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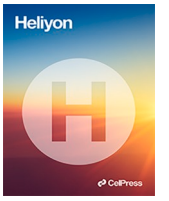

Research article

Ephedra alte extracts' GC-MS profiles and antimicrobial activity against multidrug-resistant pathogens (MRSA)

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ABSTRACT

The extracts of *E. alte* offer promising potential as renewable resources for various chemical derivative products aimed at addressing antibiotic resistance. These extracts exhibited significant activity against methicillin-resistant *Staphylococcus aureus* (MRSA), a strain known for its resistance to multiple antibiotics. The extracts were found to be effective against several common antibiotics, including Imipenem, Ampicillin, Penicillin G, Oxacillin, and Amoxicillin-clavulanate. GC-MS analysis revealed that the phytoconstituents of *E. alte* extracts, obtained using both methanol and ethyl acetate, consist of a diverse range of 83 and 160 phytocompounds, respectively. These organic compounds serve as important biochemical precursors for the synthesis of vitamins E and K1, and exhibit antioxidant, antimicrobial, and anti-inflammatory properties in both plants and microorganisms. Notable compounds identified include fatty acids (such as palmitic acid, dodecanoic acid, sebacic acid, pentadecanoic acid, myristic acid, stearic acid, behenic acid, and linoelaidic acid), phytosterols (Campesterol, β-sitosterol, Stigmast-5-ene), sugars (D-fructose, Fructofuranans), terpenoids (Phytol, citronellol), and phenolic acids (Protocatechoic acid, shikimic acid). The antimicrobial activity of all *E. alte* extracts was found to be superior to that of mupirocin and ciprofloxacin, as observed in susceptibility testing against MRSA ATCC 43300 and other pathogenic bacteria and fungi. It is likely that the combined action of the antimicrobial components within the *E. alte* extract bypasses the mechanisms employed by MRSA to protect itself from antibiotics. Further experiments are needed to investigate the individual effects of each pure compound and their potential synergistic interactions, which may enhance their overall performance.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is an incredibly common and dangerous type of bacteria. It is a gram-positive coccus with coagulase activity, meaning it is very resistant to antibiotics and other medications. It is a major cause of skin infections, such as boils, and can cause severe illnesses like pneumonia, meningitis, and toxic shock syndrome. Furthermore, it is a major cause of food poisoning and other food related illnesses [1]. *S. aureus* spreads through contact using an infected surface or person. It can additionally spread through the sharing of personal items, such as towels and eating utensils. It is critical to maintain good hygiene and to wash hands continually to prevent the spread of this bacteria. Additionally, ensure that meat, milk, fruit, and vegetables are properly cooked and stored to reduce the likelihood of food poisoning. It is essential to seek medical advice if any signs of infection occur [2]. The number of incidents reported varies due to deviations in the study populations' size as well as demographics, the reliability of sampling, as well as the culture techniques used [3,4].

The public's health is seriously threatened by MRSA, also known as methicillin-resistant *S. aureus*. Since this bacterium is resistant to widely used antibiotics, they are useless for treating infections. MRSA infection increases the risk of developing related complications like fever as well as abscesses in infected individuals. Additionally, it has been discovered to be highly contagious, meaning that it can be transmitted from person to person with ease in a variety of settings, which would include nursing homes and hospitals. MRSA's emergence has called for careful management. As it can lessen the likelihood of the bacteria being transmitted, maintaining proper hygiene is among the most crucial steps. Healthcare facilities must also take additional precautions to stop outbreaks [5–9].

Saudi studies on MRSA aim to improve the knowledge about this major health threat. The scientific community in Saudi Arabia is aiming to develop novel strategies to diagnose, treat and prevent infections caused by MRSA. Various studies have been conducted to understand the epidemiology of MRSA in the country, the genetic and molecular characteristics of the isolates, and their antibiotic resistance profiles [10]. A number of studies have focused on exploring the prevalence and risk factors associated with MRSA colonization in the country. These studies highlighted the fact that MRSA is a common cause of healthcare-associated infections in Saudi Arabia, and that it is an increasing threat in the community [11]. Moreover, they emphasized the need to understand and address the risk factors associated with MRSA colonization. In addition to prevalence studies, genotyping and molecular studies have been conducted to understand the genetic characteristics of MRSA isolates circulating in the country [12,13].

