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Synergistic Profile of *Syzygium aromaticum* Aqueous Extract with Standard Antibiotics or Rotaxane Derivatives against Multi-Drug Resistant, Biofilm Forming Pathogenic Bacteria, Fungi, and Aflatoxin Biosynthesis

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THE increasing resistance of pathogenic bacteria and fungi to remedies and their biofilm formation made it is obligatory to search for new, safer and more effective alternatives using medicinal plants. The present investigation aims to study the potency of *Syzygium aromaticum* (clove) aqueous extract mixture with antibiotics or rotaxane derivatives against 3 multi-drug resistant bacteria, 2 fluconazole resistant fungi strains, their biofilm, 3 aflatoxigenic *Aspergillus flavus* strains and aflatoxin biosynthesis. Some bioactive compounds and elements were obtained for clove extract by Fourier transform infrared (FTIR) spectroscopy and energy dispersive X-ray fluorescence (EDXRF) analysis and gas chromatography-mass spectroscopy (GC-MS). A 33.3 - 60 mg/mL concentrations of *S. aromaticum* aqueous extract were sufficient as MBC against *E. coli* O157:H7, *E. coli* O121, and *Staphylococcus aureus* (MRSA) strains, respectively. In practically all combinations of extract, antibiotics, and rotaxane derivatives, synergistic or indifferent relations were detected. No antagonistic interactions were observed against bacteria. For *C. albicans* and *C. glabrata* concentrations 24.64 and 49.28 mg/mL of clove were observed as MFC, respectively. Conversely, *A. flavus* strains were not repressed. Combination of clove with fluconazole exposed synergistic interactions against *C. albicans*. Clove considerably suppressed biofilm formation with inhibition percentage of 81.99, 82.2, and 72.89% for *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA), respectively. Furthermore, *C. albicans* biofilm was reduced and the inhibition percentage was 54%. The highest inhibition percentage of aflatoxin production using clove was 31.7%. This investigation suggests the clove aqueous extract to be a prospective broad spectrum antimicrobial composite alone or in combination.

Keywords: Antibiotics; Combination; Biofilm .

Introduction

Over the past few decades, microbial infections have drastically increased. Furthermore, the continuous use and misuse of antimicrobial drugs to treat these infections has led to the development of resistance in diverse strains of microorganisms (Elamary et al., 2020; Tanwar et al., 2014), amongst them, *Escherichia coli* and *Staphylococcus aureus* (WHO, 2014; Elamary et al., 2021; Elamary et al., 2018; Salem et al., 2017). High mortality proportions, high medical prices, and the inactive therapies. Antimicrobial resistance is associated with and significant impacts on the efficacy of antimicrobial agents (Fishbain & Peleg, 2010). Several antifungal drugs, such as fluconazole, intraconazole, polyenes, pyrimidines, echinocandins, and voriconazole are commercially available to treat candidiasis. However, pathogenic fungi become

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resistant to these antifungal agents (Sanglard, 2016). The molecular mechanisms comprise altered drug affinity, biofilms formation, reduced drug concentration caused by efflux pumps, and Finally, alteration in target abundance (Cowenet al., 2014). Antibiotic resistance owing to beta-lactamase assembly poses a proven problem which may be eliminated by using drug synergy. Synergism, an optimistic interaction between two chemicals, where phytochemicals exhibit their antibacterial prospective (Silva et al., 2019). The synergistic effect of two substances can increase the antifungal activity of their combination. Each compound may have a different growth inhibitory mechanism, which is complemented by the other compound (Mukherjee et al., 2005). Furthermore, the current inadequacy of antibiotics owing to the appearance of multidrug- resistant pathogens and the quick spread of new infections, call for a change in strategy to investigate new, promising, safe alternatives.

Biofilm formation by bacteria is a major threat to human life (Yassein et al., 2021, Elamary and Salem, 2020, Elamary et al., 2020). A biofilm is aggregation of bacteria surrounded by a microbial-derived matrix, which cares their persistence. They are described by high grades of conflict to antibiotics and immune replies and contribute pathogenesis of several chronic human infections (Rabin et al., 2015). Nosocomial *Candida* infection are often associated with biofilm formation on mucosal surfaces and embedded medical devices (Cavalheiro &Teixeira, 2018).

Aflatoxins are a type of fungal toxins produced by *Aspergillus* and *Emerciella* species*,* Presently, more than 18 diverse types of aflatoxins have been described but still the most public are aflatoxin B_1, B_2, G_1 and G_2 (Benkerroum, 2020; Kumar et al., 2017).

Medicinal plants are a good source of natural antibiotics (Elamary et al., 2021; Elamary et al., 2020). Plants exhibited antimicrobial potency against bacteria, fungi, and yeasts (Karkosh, 2012). *Syzygium aromaticum* (clove), from the Myrtaceae family that have been used for centuries as a food conserving and for therapeutic resolves (Pandey &Singh, 2011). S. *aromaticum* is commonly used while preparing meats, salad dressings, and desserts, and in the daily diet as spice and flavoring agent (Chniguir et al., 2019). It displays antimicrobial, antiviral, antioxidant, and anti-inflammatory efficiencies (Batiha et al., 2020, Dacrory et al., 2022). *S. aromaticum* volatile oil and its component, eugenol have been shown to exhibit their antimicrobial activities by inhibiting biofilm synthesis, expression of virulence factors (Hu et al., 2018; Rajkowska et al., 2019; Hamzah et al., 2022).

Cyclodextrins (CDs) are extensively incorporated in the synthesis of diverse macromolecular compounds, like rotaxanes (Harada &Kamachi, 1993). Rotaxanes are widely used for diverse applications in different fields, like as sensory polymers (Kwan et al., 2004), molecular switches, crossbar devices (Jang et al., 2005), bioelectronics (Willner and Katz, 2000), and antimicrobial agents (Özkan et al., 2019).

This study aims to find out new substituents for incidence of multidrug resistant bacteria and fungi using *S. aromaticum* aqueous extracts alone or in combination with pharmaceuticals and polymeric rotaxane to inhibit the development of virulent microorganisms.

Materials and Methods

Tested microorganisms

Bacterial strains

Tested bacterial strains [Two *Escherichia coli* strains and one *Staphylococcus aureus* (MRSA)] were taken kindly form Luxour international hospitals, Luxour governorate, Egypt. The strains were obtained from patients suffering from septicemia. Identification was confirmed by biochemical tests and *E. coli* strains were identified serologically as described by Kok et al. (1996). ORSAB (oxacillin resistant screening agar base) was used to confirm that *S. aureus* was MRSA (Becker et al., 2002). Strains were then identified by 16sRNA gene sequencing (Altschul et al., 1990).

Tested fungal strains

Two strains of pathogenic *Candida* were bought from Assiut University Mycological Center (AUMC); *C. albicans* (MH534933) and *C. glabrata* (MH534928) were used in this work. The cultures were preserved on SDA medium. The suspension turbidity was adjusted to 1.5×10^8 CFU/ mL using 0.5 McFarland standards (McFarland, 1907). In addition, three strains of aflatoxigenic *A. flavus* isolated from spices. The cultures were maintained on PDA medium and the spore suspension concentration was set to 10^7 spores/mL using a hemocytometer (Liang, 2008).

Antimicrobial susceptibility testing

The antibiogram of the tested bacterial strains was estimated using disc diffusion test (Bauer et

al., 1966). The used antibiotics (Bioanalyse®) were chloramphenicol, ampicillin, ampicillin/sulbactam, meropenem. Amoxicillin-clavulanic acid, vancomycin and oxacillin and the resulting zones of inhibition were measured using a ruler. Clarification of the results was completed according to CLSI (2017, 2020) to decide if the strain is resistant, intermediate, or susceptible to the tested antibiotics.

Preparation of clove aqueous extract

Dried clove seeds samples were obtained from commercial spice-seller, Qena governorate, Egypt. Clove seeds were ground with laboratory grinder (IKA A10, Germany) to fine powder. Samples were kept in close container till use. To prepare the extract 40 g of fine powder was added to 100 mL sterile distilled water under stirring condition (Bigger bill shaker (USA), 150 rpm) for 1 day at room temperature. All the solutions were filtered through sterilized cheesecloth. The extract was sterilized using syringe filter holder armed with a 0.45µm filter and was stored at 4°C (Wilson, 1995).

Characterization of clove

Bioactive functional groups in clove material was detected by FTIR. The analysis was carried out using a Magna-FTIR 560 (USA) instrument. Clove extract was also subjected to elemental analysis by EDXRF (JEOL JSX 3222, Japan).

