped bacteria, respectively. To investigate the effect of the extract on the

morphological shape of bacterial cells, bacteria were grown in nutrient broth supplemented with the extract (1 mg/mL) and incubated at 37 ºC for 24 h. Bacteria that were grown in nutrient broth without the extract were used as controls. Cells were smeared on glass slides and stained with 0.1% safranin solution. The morphological shape of the cells was investigated by Novel Optics Biological Microscope (Model: N-800 M) using magnification 1000X. Cell sizes were measured using ocular and stage micrometer.

7-In Vitro Antibiofilm Activity:

Crystal violet assay according to Feoktistova *et al.* (2016) was followed to evaluate the antibiofilm activity of the extract. The test tubes contained 5 mL of nutrient broth and 1 mL of extract (1 mg/mL in methanol) were inoculated with 10 µL of bacterial suspension (10^7 CFU/mL). Tubes were incubated at 37 ºC for 24 h. 1 mL extract was replaced with 1 mL methanol and 1 mL ampicillin (1 mg/mL) in negative and positive control tubes, respectively. After incubation, the bacterial growth inhibition was determined by measuring the optical density at 600 nm. The planktonic cells were removed, the tubes were carefully washed three times with sterilized distilled water, and then left to dry. Tubes were stained with crystal violet solution (0.1 %) for 15 min and then washed with distilled water and dried. The crystal violet stain that bound to the biofilms was de-stained using 1 mL of absolute ethanol. The optical density of the de-staining solutions was read at 590 nm. The biofilm inhibition percentage was calculated using the following equation (Sandasi *et al.* 2008):

$$\text{Biofilm inhibition (\%)} = \frac{\text{OD590 NC} - \text{OD590 EXT}}{\text{OD590 NC}} \times 100$$

Where: NC is the negative control and EXT is the extract.

8-In Vitro Antifungal Activity:

The antifungal activity of the extract was assessed using Sinha and Gulati (1990) method. Autoclaved potato dextrose agar (PDA) media supplemented with two concentrations of the extract (5 mg/mL and 8 mg/mL) were poured in 5 cm diameter petri plates and left overnight at 4 ºC. Plates without extract were served as controls. Discs of 0.6 cm agar were removed from the center of the plates and replaced by 0.6 cm mycelial discs of the tested fungi. Plates were incubated at 28±2 ºC for 7 days. Three replicates were made for each treatment. The inhibition percentage of mycelial growth was calculated according to the following formula (Singh and Tripathi 1999):

$$\text{Inhibition (\%)} = \frac{\text{Avg. D. control} - \text{Avg. D. treatment}}{\text{Avg. D. control}} \times 100$$

Where Avg. D. is the average diameter of mycelial growth.

RESULTS

1. Anthocyanins, Polyphenols, Flavonoids and GC-MS Profile of The Extract:

In the present study, the extract of tropical hibiscus flower was found to has a total anthocyanin content of 2.97±1.48 mg/ g FW. The amount of polyphenols was quantified using Folin–Ciocalteu reagent and it was found that the extract contained 8.83±1.28 mg gallic acid equivalent/g FW. On the other hand, the aluminum chloride assay detected the presence of flavonoids in the extract at a concentration of 3.1±0.95 mg quercetin equivalent/g FW. Thirteen compounds were detected in the extract by GC-MS analysis (Table 1). The highest contents were for Disulfide, di-tert-dodecyl (11.378%), Tridecanol, 2-ethyl-2-methyl- (11.940%), Didodecyl phthalate (13.701%), Eicosane (14.532%), and Octane, 2-methyl- (15.242%).

Table 1. GC-MS Analysis of VOCs Released by methanolic extract of *Hibiscus* flowers.

NO	Compound name	Molecular formula	Molecular weight	Retention time	Content %	Peak Area	Peak Height
1	Heptane, 2,2,4,6,6-pentamethyl-	$C_{12}H_{26}$	170	4.426	17.923	1863723	88149
2	Nonane, 2,2,3-trimethyl-	$C_{12}H_{26}$	170	4.431	11.321	1709131	66867
3	Formic acid, 2-methylpentyl ester	$C_7H_{14}O_2$	130	5.213	4.379	661119	34707
4	Ethanamine, N-pentylidene-	$C_7H_{15}N$	113	7.374	2.767	1041370	80576
5	Octane, 5-ethyl-2-methyl-	$C_{11}H_{24}$	156	7.376	5.987	903832	69700
6	Octadecane	$C_{18}H_{38}$	254	7.736	5.787	873751	58814
7	Heptadecane, 2-methyl-	$C_{18}H_{38}$	254	9.089	17.894	2701569	145307
8	Pentacosane	$C_{25}H_{52}$	352	9.497	13.336	2013320	115811
9	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	402	11.378	11.470	1731725	89849
10	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	242	11.940	8.112	1224642	56867
11	Didodecyl phthalate	$C_{32}H_{54}O_4$	502	13.701	2.148	808381	35205
12	Eicosane	$C_{20}H_{42}$	282	14.532	10.701	1615566	40634
13	Octane, 2-methyl-	C_9H_{20}	128	15.242	11.014	1662776	52631