Many studies have recently reported that many medicinal herbs have antibacterial activity against MRSA. As a result, these medicinal plants could be effective options for treating MRSA infections.

The botanical compounds of *Ephedra alte* (*E. alte*) crude product was examined using gas chromatography-mass spectrometry (GC-MS) as well as high-performance liquid chromatography (HPLC). It was discovered that it contained many natural products like alkaloids, flavonoids, tannins, phenolic acid as well as terpenoids. *S. aureus*, *Escherichia coli* (*E. coli*), and *Klebsiella oxytoca* (*K. oxytoca*) were all susceptible to the antioxidant, antibacterial, anti-inflammatory effects and pepsin enzyme inhibitory properties of *E. alte*. All these findings suggested that *E. alte* could be used as a natural remedy for treating infectious diseases as well as GERD (Gastroesophageal reflux disease) [14,15].

The current study's objective is to investigate the effectiveness of the natural methanolic as well as ethyl acetate phytochemical constituents of *E. alte* as anti-MRSA characteristics.

2. Materials and methods

2.1. Plant materials

The *E. alte* plant's aerial components were collected from a local region within the Al-Tafila governorate of Jordan. These parts were then classified and verified by Prof. Abd Elnaser Khalil, an expert from the Botanical Herbarium of the Egyptian Ministry of Agriculture. The validation of the plant and the associated voucher specimen was conducted by the Cairo University Herbarium in Egypt (Eg-N. D50233).

2.2. Sample extraction method

An amount of 10 g of dried and grinded plant was extracted using 250 ml ethyl acetate and 250 ml methanol separately by stirring at 250 rpm at room temperature for 24 h. The extracts were then filtered through Whatman filter paper (No. 4) as well as then concentrated by rotary evaporator (Buchi R-215, Switzerland) at 40 °C until drying. An amount of 10 mg for each extract were derivatized by 150 μL of N-Methyl-N-trimethylsilytrifluoroacetamide – with 1% Trimethylchlorosilane and 150 μl of hexane then incubated at 60 °C for 30 min. The final solution was filtered by 0.22 μm syring filter before transfer into 1.5 ml HPLC vial. An amount of 1 μl were injected into GC-MS.

2.3. GC-MS method

For the extracts analysis, a GC-MS-QP 2010 Ultra System (Shimadzu, Kyoto, Japan) was used in conjunction with Lab-Solutions GC-MS program. Separation was achieved using a Restek Rtx®-5 ms column (30.0 m 0.25 mm, 0.25 μm). At a flow rate of 1.0 ml/min, the carrier gas was helium (99.9%). The initial temperature of the oven had been set to 60 °C and stayed there for 3 min. The temperature was then raised to 140 °C at a rate of 7 °C/min, following that directly raised to 300 °C at a rate of 5 °C/min, and remained there for 5 min for a total run time of 51.43 min.

The ionization temperature had been set at 250 °C, as well as the interface's auxiliary temperature. In full-scan mode, analytes between 50 and 650 amu were examined. With the help of a Shimadzu AOC-20i Auto Injector (Kyoto, Japan), an overall volume of 10 μl was injected splitlessly. The compound was located using the NIST/EPA/NIH Mass Spectral Library (NIST 17), GC total ion chromatograms (TIC), as well as fragmentation patterns.

2.4. Antimicrobial susceptibility testing (AST)

The chemical structures of natural products have a significant impact on their antimicrobial activity. Specific structural features, such as functional groups, aromatic systems, stereochemistry, and size/shape, influence the compound's ability to interact with microbial targets and inhibit microbial growth. Understanding this relationship is crucial for identifying key structural determinants and designing effective antimicrobial agents [16–19].