Gas chromatography–mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. Sample was dissolved in dichloromethane. The GC was equipped with DB-624 column (30 m x 320 µm internal diameter and 1.8 µm film thickness). Analyses were carried out using hydrogen as the carrier gas at a flow rate of 3.0 ml/min at a split 1:20 of, injection volume of 1 µl and the following temperature program: 40 °C for 1 min; rising at 7 °C/min to 250 °C and held for 5 min. The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 30-440 and solvent delay 6 min. Identification of different components was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Preparation of commercial antibiotics

Amoxicillin+ clavulanic acid, ampicillin and oxacillin were set by dissolving 1 g in 10 mL of sterile distilled water (100 mg/mL). Antifungal drug (fluconazole) 150 mg was dissolved in 2 mL sterile distilled water.

Characterization and synthesis of rotaxane compounds

The melting points were measured on a Stuart SMP11. Chemical composition of the prepared compounds was confirmed by (^{1}H-NMR), (^{13}C-NMR) and FTIR spectroscopy. The active groups of the resulted compounds were estimated by (Jasco Model 4100 – Japan) IR spectrometer at room temperature in the wave number range of 4000 to 400 cm^{-1}. Scanning electron microscope SEM (JEOL SEM model JSM – 5500 – Japan) was used to study the structure morphology of the synthesized rotaxane with accelerated voltage 10 kV.

Preparation of [2][Succinic dihydrazide]-rota-[α -Cyclodextrin] (1)

A mixture of α -Cyclodextrin (2g) and succinic dihydrazide (0.3 gm, 2.06 mmole) was stirred in DMF (20 mL) for 4 hrs. After that, the 1,4,5,6, 7, 7-Hexachloro-5-norbornene-2,3 dicarboxylic anhydride(1.52 gm, 4.12 mmole) was added with continuously stirring for 30 hrs. The solid prepared was filtered off and dried to afford (1) as white precipitate in 78 % yield; m.p. over 350°C; *Anal.* Considered for $C_{85}H_{70}N_4O_{35}Cl_{12}$ (1808): C, 38.50; H, 3.87; N, 3.09; Cl, 23.56. Found: C, 38.52; H, 3.85; N, 3.11; Cl, 23.58.FT-IR (KBr, cm^{-1}): Indicated the occurrence of peaks due to (vOH's) at 3430 cm^{-1} , (nC=O's) at the region 1790-1740 cm^{-1} and (nC-O-C) glucosydic bond at 1158 cm^{-1}; ^{1}H-NMR (400MHz/DMSO-d_6) displayed at δ 2.55 (4H, $2CH_2$) ; 3.31-3.96 (CH-protons of α -CD + 4H, CH *sp³*) ; 4.84 (H-1 of α -CD); 5.64 (broad s -OH, OH secondary). ^{13}C-NMR (100MHz/DMSO-d_6) presented at δ18.79, 50.28, 50.56, 54.72, 56.51, 60.44, 72.52, 72.90, 73.53, 80.01, 82.05,102.42, 131.84 ,168.13.

Preparation of [2][Hexamethylene diamino]-rota-[β -Cyclodextrin] (2)

By dissolving of β -Cyclodextrin (3 gm, 2.64 mmol) in DMF (20 mL). Then hexamethylene diamine (0.31 mL, 2.67 mmole) was added. Then 2,3-diphenyl maleic anhydride (1.32 gm, 5.28 mmole) was added with stirring for 27 hr. The prepared precipitate was filtered off and dried to afford (2) as yellow solid in 74 % yield; m.p. over 350°C; *Anal.* Calculated for $C_{80}H_{102}N_2O_4$(1154): C, 83.19; H, 8.84; N, 2.43. Found: C, 83.21; H, 8.81; N, 2.46; .FT-IR (KBr, cm^{-1}): exhibited the

existence of peaks due to (vOH's) at 3420cm^{-1} , (nCH-aromatic) at 2985 cm^{-1} , (nC=O's) at the region 1830-1762 cm^{-1} and (nC-O-C) glucosydic at 1159 cm^{-1}; ^{1}H-NMR (400MHz/DMSO-d$_6$) revealed at δ 1.65(8H, 4CH$_2$) ; 3.17 (4H, 2CH$_2$) ; 3.32-5.61 (CH-protons of β -CDf) 7.15-7.47 (20H, arom.H); ^{13}C NMR (DMSO-d_6): δ 18.81, 60.49, 72.52, 72.87, 73.55, 82.05, 102.41, 127.95, 128.23, 128.89, 129.15, 129.85, 130.02, 131.11, 136.55, 138.98, 165.53ppm.

Formation of rotaxane compounds for screening as antimicrobial agents

Rotaxane compounds were synthesized for studying its biological assay. A weight of 0.05 gm of each compound was dissolved in 1 mL dimethyl sulfoxide (DMSO) and completed with distilled water to 10 mL to avoid the inhibitory effect of DMSO (10-fold dilution) (Kirkwood et al., 2018).

Antimicrobial efficacy of S. aromaticum, standard antibiotics, and rotaxane compounds

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *S. aromaticum*, antibiotics and rotaxane compounds against bacteria were determined as following: 24 h incubated culture of *E. coli* (O157:H7 and O121) and *S. aureus* were adjusted to 0.001 OD$_{595}$ in tryptic soy broth (TSB) and MIC was determined according to Eloff (1998) and Elamary et al. (2020) using *p*-iodonitrotetrazolium violet (INT) indicator. The minimum bactericidal concentration (MBC) was determined by inoculating sterile tryptic soy agar (TSA) plates with 50 µl of overnight MIC plates. Viable colonies were counted after 24 h at 37°C. The limit of detection for this assay was 10 cfu /mL (Sirelkhatim et al., 2015).

For fungi, MIC and minimum fungicidal concentration (MFC) for *S. aromaticum*, antibiotics and rotaxanecompoundswere determined as described by Ivanova et al. (2013) with little modification was performed for estimation the antifungal efficacy of clove extract, fluconazole and rotaxane derivatives. Minimum inhibitory concentration (MIC) was determined in sterile 96-well plate containing 100 µL of potato dextrose broth (PDB), 20 µL of clove extract or rotaxane compounds or fluconazole at different concentrations. Then, 5 µl of resazurin was added to each well followed by 5 µl of fungal suspension adjusted to 1.5×10^8 cfu/mL for *Candida* and 10^7 spores/mL for *A. flavus* strains. The plates were enclosed in foil and *Candida* plates were incubated at 37 °C for 4 days. While, *A. flavus* plates were putted at 30 °C for a week. Formation of blue colour indicates growth inhibition, while pink to colorless indicates fungal growth. The assessments were completed in four replications. Minimum fungicidal concentration (MFC) was determined by inoculating PDA medium with 20 µL of each of the wells that were positive for MICs for 24 h at 37 °C for *Candida* and at 25±2 °C for 10 days for *A. flavus* strains. No growth indicated fungicidal activity (Alyousef, 2021; Kumar et al., 2016).

Checkerboard dilution assay

In Checkerboard method, antibacterial effects of clove aqueous extract, antibiotics and some rotaxane derivatives in addition to some combinations of them were evaluated by the checkerboard examination (Cha et al., 2007). Standard powder forms of amoxicillin+ clavulanic acid, ampicillin and oxacillin were stored at 2 – 8 °C until use. The stock solutions and serial dilutions for clove extract, antibiotics, and rotaxane derivatives were prepared. MIC and MBC were determined as described earlier. A fractional inhibitory concentration index was used to interpret the results (Lin et al., 2003). The ΣFICs (FIC index) were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of treatment in the combination/MIC of treatment alone, and FIC B is the MIC of another treatment in the mixture/ MIC of it single-handedly. The interface was defined as synergistic if the FIC index was less than or equal to 0.5, indifferent if the FIC index was greater than 0.5 and less than or equal to 4.0, and antagonistic if the FIC index (FICI) was greater than 4.0 (Prinsloo et al., 2008).

The antifungal potency of clove extract in combination with fluconazole against the tested *Candida* was determined by checkerboard titration test according to Ivanova et al. (2013) with little modification as designated above. The nature of relations was determined as described by Odds (2003).

Antibiofilm efficacy of S. aromaticum

Against bacterial biofilm

The ability of the tested strains naming *E. coli* (O157:H7 and O121) and *S. aureus* was assessed using microtitre plate as designated by Elamary et al. (2020).

Against Candida biofilm

The effect of clove extract on *Candida* biofilm was evaluated as it considerably inhibited *Candida* growth. The method described by Balasamy et al. (2019) was applied for this purpose.

Anti-aflatoxigenic potential of clove aqueous extract

The inhibitory effect of clove extract against aflatoxin levels of 3 aflatoxigenic *A. flavus* strains isolated from spices was studied following the method described by Yassein et al. (2020). Aflatoxins levels were determined fluorometrically (VICAM, chemists. Inc., U.S. America) using standards of aflatoxins (Hansen, 1993).