2. Antibacterial Activity and MIC:

The extract of tropical hibiscus flower was screened for antibacterial activity against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Klebsiella pneumonia* by well diffusion method. The extract interestingly affected both Gram positive and Gram-negative bacteria. *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Klebsiella pneumonia* exhibited considerable sensitivity towards the extract and showed inhibition zones of 36±0.1, 45±0.5, 45±0.15 and 40±0.5 mm respectively (Fig. 1). The minimum inhibitory concentration (MIC) of the extract was 1 mg/mL for *Staphylococcus aureus*, and 0.5 mg/mL for *Micrococcus luteus* and *Escherichia coli*, and 1 mg/mL for *Klebsiella pneumonia*.

3. Effect of Extract on Morphological Shape of Bacteria:

Interestingly, both *Staphylococcus aureus* and *Escherichia coli* exhibited cell morphological changes in response to the extract (Fig. 2). The cells of *Staphylococcus aureus* changed from aggregated cocci (1.5 µm in diameter) to curved rods (1 µm length×0.5 µm width). The cells of *Escherichia coli* changed from straight rods of 3 µm length×1.2 µm width to short rods of 1 µm length×0.5 µm width.

4. Antibiofilm Activity:

The extract considerably effected on the formation of bacterial biofilms. It was found that the biofilm inhibition was directly proportional with the growth inhibition (Fig. 3). The results showed that the biofilm formation was inhibited by 46.4, 47.6, 46.3 and 38.4 % in *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Klebsiella pneumonia*, respectively.

5. Antifungal Activity:

The extract inhibited the mycelial growth of the tested fungi at both the tested concentrations. *Alternaria phragmospora* and *Fusarium solani* exhibited concentration-dependent inhibition, where the percentages of inhibition were 21.13% and 26.08% at 5 mg/mL and 33.8% and 34.7% at 8 mg/mL, respectively. *Cochliobolus spicifer* showed the lowest inhibition percentage (9.09%) at both 5 mg/mL and 8 mg/mL (Fig. 4).