The biological activity of the extracts was evaluated as antimicrobial agents against *Salmonella typhimurium (S. typhimurium) ATCC 14028* (Gram-negative bacterium), MRSA, ATCC 43300 (Gram-positive bacterium resistant to antibiotics) and *Candida albicans (C. albicans)* ATCC 60193 (pathogenic yeast) using disk diffusion and two-fold broth microdilution assays according to Bavelaar et al. (2021) [16], Caso Coelho et al. (2021) [17] and Yücesoy et al. (2001) [18]. Briefly, for the disk diffusion method, sterile disks (6 mm) were loaded with 10 mg of the extract and the disk gently was placed on the surface of Mueller–Hinton agar (Scharlab, S.L., Spain) inculcated by tested microorganisms. The standard antibiotic disk (Tobramycin, 10 mg/desk, Becton, Dickinson and Company-Cockeysville) was used as control and the media, biological solutions, and microorganisms used in this work were carried out in accordance with the manufacturer's instructions as well as standard procedures. The inhibition areas that formed around the disks on all plates had been measured (mm) after 24 h of incubation at 35 °C ± 1.

2.5. Susceptibility test of MRSA

For two-fold-microdilution method, each well of the sterile 96 microplates received 100 μl of nutrient broth (Scharlab, S.L., Spain) inculcated by the tested microorganisms (total microbial cells of each well were 4.5–5 × 105 cells as colony form unit per ml (CFU/ml)). The first well received 100 μl of the extract then the two-fold serial dilutions were done so that each well received half of the concentration in the well preceding it. The minimal inhibitory concentration (MIC) (mg/ml) was calculated from the well that received the least amount of extract required to effectively prevent microbial growth in a well. The MBC, or minimum bactericidal concentration (mg/ml) was determined using re-cultivation method according to Chikezie (2017) [19]. For the sake of comparison, the susceptibility test for MRSA was carried out using the VITEK® 2 system (BioMérieux, USA) (Table 1).

2.6. Statistical analysis

The data analysis in this study was conducted using Microsoft Excel 2013. The results were reported as the mean of three samples ± standard deviation (SD) of three samples (n = 3).

3. Results

3.1. Phytochemical profiling of the extracts using GC-MS

Table 2 provides a summary of the findings for both the ethyl acetate and methanol extracts. The chromatograms for these extracts are depicted in Fig. 1(a and b). The analysis identified approximately 190 compounds in the ethyl acetate extract and 160 compounds in the methanol extract. Interestingly, 83 compounds were found to be present in both extracts. Table 2 lists the main components with a peak area percentage above 0.5%. The phytoconstituents of the *E. alte* extract were evaluated using GC-MS, comparing the mass fragmentation patterns to standards such as Wiley 9 library spectral information and NIST. This allowed for the quantification and

Table 1
Susceptibility test of MRSA using VITEK® 2 system.

Antibiotic	MRSA	Interpretive Criteria (in μg/ml) of some antibiotics[a]	
		Susceptible	Resistant
Gentamycin	Susceptible		
Imipenem	Resistant		
Cefoxitin	Positive	≤4 μg/ml	≥8 μg/ml
Ampicillin	Beta-lactamase positive		
Penicillin G	Beta-lactamase positive		
Oxacillin	Resistant	≤2 μg/ml	≥4 μg/ml
Amoxicillin-Clavulanate	Resistant		
Trimethoprim-Sulfamethoxazole	Susceptible	≤2/38	≥4/76
Mupirocin	Susceptible	≤1 mg/ml	≥265 mg/ml
Ciprofloxacin	Susceptible	≤1 mg/ml	>1 mg/ml

[a] According to https://www.cdc.gov/mrsa/lab/index.html [20].

Table 2
E. alte chemical components list.