Scanning electron microscopy (SEM) analysis of antimicrobial efficiency of S. aromaticum

Formation of compounds for antibacterial potency was completed as defined by Wang et al. (2018), following which, the samples were fixed in 4% (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) at 4 °C for 12 hr. Subsequently, washing three times in phosphate buffer, dehydration through a graded ethanol series, dried in a critical-point drying apparatus with liquid carbon dioxide; slides coated with gold and viewed using (JEOL JSM-5500LV, Japan), while antifungal was performed using method of Benli et al. (2008).

Statistical analysis

The variability degree of results was expressed in form of Means ± Standard Deviation (Mean ±S.D) based on three independent determinations (n=3). The data were statistically analyzed by one-way ANOVA analysis and compared using the least significant difference (LSD) test at 0.05 (*) levels. It was done to compare between control and treatments.

Results

The phylogenetic tree and relationship between tested fungal strains was illustrated in Fig. (1A) and tested bacterial strains (Fig. 1B).

Antimicrobial susceptibility testing

The tested bacteria resistance degree to a board of antibiotics showed in Table 1, the results revealed that *E. coli* (O157:H7 and O121) were sensitive to chloramphenicol, meropenem, and amoxicillin-clavulanic acid and resistant to ampicillin, ampicillin/sulbactam. Finally, *S. aureus* (MRSA) were sensitive to chloramphenicol and amoxicillin-clavulanic acid and resistant to vancomycin and oxacillin.

Characterization of S. aromaticum

The FTIR spectrum of *S. aromaticum* extract was shown in Fig. (2A).It showed absorption band at 3642, 3431, 2926, 2858, 2511, 2358, 1796, 1420, 1116, 994, 874, 712, 521, and 426 cm^{-1}. The EDXRF results revealed that the extract contains potassium (K), calcium (Ca), and iron (Fe) with ms percentages of 54.75, 41.18, and 2.89, respectively Fig. (2B).

Gas chromatography–mass spectrometry analysis (GC-MS)

Extraction and separation of plant material plays an important role in formulation of herbs. So, one of important goals of the current study was to detect active components by GC-MS. This shows 16 active phytochemical constituents. The highest percentages were as follows: eugenol (80.72%); phenol, 2-methoxy-4-(2-propenyl)-, acetate (9.95%); caryophyllene (6.22). Other components with their percentages and molecular formula are listed in Table 2.

Synthesis and spectral analysis of two rotaxane compounds

[2] [Succinic dihydrazide]-rota- [α -Cyclodextrin] (1) was resulted by threading of α-CD (host molecule) into succinic dihydrazide (axis) then stoppered by using 1,4,5,6,7,7-Hexachloro-5-norbornene-2,3 dicarboxylic anhydride (See Supplementary Data Fig. S1). The chemical compositon of the rotaxane (1) was recognized by its spectra. FTIR spectra of [2] rotaxane (1) give good indication for the preparation of [2] rotaxane (1) owing to presence of distinctive absorption bands for (νOH) groups at 3398.43 cm^{-1} and characteristic band for (νC–O–C) glucosydic at 1159.56 cm^{-1} (Table S1, Fig. S2). The change in the frequencies of pure α-CD after threading, this variation in the absorption bands is owing to the intermolecular hydrogen bonds among (NH, C=O) groups of succinic dihydrazide and the (OH) groups of α-CD ring (Table S1) (Dardeer and Ebnalwaled, 2019, Dardeer, 2018, Dardeer and Hassan, 2015). We observed that there is minor enhancement and reducing in the intensity change Δδ. Increasing Δδ is as a result of entering of the aliphatic chains inside the cavity of α-CD (Table S1), in contrast, the reducing of intensity change is owed to the development of van der Walls forces and hydrogen bonding. The ^{1}H-NMR of the pure α -CD (Fig. S3). Figure 3 (A), shows the ^{1}H-NMR spectra of [2] rotaxane (1). The NMR spectra give good indication for the creation of compound (1), the appearance of the aliphatic protons of α

-CD in the region 3.31-3.96 ppm., in addition to presence of peaks for secondary OH groups at 5.64 ppm., and singlet peak for H-1 at 4,48 ppm. Also, appearance signals for (2 CH_2) at 2.55 ppm. From ^{1}H-NMR spectra, the stoichiometry ratio for the preparation the inclusion complex is 1:1.

^{13}C NMR spectra of [2] rotaxane (1) providing respectable confirmation for the chemical structure of compound (1), displaying peak because of two (CH_2) at 18.97 ppm, in addition to four signals due to 4CH *sp*3 at 50.28, 50.56, 54.72 and 56.51 ppm. Also, appearance of six diverse carbon atoms attributable to β-CD at 60.44, 72.52, 72.90, 73.53 and 80.10, as well as the attendance of a signal due to two carbons (C=C) at 82.05 ppm, a band for two C–Cl at 102.42 ppm, a peak due to [C–(Cl)2] at 131.84 ppm, and a signal owing to (C=O) groups at 168.13 ppm (Fig. 3C).

The [2] [Hexamethylen diamino]-rota- [β -Cyclodextrin] **(2)** was prepared by two steps (Fig. S4) in which β-CD threaded into the aliphatic chain (hexamethylene diamine**)** to give the [2] pseudorotaxane, then stoppered by adding 2,3-diphenyl maleic to give the [2] rotaxane **(2)** in good yield. FTIR spectra discovered the incidence of representative absorption bands at 3437cm^{-1} in arrears to (OH), CH-aromatic at 2930 cm^{-1}, also, peaks due to (C=O) at the region 1840-1760 cm^{-1}, glycosidic group at 1159 cm^{-1} (Table S2, Fig. S5). ^{1}H-NMR and ^{13}C-NMR spectra confirmed the composition of [2] rotaxane **(2)** (Fig. S6, Fig. 3B). ^{1}H-NMR displays a triplet for two methylene protons look at δ 1.53 ppm, a triplet owing to two methylene protons at 2.55 ppm, aliphatic protons of β –CD appear at 3.15-3.67 ppm. Moreover, the primary (OH) groups at δ 4.31 and secondary hydroxyl groups at δ 5.61 ppm, the aromatic protons at chemical shift 7.15-7.47 ppm. The ^{13}CNMR spectrum of [2] rotaxane **(2)** in dimethyl sulfoxide confirmed its construction (Fig. 3D).

Surface morphology

The surface morphology of pure α-CD, β-CD and the prepared [2] rotaxanes (1), (2), was studied by scanning electron microscopy (SEM) (Fig. 4). The morphology of [2] rotaxane (1) has a regular crust-like shape, while [2] rotaxane **(2)** is similar to cutting glass. Hence, the difference in the morphologies of the obtained [2] rotaxanes (1) and (2) from α-CD and β-CD indicates the formation of the inclusion complexes.

Determination of MIC, (MBC and MFC) for S. aromaticum, antibiotics, and rotaxane compounds.

Effective antibacterial activities of *S. aromaticum*, different antibiotics (amoxicillin+ clavulanic acid, ampicillin, oxacillin), and rotaxane derivatives (α and β-CDs–rotaxane 1 and 2) against *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA) was established by the colorimetric INT- formazon assay, as shown in Table 3. The MIC of *S. aromaticum* aqueous extract was 60, 40, and 33.3 mg mL^{-1} against *E. coli*O157:H7, *E. coli* O121, and *S. aureus* (MRSA) strains, respectively, while that of amoxicillin+ clavulanic acid antibiotic was 100, 100, and 90 mg mL^{-1} against the three tested pathogens, respectively. Ampicillin showed an MIC of 90 mg mL^{-1} against *E. coli* O157:H7, and *E. coli* O121, while oxacillin exhibited an MIC of 70 mg mL^{-1} against *S. aureus* (MRSA) strain. α –CDs showed the same MIC of 0.373 mg mL^{-1} against the three strains while β-CDs exhibited an MIC of 0.9, 0.81, and 0.72 mg mL^{-1} against them, respectively. Rotaxane 1 and 2 displayed MICs of (0.127, 0.1, 0.127 mg mL^{-1}) and (0.593, 0.534, 0.415 mg mL^{-1}) against the three strains, respectively. The MBC was also determined against these three strains and the results were displayed in Table 3. The following were the MBCs against *E. coli* O157:H7, *E. coli* O121,and *S. aureus* (MRSA) strains, respectively: 60, 46.6, and 33.3 mg mL^{-1} for *S. aromaticum* aqueous extracts; 5, 8.3, and 6.6 mg mL^{-1} for amoxicillin+ clavulanic acid. On contrast, ampicillin antibiotic exposed no bactericidal effect against *E. coli* O157:H7, *E. coli* O121 strains and oxacillin antibiotic has no bactericidal outcome against *S. aureus* (MRSA) strain. α –CDs and β-CDs, as well exhibited no efficacy against *E. coli* O157:H7, *E. coli* O121, and *S. aureus* (MRSA) strains, respectively. Although, rotaxane 1 and 2 revealed MBC of (0.127, 0.1, and 0.127) and (0.593, 0.475, and 0.475) mg mL^{-1} against *E. coli* O157:H7, *E. coli* O121, and *S. aureus* (MRSA) strains, respectively.