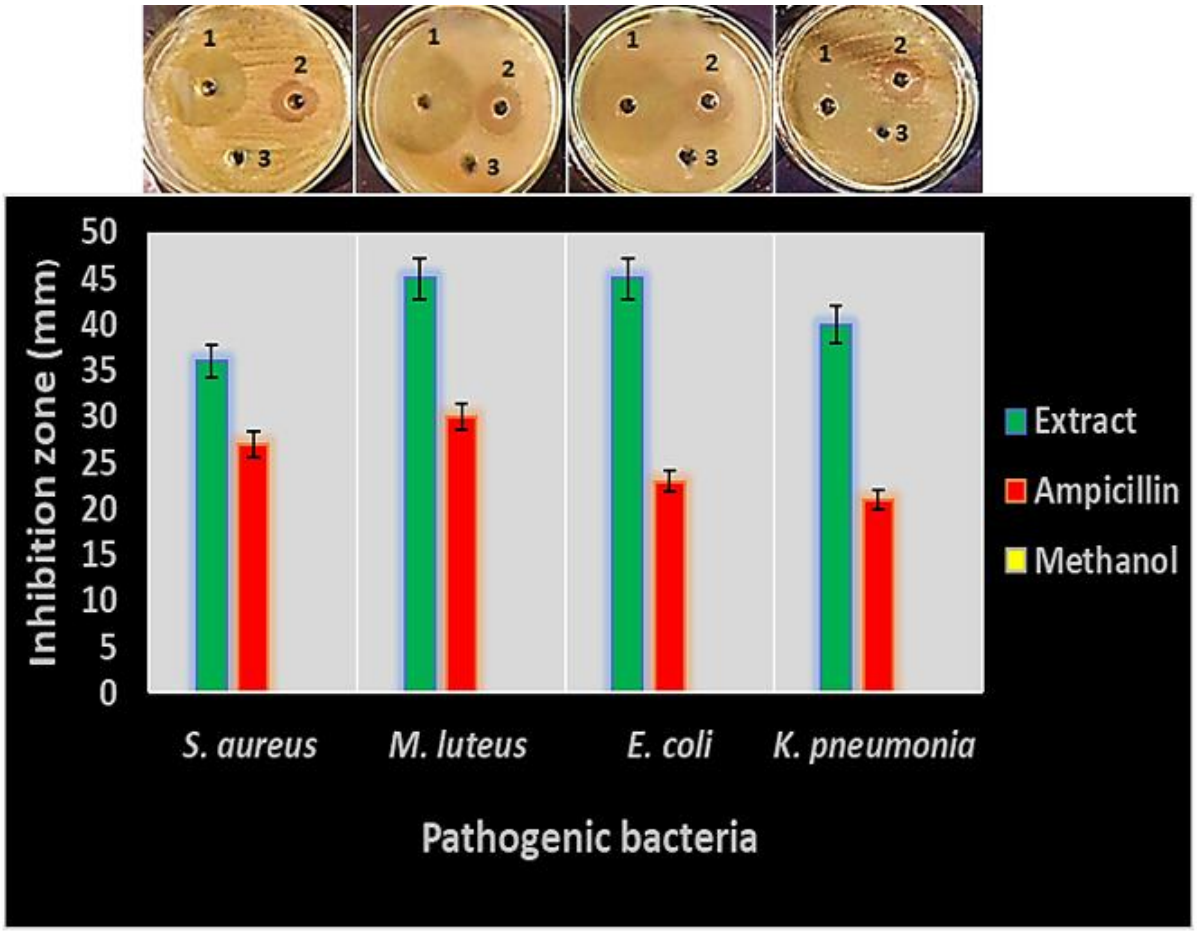

Fig.1 Antibacterial activity of tropical hibiscus flower extract. **1**: the extract, **2**: the positive control (ampicillin), **3**: the negative control (methanol).

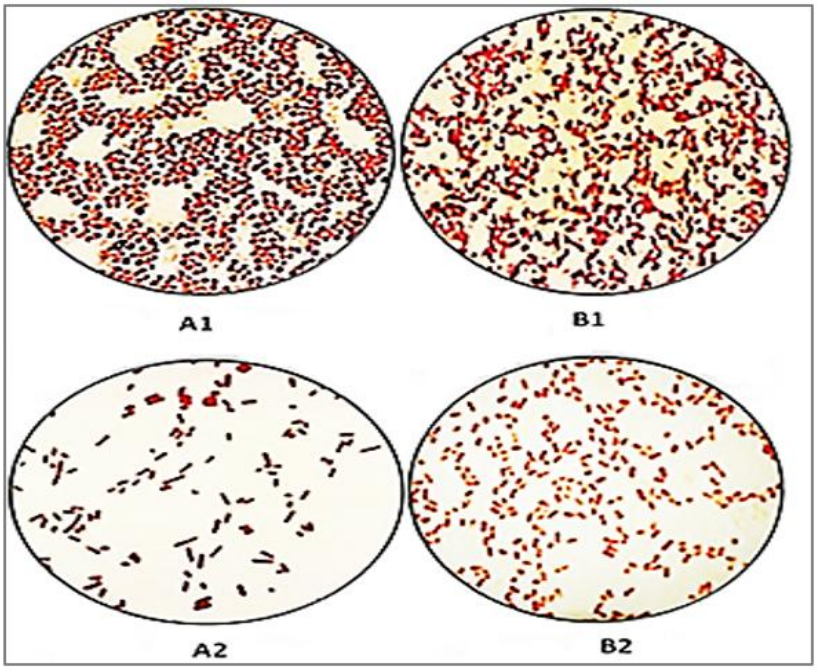

Fig.2 The effect of tropical hibiscus flower extract on bacterial cell shape. **A1**: control cells of *Staphylococcus aureus*, **B1**: treated cells of *Staphylococcus aureus*, **A2**: control cells of *Escherichia coli*, **B2**: treated cells of *Escherichia coli*.