Chemical class	Compound name	Retention time (RT)	Ethyl acetate extract	Methanol extract
			Area %	Area %
Fatty acid	Dodecanoic acid	21.33	1.72	0.92
Polyalcohol	Xylitol	22.27	0.07	1.6
Sugars	D-Fructose	24.31	1.69	ND[a]
	D-Fructofuranose	24.47	0.66	1.1
Phenolic acid	Shikimic acid	24.52	0.57	ND[a]
	Protocatechoic acid	24.70	0.52	0.21
Fatty acids	Myristic acid	25.34	1.26	0.34
	Sebacic acid	26.18	0.1	0.77
Sugar alcohol	D-Glucitol	26.88	1.66	4.25
Sugar Acid	Arabino-Hexonic acid	27.19	ND[a]	0.95
Fatty acid	Pentadecanoic acid	27.25	1.04	ND[a]
Pentoses	β-Arabinopyranose	28.09	0.85	ND[a]
Fatty acid	Palmitic acid	29.19	ND[a]	15.22
Terpenoid	Phytol	30.70	0.03	1.16
	Citronellol	31.45	2.4	ND[a]
Fatty acids	5,8,11-Eicosatrienoic acid	31.82	0.89	0.21
	Linoelaidic acid	32.07	ND[a]	3.72
	9-Octadecenoic acid	32.24	ND[a]	10.68
	Stearic acid	32.82	3.8	2.49
	9-Decenoic acid	35.10	ND[a]	1.25
	Eicosanoic acid	36.03	3.26	1.37
β-hydroxycarboxylic acid	3-Hydroxydodecanedioic acid	37.51	ND[a]	1.29
Fatty acid	Behenic acid	39.07	4.06	1.67
	Lignoceric acid	41.84	2.13	0.36
Fatty alcohols	1-Hexacosanol	43.27	1.6	ND[a]
Fatty acid	Hexacosanoic acid	44.44	0.61	0.04
Fatty alcohols	1-Octacosanol	45.79	1.2	0.09
Phytosterols	Campesterol	47.28	1.31	0.82
Terpenoids	Stigmast-5-ene	48.45	ND[a]	2.68
	β-sitosterol	48.59	3.71	ND[a]
summary of the area %			35.14	53.19

[a] ND: Not determined.

determination of each constituent in the methanolic and ethyl acetate extracts. Fig. 2 illustrates the structures of some of the identified compounds in both extracts as determined by GC/MS analysis.

3.2. Antimicrobial activity

The antimicrobial testing results, as shown in Fig. 3(A-D), revealed that the *E. alte* extract exhibited significant antimicrobial activity against MRSA. However, no bioactivity was observed against *C. albicans* ATCC 60193 or *S. typhimurium* ATCC 14028. Furthermore, neither of the tested extracts displayed bioactivity against the other pathogenic bacterial or yeast strains. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the *E. alte* extract against MRSA were determined to be 0.16 mg/mL and 0.31 mg/mL, respectively, as shown in Fig. 4. Additionally, the disk diffusion test conducted with 4 mg/disk of the *E. alte* extract resulted in a zone of inhibition measuring 13 mm.

4. Discussion

Fatty acids, which are organic compounds comprising carboxylic acids and characterized by long aliphatic chains that can be straight or branched, saturated or unsaturated [21], have long been acknowledged for their antimicrobial properties. Notably, plants and algae produce fatty acids as a defense mechanism against various pathogens, including multidrug-resistant bacteria (MDRB) [22]. The Centers for Disease Control and Prevention (CDC) are particularly intrigued by the antibacterial activity of fatty acids against MDRB, as it positions them as potential next-generation antibacterial agents for treating and preventing bacterial infections [23]. While numerous reviews have discussed the antibacterial activity of fatty acids [24–26], there is still a need for a comprehensive understanding of the diverse mechanisms underlying their antibacterial effects. Additionally, the practical application of fatty acids has been explored through their combination with other antibiotics [27]. Recent studies have indicated synergistic effects when fatty acids are combined with penicillins, fluoroquinolones, and aminoglycosides against both Gram-positive and Gram-negative bacteria [28,29]. Furthermore, fatty acids have demonstrated notable anti-inflammatory and wound healing properties [30,31]. Overall, these findings emphasize the potential of fatty acids as promising candidates for the development of innovative antibacterial agents.