The efficacy of *S. aromaticum* aqueous extract to inhibit the growth of *C. albicans*, *C. glabrata*, *A. flavus* (MW040477), *A. flavus* (MW040478), and *A. flavus* (MW040479) was compared with that of fluconazole, rotaxane derivatives (α- and β-CDs–rotaxane 1 and 2) and the negative and positive controls by evaluating the MIC and MFC using a microtiter plate assay and resazurin indicator. The obtained MICs against *C. albicans* (MH534933) and *C. glabrata* (MH534928), respectively were: 18.5 and 49.28 mg / mL for *S. aromaticum* extracts; 11.55 and 5.775 mg / mL

for fluconazole. However, rotaxane compounds and their derivatives (α -CDs and β –CDs) did not exhibit inhibitory effect against the growth of tested *Candida* strains, as illustrated in Table 2. For MFC determination, as shown in Table 3; concentrations 24.64 and 49.28 mg / mL of *S. aromaticum* extract were the MFC against *C. albicans* and *C. glabrata*, respectively. Although, fluconazole did not reveal any fungicidal effect against *C. albicans*, its MFC against *C. glabrata* was 5.775 mg / mL. On the contrary, *Aspergillus flavus* strains were not affected by the different concentrations of *S. aromaticum*, fluconazole, and rotaxane derivatives (Table 3).

Checkerboard dilution assay

For bacteria, the effects and interactions of combinations of *S. aromaticum*, different antibiotics (amoxicillin+ clavulanic acid, ampicillin, oxacillin), and rotaxane derivatives (β-CDs -101) against *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA) were determined by the checkerboard method. The results were presented in Table 3. The fractional inhibitory concentration index (FIC) and the consequence of the interaction were determined. The results are shown in Table 3. In almost all combinations of extract, antibiotics, and rotaxane derivatives, either synergistic or indifferent interactions were observed; antagonistic interactions were not observed.

The combination of *S. aromaticum* and amoxicillin+ clavulanic acid antibiotic was synergistic, lowering the MIC of each individual treatment by 90.5% and 91.5% against *E. coli* (O157:H7), 88.6% and 82.86 % against *E. coli* (O121), and 84.6% and 71.4% against *S. aureus* (MRSA), respectively. The combination of *S. aromaticum* and ampicillin antibiotic lowered the MIC of the individual treatments by 81% and 80.96% against *E. coli* (O157:H7), 85.8% and 71.4% against *E. coli* (O121), respectively. Synergistic effect was also detected for the combination of the extract with oxacillin antibiotic and MIC was reduced by 88% and 87.9% against *S. aureus* (MRSA), respectively. The combination of α and β-CDs with the extract was indifferent, although the MIC of α -CDsdecreased by 91.4%, 65.7%, and 39.9% and that of β–CDs reduced by 57.1%, 81%, and 89.3% against *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA), respectively. The MIC of the extract against the three strains reduced by 4.8%, 28.6%, and 65.8 %, respectively, in combination with α-CDs and by 61.9%, 14.3%, and 48.5%, respectively in combination with β-CDs. Interestingly, the synthesized rotaxane products ([1] and [2]) exhibited synergistic efficacy when combined with *S. aromaticum* extract. The MICs of rotaxane [1] and [2] reduced by 91.3%, 67%, and 91.3% and by 91.6%, 99.3%, 75.9 %, respectively, against the three pathogenic strains. Meanwhile, the MIC of *S. aromaticum* reduced by 61.9%, 88.6%, and 93.2%, respectively when combined with rotaxane [1] and by 94.3%, 87.2%, and 98.3%, respectively when combined with rotaxane [2].

For fungi,the antifungal efficiency of *S. aromaticum* aqueous extract in combination with fluconazole against *C. albicans* and *C. glabrata* was analyzed by the checkerboard method. The combination of *S. aromaticum* with fluconazole was synergistic against *C. albicans*, reducing their MICs by 71.24% and 83.13%, respectively. Though, this combination showed indifferent interaction against *C. glabrata*, decreasing their MICs by 56.8% and 65.5%, respectively. On the other hand, *A. flavus* strains were not affected by the combination of *S. aromaticum* with fluconazole (Table 3).

Antibiofilm efficacy of S. aromaticum

The antibiofilm properties of *S. aromaticum* were tested against some pathogens. Figure 5 shows the capability of *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA) to form biofilms before treatment with the extract. *S. aromaticum* extract inhibited biofilm formation in the three tested strains. The percentage of inhibition was 81.99%, 82.2%, and 72.89% for the three strains, respectively. The biofilm of *C. albicans* significantly inhibited with percentages of 54% after treatment with *S. aromaticum* extractbut the extract weakly inhibited *C. glabrata* biofilm by merely 12.35 % (Fig. 5).

Scanning electron microscope analysis (SEM)

The morphological modifications in bacterial cells treated with *S. aromaticum* were examined by SEM. The untreated *E. coli* cells had a typical striated wall structure; however, their morphology underwent severe detrimental changes after *S. aromaticum* treatment. The cell shape distorted from rod-shaped to curve. Cells began to swell up to form irregular shapes, lysed and excreted their protoplasm. The untreated *C. albicans* cells SEM images showed that the ultrastructure of the cells had a well-developed structure, fixed with a round to ovate shape, and polar bud scars. After treatment with the extract, the cells showed irregular shapes having pores on the surface. Autolysis was observed, cytoplasmic contents retreated from the cell membrane, and hyphae formation was hindered (Fig. 6).

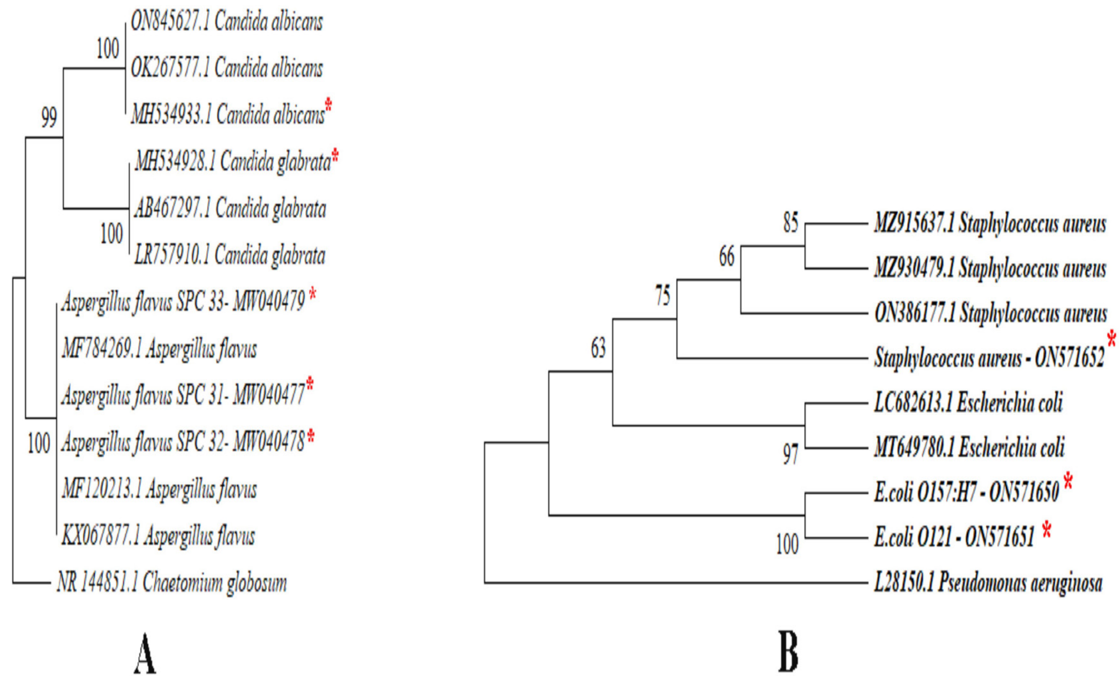

Fig. 1. Phylogenetic tree and relationship among fungal strains (A), bacterial strains (B) compared with some strains at NCBI.