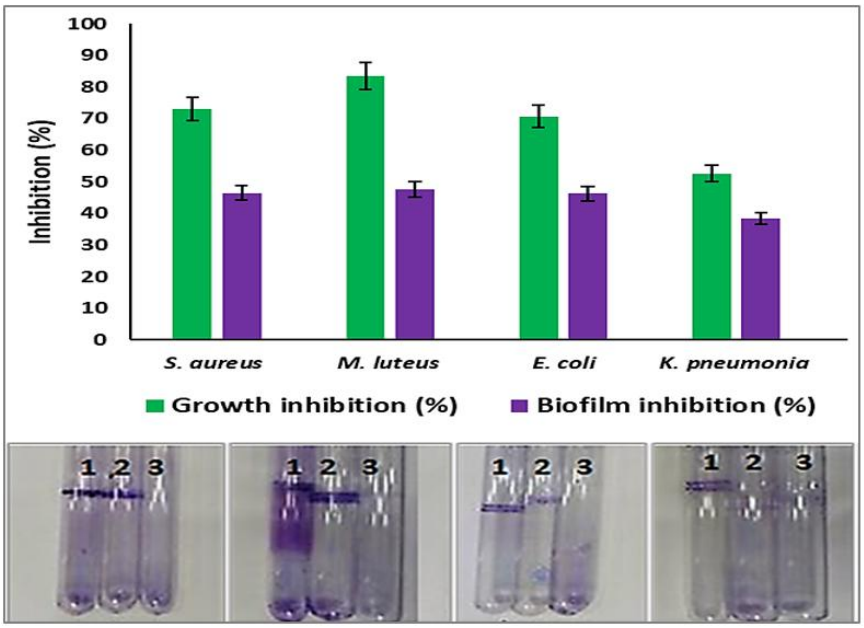

Fig.3 Growth and biofilm inhibition of pathogenic bacteria in response to tropical hibiscus flower extract. 1: the neative control (methanol), 2: the positive control (ampicillin), 3: the extract.

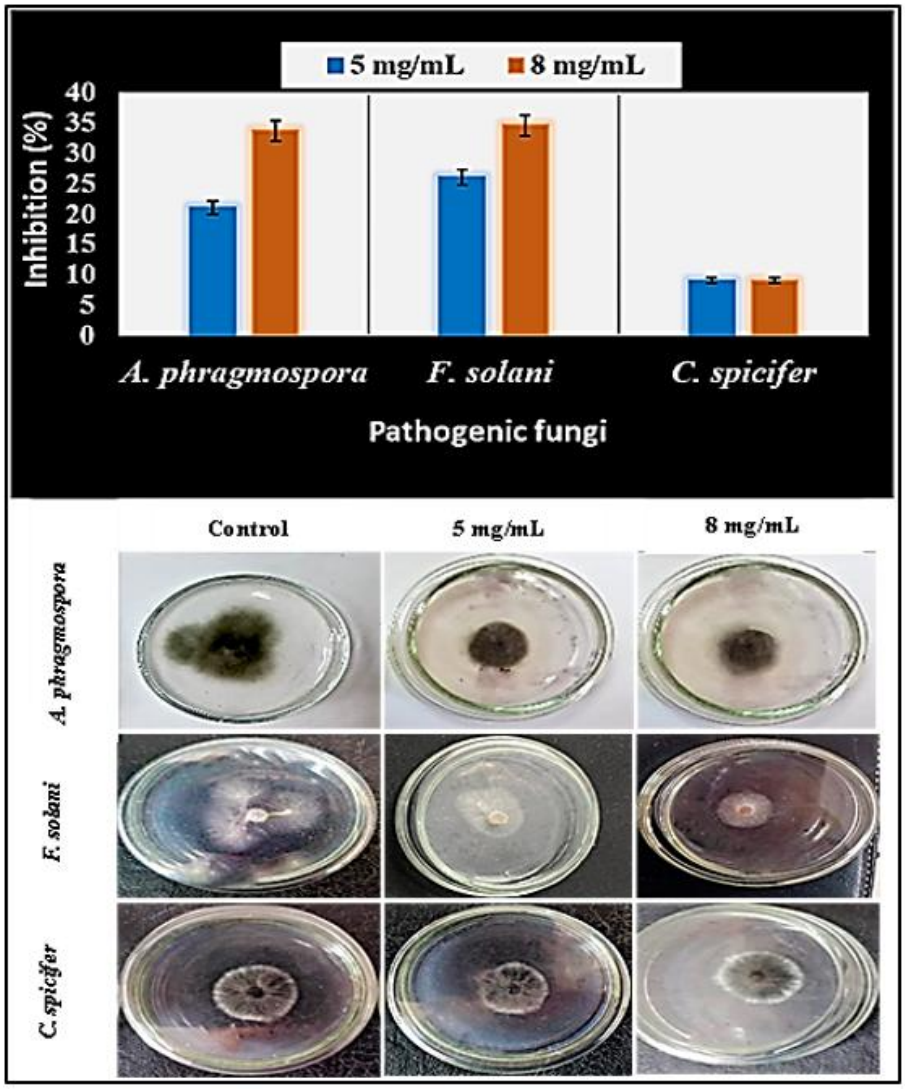

Fig. 4 Antifungal activity of tropical hibiscus flower extract.