The analysis revealed that the intended extract is mostly made up of fatty acids like dodecanoic acid, sebacic acid, myristic acid, stearic acid, pentadecanoic acid, behenic acid, linoelaidic acid, as well as palmitic acid. Phenolic acids were also detected: Protocatechoic acid which has antioxidant and anti-inflammatory activities, shikimic acid (an important biochemical metabolite in plants

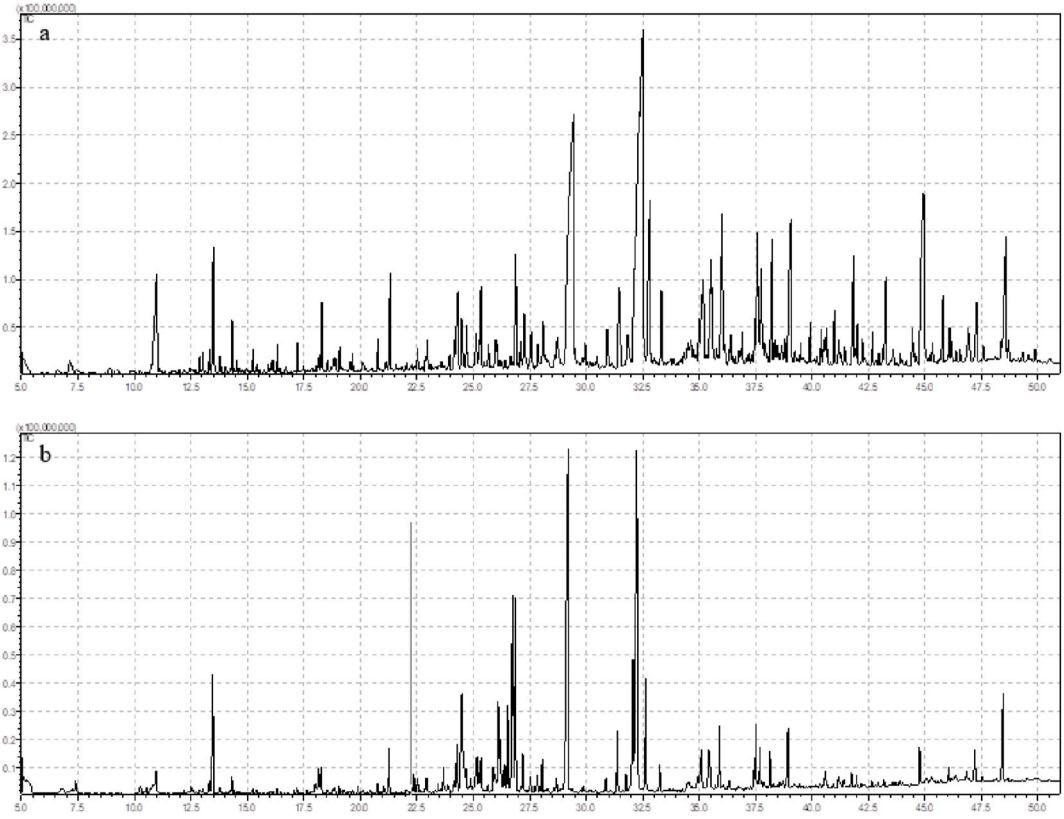

Fig. 1. GC–MS profile of the plant extracts by **a**-ethyl acetate and **b**-methanol.