TABLE 1. Antimicrobial susceptibility testing[a].

Tested bacteria	Code[b]	Diameter of clear zone in mm	Resistant	Intermediate	Susceptible
	C	21	≤ 12	13-17	≥ 18
E. coli	AM	6	≤ 13	14-16	≥ 17
(O157:H7)	SAM	4	≤ 11	12-14	≥ 15
	MEM	24	≤ 19	20-22	≥ 23
	AMOX-CLAV	22	≤ 13	14-17	≥ 18
	C	22	≤ 12	13-17	≥ 18
	AM	5	≤ 13	14-16	≥ 17
E. coli	SAM	7	≤ 11	12-14	≥ 15
(O121)	MEM	25	≤ 19	20-22	≥ 23
	AMOX-CLAV	19	≤ 13	14-17	≥ 18
	C	25	≤ 12	13-17	≥ 18
	AMOX-CLAV	24	≤ 13	14-17	≥ 18
S. aureus	VA	0	≤ 14	15-16	≥ 17
	OX	0	≤ 10	11-12	≥ 13

[a]: Antimicrobial susceptibility of a group of standard antibiotics according CLSI, 2017, 2020 against three human pathogenic strains. [b]:C: Chloramphenicol; AM: Ampicillin; SAM: Ampicillin/Sulbactam; MEM; Meropenem; AMOX-CLAV: Amoxicillin-clavulanate; VA: Vancomycin; OX: Oxacillin.

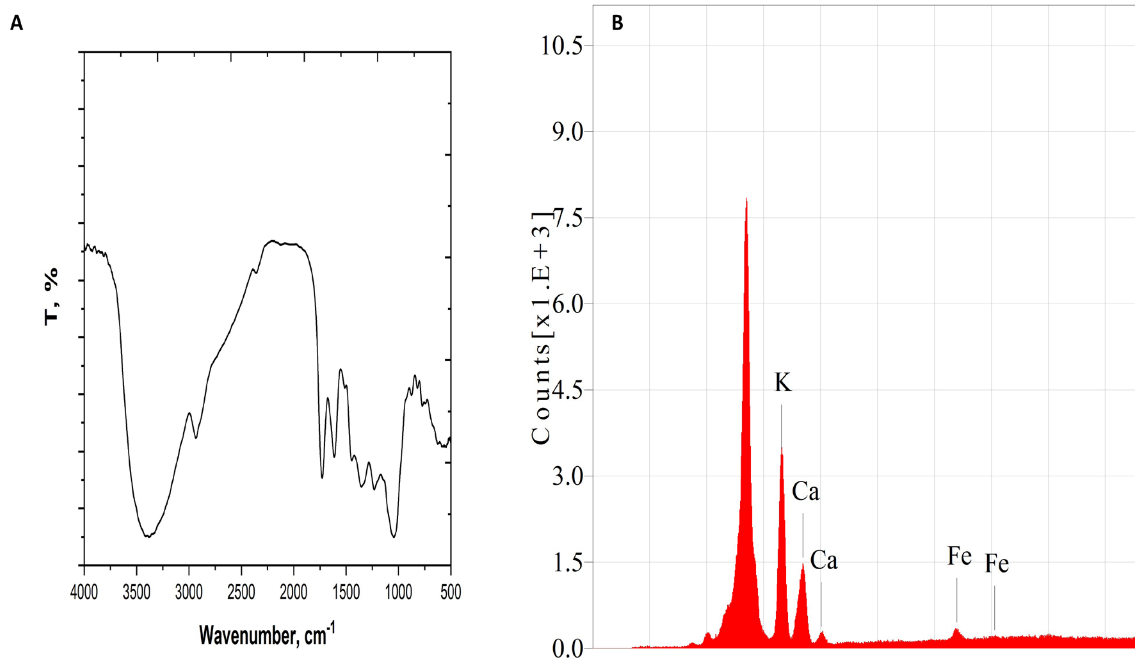

Fig. 2. FTIR (A) and EDXRF (B) analysis of *S. aromticum*

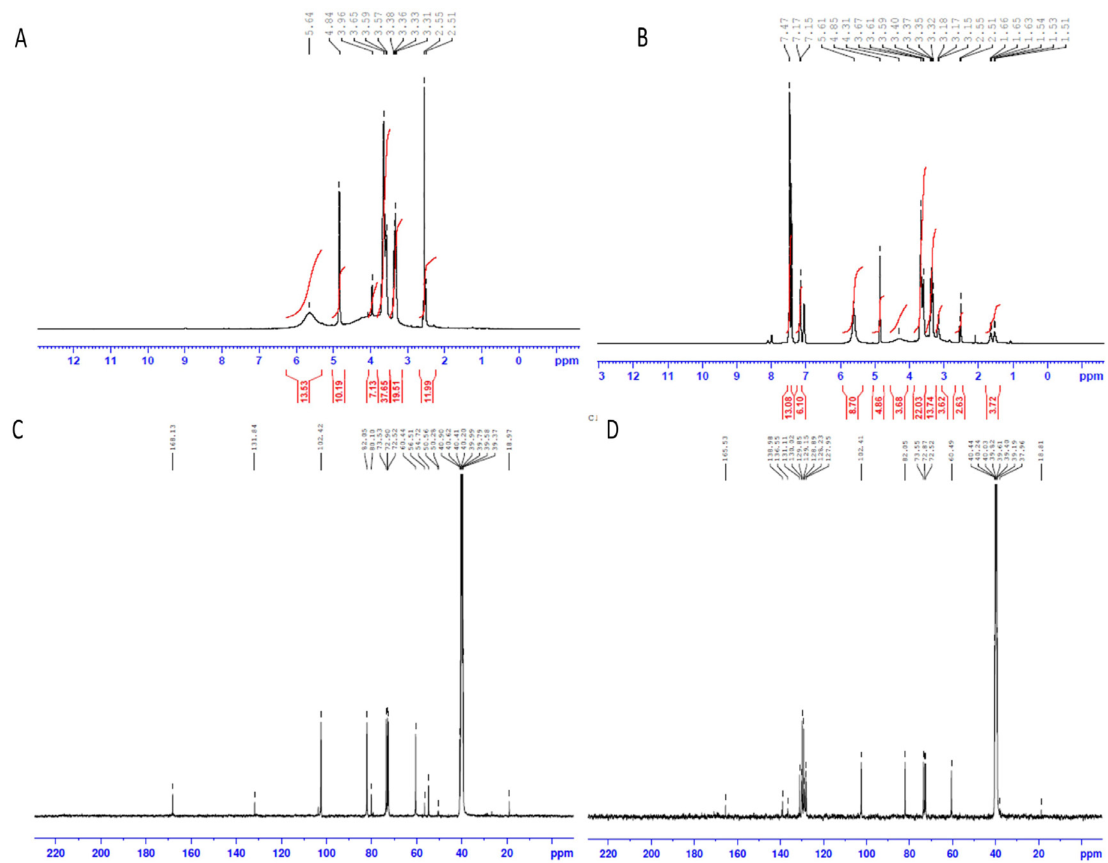

Fig. 3. A ^{1}H-NMR spectra for [2] Rotaxane (1); B: ^{1}H-NMR spectra of [2] rotaxane (2);C: ^{13}C-NMR spectra [2] Rotaxane (1); D: ^{13}C-NMR spectra of [2] rotaxane (2)..

TABLE 2. Gas chromatography–mass spectrometry analysis (GC-MS) analysis of *S. aromaticum*

Peak	RT	Name	Formula	Area	Area Sum %
1	18.183	Phenol, 4-(2-propenyl)-	$C_9H_{10}O$	63479.26	0.11
2	18.712	Copaene	$C_{15}H_{24}$	94159.06	0.16
3	19.24	Eugenol	$C_{10}H_{12}O_2$	46342762	80.72
4	19.769	Caryophyllene	$C_{15}H_{24}$	3572917.2	6.22
5	20.421	Humulene	$C_{15}H_{24}$	390052.35	0.68
6	20.626	Tetracyclo[6.1.0.0(2,4).0(5,7)] nonane, 3,3,6,6,9,9-hexamethyl-, cis,cis,trans-	$C_{15}H_{24}$	35966.99	0.06
7	21.002	1,9-Decadiyne	$C_{10}H_{14}$	23115.04	0.04
8	21.083	4-[5-Methyl-2-(1-methylethylidene) cyclohexyl]-3-cyclohexen-1-one	$C_{16}H_{24}O$	6888.66	0.01
9	21.393	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	$C_{15}H_{24}$	131515.08	0.23
10	21.607	1H-Cyclopropa[a]naphthalene, 1a,2,6,7,7a,7b-hexahydro-1,1,7,7a-tetramethyl-, [1aR-(1aα,7α,7aα,7bα)]-	$C_{15}H_{22}$	58870.98	0.1
11	22.316	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	5713745.8	9.95
12	23.331	Preg-4-en-3-one, 17α-hydroxy-17β-cyano-	$C_{20}H_{27}NO_2$	623655.09	1.09
13	23.75	Longipinene epoxide	$C_{15}H_{24}O$	57142.02	0.1
14	24.55	Adamantane-1-carboxamide, N-benzyl-	$C_{18}H_{23}NO$	42186.74	0.07
15	24.812	5,9-Tetradecadiyne	$C_{14}H_{22}$	127923.71	0.22
16	25.174	1-(4,7-Dimethoxy-2H-1,3-benzodioxol-5-yl)-2-nitroethanone	$C_{11}H_{11}NO_7$	128444.26	0.22

Peak: no. of peaks present; RT: Retention time per minute; Name: active compounds detected by GC-MS; Formula: Molecular formula; Area (%): percentage of compound.