DISCUSSION

Antibiotic resistance is a serious growing problem threats the public health worldwide (Vaou *et al.,* 2021). Therefore, searching for promising natural antimicrobial agents has become necessary. Plants having customary curative uses are being extensively screened for their antimicrobial activities to be considered as alternatives for chemical-based antibiotics (AlSheikh *et al.,* 2020). Utilizing naturally occurring antimicrobials obtained from plants can be a highly effective approach to reducing the need for traditional antibiotics and minimizing the risk of antibiotic resistance (Abreu *et al.,* 2012). In the present work, we investigated the antimicrobial and antibiofilm activities of tropical hibiscus flower extract against several common pathogenic bacteria and fungi.

The antibacterial activity of the extract was estimated towards four bacterial pathogens: *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Klebsiella pneumonia*. Interestingly, Gram-positive bacteria showed high sensitivity to the extract with the lowest MIC values compared to Gram-negative bacteria (Fig. 1). Our findings agreed with those of others who found that plant extracts have higher antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria (Zampini *et al.,* 2009; Sánchez *et al.,* 2016). This may be attributed to the differences in their cell wall structures and membranes, where Gram-negative bacteria have outer membranes and efflux pump systems that act as barriers for many molecules, preventing them from entering the cell (Lambert, 2002; Wendakoon *et al.,* 2012). In this study the antibacterial activity of the extract may be due to its high contents of anthocyanin, polyphenols and flavonoids that can destroy the cell wall, modify cell membrane permeability and complex with extracellular and soluble proteins of the bacterial cell and consequently inhibit its growth (Xie *et al.,* 2015; Bae *et al.,* 2022). On the hand, many hydrocarbons were detected in the extract by GC-MS analysis (Table 1). Previous studies indicated that hydrocarbons have antibacterial properties (Rahbar *et al.,* 2012; Begum *et al.,* 2016).

In the present study, it was observed that the bacterial cells changed their shape and size in response to the extract (Fig. 2). Our finding agreed with other studies that have demonstrated that in response to antibiotics, bacteria alter their cell morphology by changing cell size or curvature (Ojkic *et al.,* 2022). It was reported that *Escherichia coli* and *Pseudomonas aeruginosa* shifted their cell shape by reducing size and changing from rod to spherical upon treatment with antibiotics (Monahan *et al.,* 2014; Harris and Theriot 2016). Therefore, in the current investigation, changes in the size and shape of the treated bacterial cells may be a means to inhibit the action of the extract by reducing the flow of the extract into the cells.

Many pathogens form biofilms as a defense strategy against antibiotic action; this makes their treatment more complicated than that of their planktonic cells (De La Fuente-Núñez *et al.,* 2012). Bacterial biofilm is a continuing health threat worldwide, where over 60% of bacterial infections are caused by biofilms (Adeyemo *et al.,* 2022; Olawuwo *et al.,*2022). Therefore, the search for natural antibacterial agents to tackle this problem is of high priority (Zeng *et al.,* 2022). In the present study, the extract of tropical hibiscus s inhibited biofilms of all tested pathogenic bacteria by 38.4 – 47.6% (Fig. 3). The antibiofilm activity of the present extract may be due to its interference with Brownian motion and sedimentation force, which represent important factors in the adhesion of bacteria to surfaces (Li *et al.,* 2008; Ma *et al.,*2022).

On the other hand, the results of antifungal assay revealed that the present extract inhibited the mycelial growth of

Alternaria phragmospora and *Fusarium solani* by 33.8% and 34.7% at a concentration of 8 mg/mL, respectively, but the growth of *Cochliobolus spicifer* was slightly affected by the extract (9.09%). The antifungal effect of the extract may be attributed to its polyphenolic content (Simonetti *et al.,* 2020).

Conclusion:

In the current study, a phytochemical-rich extract was derived from tropical hibiscus flower. The extract had potential antibacterial and antibiofilm activities against Gram-positive and Gram-negative pathogenic bacteria, as well as antifungal activity. Therefore, it offers hope for the future development of natural antimicrobial alternatives, which may contribute to tackling the global issue of antibiotic resistance.

DECLARATIONS:

Ethical Approval: Not applicable.

Authors' Contributions: N.Sh.A.H. study design, bacterial experimental, data analysis and writing the original manuscript; **D.M.A.K.** antifungal assay and data analysis; **U.M.A-R.** edited and reviewed the final manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

Data availability Statement: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request

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