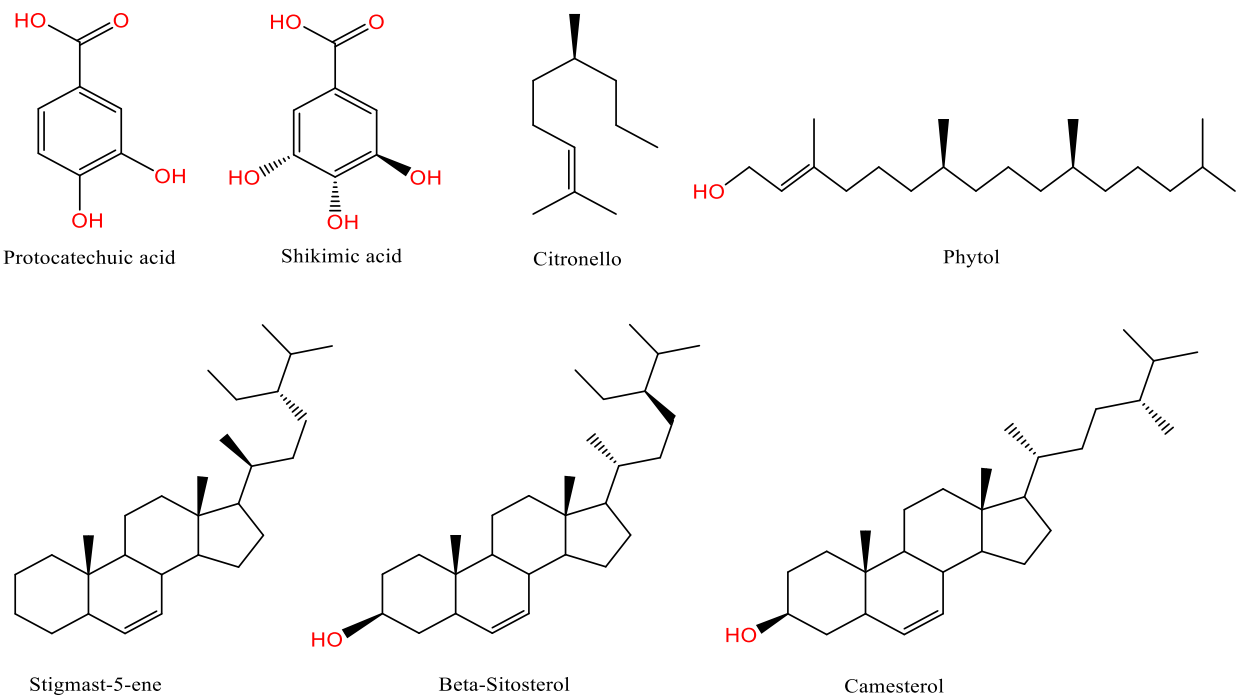

Fig. 2. Chemical structures of identified compounds in methanolic and ethyl acetate extracts by GC-MS.

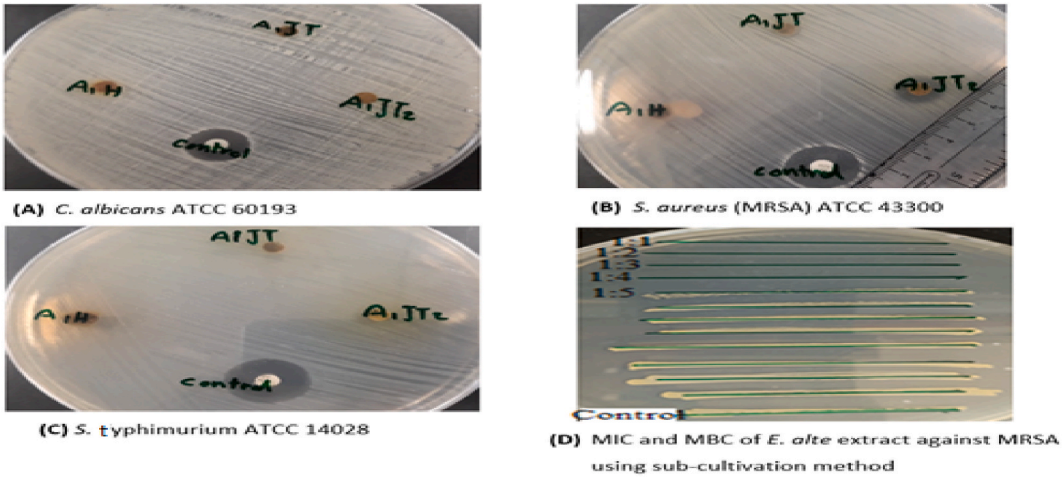

Fig. 3. The biological activity of the *E. alte* extracts against selected pathogenic microbes. (A) *C. albicans* ATCC 60193 using disk diffusion assay, (B) *S. aureus* (MRSA) ATCC 43300 using disk diffusion assay, (C) disk diffusion assay, and (D) MIC and MBC of *E. alte* extract against MRSA using re-cultivation method.