TABLE 3. Synergistic effect of *S. aromaticum*, antibiotics and rotaxane derivatives on some human pathogenic microbes .

Microbial strains	Agent	Alone		Combination	% MIC	FICA FICB	FIC	Outcome
		MIC mg/ml	MBC or MFC mg/ml	MIC mg/ml				
E. coli (O157:H7)	*S. aromaticum*	60	60	5.7	90.5	0.095	0.18	Synergism
	Amoxicillin+ clavulanic acid	100	100	8.5	91.5	0.085		
	S. aromaticum	60	60	11.4	81	0.19	0.38	Synergism
	Ampicillin	90	-ve	17.14	80.96	0.19		
	S. aromaticum	60	60	57.1	4.8	0.952	1.038	Indifference
	α -CDs	0.373	-ve	0.032	91.4	0.086		
	S. aromaticum	60	60	22.86	61.9	0.381	0.81	Indifference
	β-CDs	0.9	-ve	0.386	57.1	0.429		
	S. aromaticum	60	60	22.86	61.9	0.381	0.468	Synergism
	Rotaxane 1	0.127	0.127	0.011	91.3	0.087		
	S. aromaticum	60	60	3.42	94.3	0.057	0.141	Synergism
	Rotaxane 2	0.593	0.593	0.05	91.6	0.084		
E. coli (O121)	*S. aromaticum*	40	46.6	4.57	88.6	0.114	0.284	Synergism
	Amoxicillin+ clavulanic acid	100	100	17.14	82.86	0.17		
	S. aromaticum	40	46.6	5.7	85.8	0.143	0.429	Synergism
	Ampicillin	90	-ve	25.7	71.4	0.286		
	S. aromaticum	40	46.6	28.57	28.6	0.714	1.057	Indifference
	α -CDs	0.373	-ve	0.128	65.7	0.343		
	S. aromaticum	40	46.6	34.3	14.3	0.858	1.048	Indifference
	β-CDs	0.81	-ve	0.154	81	0.19		
	S. aromaticum	40	46.6	4.57	88.6	0.114	0.444	Synergism
	Rotaxane 1	0.1	0.1	0.033	67	0.33		
	S. aromaticum	40	46.6	5.14	87.2	0.129	0.136	Synergism
	Rotaxane 2	0.534	0.475	0.004	99.3	0.007		

TABLE 3. Continued

Microbial strains	Agent	MIC mg/ml	MBC or MFC mg/ml	MIC mg/ml	% MIC	FICA FICB	FIC	Outcome
S. aureus (MRSA)	*S. aromaticum*	33.3	33.3	5.14	84.6	0.154	0.44	Synergism
	Amoxicillin+ clavulanic acid	90	90	25.7	71.4	0.286		
	S. aromaticum	33.3	33.3	4	88	0.12	0.241	Synergism
	Oxacillin	70	-ve	8.5	87.9	0.121		
	S. aromaticum	33.3	33.3	11.4	65.8	0.342	0.943	Indifference
	α –CDs	0.373	-ve	0.224	39.9	0.601		
	S. aromaticum	33.3	33.3	17.14	48.5	0.515	0.622	Indifference
	β-CDs	0.72	-ve	0.077	89.3	0.107		
	S. aromaticum	33.3	33.3	2.28	93.2	0.068	0.155	Synergism
	Rotaxane 1	0.127	0.127	0.011	91.3	0.087		
	S. aromaticum	33.3	33.3	0.571	98.3	0.017	0.258	Synergism
	Rotaxane 2	0.415	0.475	0.1	75.9	0.241		
C. albicans	*S. aromaticum*	18.5	24.64	5.32	71.24	0.287	0.459	Synergism
	Fluconazole	11.55	-ve	1.99	83.13	0.172		
C. glabrata	*S. aromaticum*	49.28	49.28	21.28	56.8	0.432	0.777	No interaction
	Fluconazole	5.775	5.775	1.99	65.5	0.345		

Anti-aflatoxigenic potency of S. aromaticum

Clove extract considerably suppressed aflatoxin production in the three tested aflatoxigenic *A. flavus* by 31.7% for *A. flavus* MW040478, by 30% for *A. flavus* MW040477 and by 10% for *A. flavus* MW040479 (Table 4).

Discussion

Pathogenic bacteria, in general are acquiring resistance to commonly used antibiotics. Moreover, the unused antibiotics may be disposed in the sewage system and if not degraded during sewage treatment, they may seep into ground, surface, and drinking water which can be a great environmental risk (Elamary et al., 2021). Thus, searching for novel antimicrobials has become imperative. Several medicinal plants produce antibiotics that studies have been used to treat infectious illnesses (Elamary et al., 2020, Salem et al., 2017). The beta-lactamase antibiotics (Singleton & Willey, 1999), disrupt the synthesis of the cell envelope in growing cells by incapacitating penicillin-binding proteins, consequently preventing the synthesis of peptidoglycan. Varying levels of antimicrobial resistance are found in human and veterinary strainsof *Staphylococci*. Methicillin-resistant strains of *Staphylococci* need special consideration because they show co-resistance to other beta-lactam antibiotics including oxacillin/methicillin (Bzdil et al., 2021). The current study revealed that *E. coli* (O157:H7 and O121)and *S. aureus* (MRSA) were sensitive to chloramphenicol and amoxicillin-clavulanic acid. *E. coli* (O157:H7 and O121) were sensitive to meropenem but resistant to ampicillin, ampicillin/sulbactam. Finally, *S. aureus* (MRSA) was resistant to vancomycin and oxacillin (Table 1). Our study focused on finding new safe alternatives to antibiotics using *S. aromaticum* and rotaxane compounds. Rotaxanes have an excellent ability to dissolve in water and generate singlet oxygen, which could damage the bacterial outer membrane (Özkan et al., 2019). Given the complexity of the disease on a metabolic, genetic, and physiological level, as well as the rapid spread of resistant microorganisms and the lack of a systematic management plan, researchers are considering plant-derived substances as an alternative to or in addition to antibiotics. The current study revealed an excellent efficacy of *S. aromaticum*, displaying MBCs in the range of 33.3 – 60 mg mL^{-1} against *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA) (Table 3). The antibacterial effect of *S. aromaticum* is becauseit's enhanced oxidative stress and induces membrane permeability (Ajiboye et al., 2016). Secondary metabolites are usually the active ingredients of plants against microorganisms (Raj et al., 2016). Several compounds from *S. aromaticum* such as biflorin, kaempferol, rhamnocitrin, have been reported to destroy oral pathogens (Shan et al., 2005). The efficacy of *S. aromaticum* extract might be owed to the presence of some functional groups that were detected by the Fourier transform infrared spectroscopy (Fig. 2A). The highly intense bands at 3642 and 3431 cm^{-1} represent the OH group, while the alkyl CH stretch was noted at 2926, 2858, 2511, and 2358 cm^{-1}. A sharp peak at 1796 cm^{-1} represented the frequency pattern of the ester group C–O. A peak at 1420 cm^{-1} indicates the presence of the methylene group (CH_2), sharp peaks at 1116 cm^{-1} belong to the methyl groups (CH_3). Moderate bands at 994, 874, and 712 cm^{-1}indicate the presence of CH_2 and C=C, respectively. This spectrum was in harmony with previous studies (Parthipan et al., 2021). These specific functional groups match with the standard eugenol functional groups, confirming its presence in the clove extract (Chowdhry et al., 2015). Energy dispersive X-ray fluorescence analysis (Fig. 2B), confirmed that *S. aromaticum* is a valuable source of potassium, calcium and iron. Potassium is important as a diuretic, calcium is important for muscles, teeth, bones and the heart (Brody, 1994) and iron is important for the formation of hemoglobin, oxygen and electron transfer in the human body, oxidation of fats, proteins, and carbohydrates, also, the function of the central nervous system, and (Mohammed et al., 2013). GC-MS confirmed presence of eugenol with percentages of (80.72% as listed in Table 2.

TABLE 4. Anti-aflatoxigenic activity of clove aqueous extract.

Aflatoxingenic strain	Mycotoxin level (C)	Mycotoxin level (T)	Inhibition %
A. *flavus* MW04047	2.8±0.058	2.5±0.1	30%
A. *flavus* MW040478	8.5±0.1	5.8±0.98	31.7%
A. *flavus* MW040479	2±0.2	1.8±0.1	10%

C= control, T= treatment, values were represented as mean values± standard deviation

Although various plant secondary metabolites exhibited potent antimicrobial activity, most of them have weak activities compared with bacterial or fungal antibiotics (Hemaiswarya et al., 2008). Nevertheless, these compounds can act in synergistically with antimicrobials to strengthen their effect and help the host overcome the infection (Silva et al., 2019). Antimicrobial combination therapy is commonly used in diagnosis to improve spectrum coverage, inhibit the formation of resistant mutants, and gain antimicrobial synergy for the management of infections and illnesses (Akinyele et al., 2017). In our study, all the combinations tested either displayed mutual stimulation or indifference; no antagonism was observed.

Nowadays, the treatment of *Candida* infections has become difficult due to the restricted number of presented antifungal drugs, the enlarged fight to these drugs, and adverse effects on human health. In our investigation, the MICs of fluconazole against *C. albicans* and *C. glabrata* strains were 11.55 and 5.775 mg/mL, and they are categorized as resistant strains according to the CLSI guidelines (CLSI, 2020). Azoles are the most universally used antifungal medicines since they are low-priced, low toxic, obtainable for oral use, and they reduce *C. albicans* ergosterol levels (Bhattacharya et al., 2020). The long-standing use of fluconazole resulted in the appearance of fluconazole-resistant *Candida* isolates (Khan et al., 2020; Sanglard, 2016). So, attempts were made to find safer alternatives in plant extracts rich in phytochemicals, less toxic and do not promote the development of resistance like synthetic antibiotics do. In our study, the MIC and MFC of *S. aromaticum* extract against *C. albicans* were 21.8 and 29 mg/mL, respectively. While, those values against *C. glabrata* were 58.18 mg/mL. Saracino et al. (2022) have similarly reported that eugenol suppressed the growth of the tested *Candida* with mean MIC and MFCs of 455.42 and 690.09 mg/L, respectively, while those values against *C. glabrata* were 1325 mg/L (Table 3). An ethyl acetate extract of clove showed the maximum antifungal effectiveness against vaginal *C. albicans* and *C. glabrata* with an MIC and MFC of 500 µg/ disc and 1 mg/ disc, respectively (Yassin et al., 2020). The difference in the results may be specific to the nature and the source of the tested *Candida*. Further, the different concentrations of the active constituents of clove correlated directly with geographical areas of the plant origin, plant age, growth stage and climatic conditions (Andrade et al., 2017). Therefore, the anti-candidal effectiveness of clove aqueous extract was tested in combination with fluconazole. In our investigation, clove extract synergistically interacted with fluconazole against *C. albicans* with MICs of 5.81 and 2.72 mg/mL, respectively. The combination of clove extract with fluconazole exhibited no interaction against *C. glabrata*. Correspondingly, Ahmad et al. (2010) found that the combination of fluconazole with eugenol and fluconazole with methyleugenol exhibited synergistic effects in 29 and 31 out of 34 fluconazole-resistant *Candida* strains, respectively. No antagonistic interaction was observed in this combination (Pootong et al., 2019). Recently, Shokri et al. (2021) reported that the eugenol and fluconazole mixture reduced the MIC of fluconazole by 81.81% and showed significant synergistic effects against *C. krusei*. On the other hand, the growth of tested aflatoxigenic *A. flavus* strains was not affected by *S. aromaticum* aqueous extract, fluconazole or rotaxane derivatives (Table 4). Recent studies reported that the aqueous extracts of different medicinal plants exhibited low or no antifungal activity but their organic solvent extracts and essential oils did (Ahamdi et al., 2020).

Bacterial biofilms have become a major threat to human health. Biofilm removal is a particularly difficult task, despite several strategies being used (Yassein et al., 2021; Elamary et al., 2021; Elamary et al., 2020; Elamary & Salem, 2020). In the current study, *S. aromaticum* displayed a brilliant efficiency against *E. coli* (O157:H7 and O121) and *S. aureus* (MRSA) biofilms with inhibition percentages of 81.99%, 82.2%, and 72.89%, respectively (Fig. 5). Kačániová et al. (2021) confirmed the inhibitory effect of *S. aromaticum* essential oil on biofilms. *Candida* biofilms are assorted well- arranged structures comprising planktonic and mycelial yeast cells enclosed by extracellular polymeric substances that make them resistant to antibiotics and reduce the efficiency of host defenses (Simoes et al., 2010). So, in this study, we used *S. aromaticum* as a natural and safer therapeutic to suppress *Candida* biofilms and it successfully repressed biofilm formation in *C. albicans* by 54%. Contrariwise, *C. glabrata* biofilms were slightly repressed by 12.35 % (Fig. 5). The effect of clove on *Candida* biofilms was shown to depend on the material used and the inhibition percentage ranged from 68.4% - 84.2% against tested yeast, including *C. albicans* (Rajkawska et al., 2019).

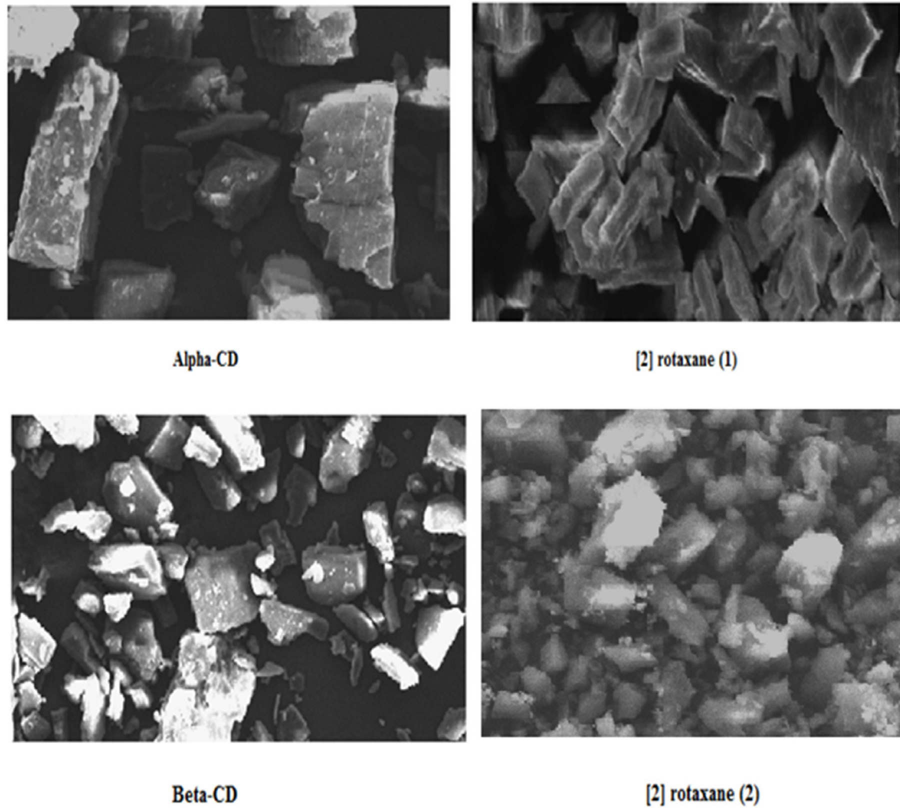

Fig. 4. Scanning electron microscopy micrographs of α-CD, β-CD, [2] rotaxanes (1), (2).

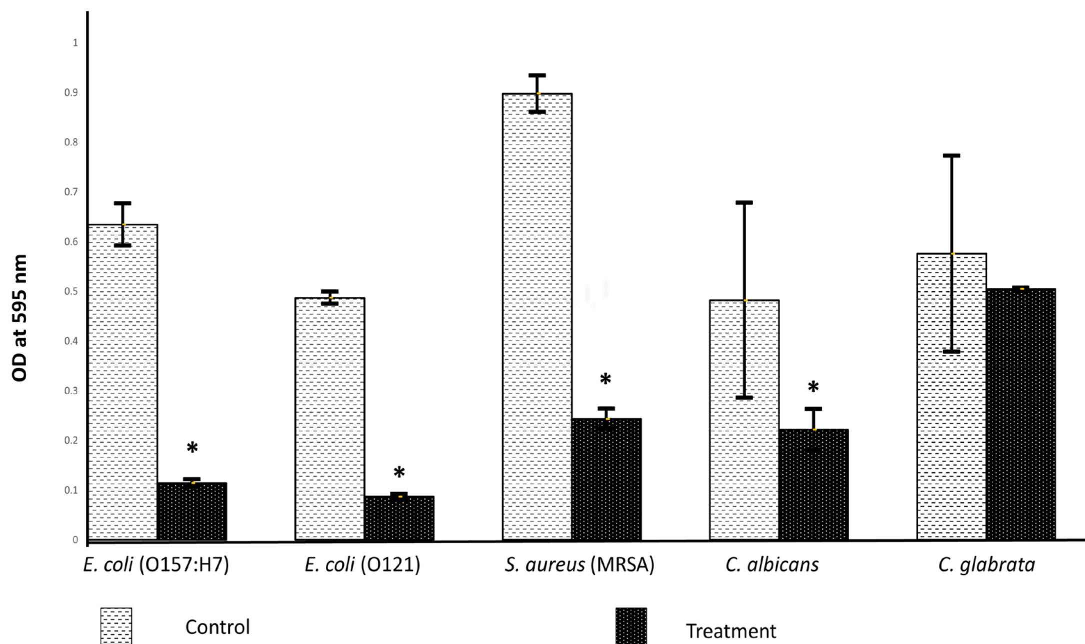

Fig. 5. Antibiofilm activity of *S. aromaticum* against some human pathogenic microbes *(E. coli* (O157:H7 and O121); methicillin- resistant *S. aureus* (MRSA)); *C. albicans* and C. *glabrata.* C: control (amount of biofilm of the tested microbe). Crystal violet and resazurin assay to determine biofilm biomass as a measure of inhibition of biofilm formation. Shown are the averages from at least three independent measurements. The error bars indicate the standard deviations. Asterisk: means values are highly significant compared with control.

The SEM micrographs (Fig. 6) revealed that the *S. aromaticum* treatment noticeably decreased the membrane integrity and disrupted cell permeabilization. The treated *E. coli* cells had a deformed, incomplete/sunken shape, and lacked cell walls. The entirety of the cell membrane was affected by *S. aromaticum* leading to cytoplasm exudation and succeeding cell death. Similar findings were reported by Behbahani et al. (2019 a), who studied the antibacterial mechanisms of cumin essential oil against some pathogenic and spoilage bacteria and by Behbahani et al. (2019 b), who examined the effect of *S. aromaticum* essential oil on foodborne pathogens. The untreated *C. albicans* cells had a normal oval shape with a smooth surface and the appearance of hyphae, whereas *S. aromaticum* extract treated cells exhibited disrupted cell walls causing leakage of cell contents. The cells had irregular shapes and appeared elongated and flaccid. Latifah-Munirah et al. (2015) similarly found that eugenol disrupted *C. albicans* cell wall and released the cellular materials. The cells were found to be flaccid. Exposing *C. albicans* to 4×MIC of eugenol led to 50.9% cell death but a 0.5×MIC dose caused a 76.23% decrease in ergosterol biosynthesis (Khan et al., 2013).

Aflatoxins, secreted generally by *A. flavus* and *A. parasiticus*, are extensively disseminated toxigenic secondary metabolites with a considerable carcinogenic property (Zhao et al., 2013). Detoxification of aflatoxin by essential oils is related to the downregulation of aflatoxin biosynthesis genesand the chemical conversion of aflatoxin B1 maternal compound to another compound (Xiang et al., 2020). In our study, *S. aromaticum* aqueous extract significantly suppressed the production of aflatoxins up to 31.7% (Table 4) and the variation in the percentage may be due to the nature of the fungus and its genetic structure. Vijayanadraj et al. (2014) showed that the *S. aromaticum* aqueous extract reduced aflatoxin B1 by was 36.4%. Also,clove oil and clove extract completely inhibited aflatoxin production by *A. flavus* (Elsamra et al., 2012).

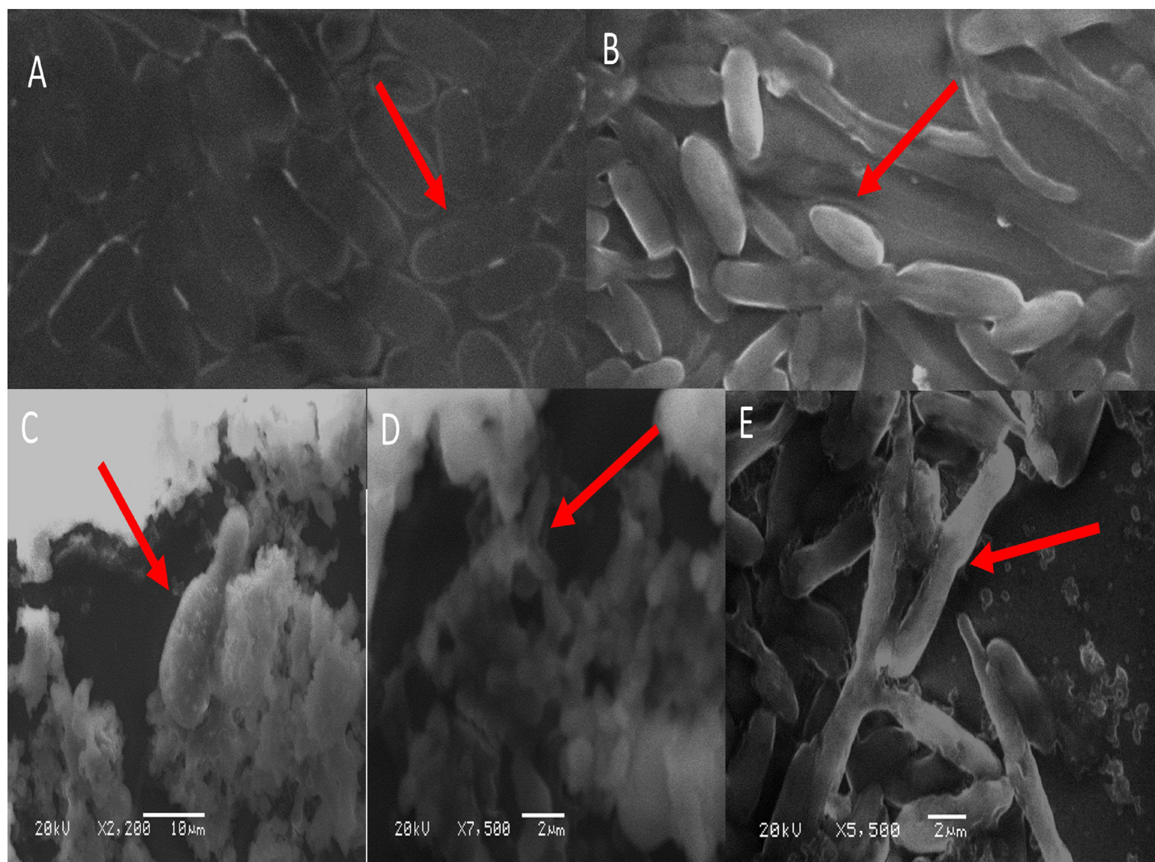

Fig. 6. Scanning electron microscopy micrographs of untreated *E. coli and Candida with S. aromaticum* (A, B); Treated *E. coli* (C, D); Treated Candida (E).

Conclusion

The current study recommended using clove aqueous extract as a new-fangled therapeutic against multidrug-resistant microorganisms. More interest in medicinal herbs as they are rich in antimicrobial compounds. Rotaxane can be excellent substituent for antibiotics for decreasing dissemination of multidrug resistant microbes. Clove, alone or in combination with some commercial antibiotics or rotaxane derivatives inhibits microbial growth, biofilm formation, and aflatoxin biosynthesis for declining the spreading of these pathogens. Future perspective for separation of the largest concentration of compounds detected by GC-MS for studying its effect alone also, conducting *in vivo* experiments.

Conflict of Interests

All authors declare that they have no conflicts of interest.

Funding Intformation

Not applicable

Availability of Data and Materials

All data and materials as well as software application or custom code support published claims and comply with field standards. All data generated or analysed during this study are included in this published article.

Fthics Approval

The protocol was accepted by Research Ethics Committee, Faculty of Science, South Valley University, Egypt (REC-FScSVU). Code No. was 025/11/22.

Consent for Puplication

Not applicable

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