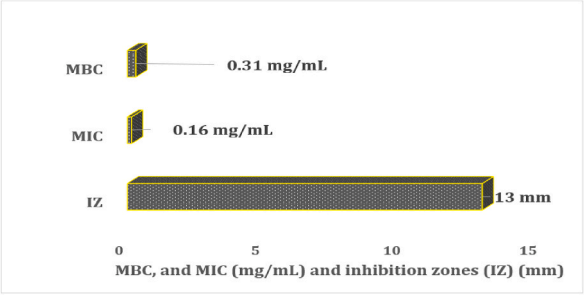

Fig. 4. *E. alte* extracts' antimicrobial activity against Methicillin-resistant *S. aureus* (MRSA) ATCC 43300. MBC (minimum bactericidal concentration), MIC (minimal inhibitory concentration), MBC (minimum bactericidal concentration) as well as IZ (inhibition zone).

and microorganisms). Sugars were also found in both extracts (D-fructose, Fructofuranose). Terpenoids were also detected in the plant extraxts: Phytol, an acyclic hydrogenated diterpene alcohol, is able to be employed as a precursor in the production of synthetic types of vitamin E as well as vitamin K1, as well as citronellol (natural acyclic monoterpenoid). phytosterols were also present e.g. Campesterol whose chemical structure is similar to that of cholesterol, and found in many fruits, vegetables and plants and have anti-inflammatory effects. A different approach phytosterol (β-sitosterol) along with a molecular structure similar to cholesterol has been identified. The plant kingdom is rich in β-sitosterol. It can be found in olive oil, nuts, as well as avocados. Furthermore, Stigmast-5-ene was also detected.

The data resulted from primarily antimicrobial susceptibility testing showed that all extracts have no biological activity against *S. typhimurium* ATCC 14028, MRSA ATCC 43300, and *C. albicans* ATCC 60193 except the *E. alte* extract that showed bio-ability to inhibit MRSA ATCC 43300 (Figs. 3 and 4). The biological activity of *E. alte* extract against MRSA ATCC 43300 is good compared to the results given in Table 1 regarding mupirocin and ciprofloxacin. The work requires further investigations to produce pure compounds from the extract and to evaluate their effectiveness for fighting drug-resistant pathogens such as MRSA, and determining the mechanism of action of those compounds. There are many studies [14] that reported that crude extracts obtained from *E. alte* have biological activity as antibacterial agents. In the present work, the GC-MS analysis of *E. alte* extract (Table 2) confirmed that the extract contains several antimicrobial compounds such as D-glucopyranoside derivatives (30.8% of the total components) and phenobarbital derivatives (16.8% of the total components). Kawsar et al. (2018) [32] and Fahad et al. (2021) [33] reported that D-glucopyranoside and phenobarbital derivatives have the ability to inhibit many bacteria and fungi. The chemical analysis in Table 2 showed that 0.5% of the chemical compositions of the extract were propanediols. It has been reported that some propanediols have high activity against some pathogenic bacteria strains [34].

The *E. alte* extract is a bio-renewable source for many chemical derivatives with which we can fight the problem of antibiotic resistance, for example, this extract showed high activity against MRSA that can resist many standard antibiotics such as Imipenem, Ampicillin, Penicillin G, Oxacillin and Amoxicillin-Clavulanate. In general, MRSA can resist β-lactam antibiotics due to the mecA gene encoded to produce a penicillin-binding protein (PBP2a) that is a distinctive transpeptidase (The enzyme responsible for forming

peptidoglycan chains in bacterial cell walls) that is not inhibited completely by β-lactams [35].

5. Conclusions

In conclusion, the extracts obtained from *E. alte* exhibit significant potential as bio-renewable components for a range of chemical derivative products aimed at addressing the challenge of antibiotic resistance. These extracts have demonstrated strong activity against methicillin-resistant *Staphylococcus aureus* (MRSA), a strain known for its resistance to several common antibiotics. The GC-MS analysis of the extracts revealed the presence of various phytoconstituents, including fatty acids, phytosterols, sugars, terpenoids, and phenolic acids. These organic substances serve as essential biochemical precursors for the synthesis of synthetic forms of vitamins E and K1, while also possessing antioxidant, antimicrobial, and anti-inflammatory properties in both plants and microorganisms. Notably, the *E. alte* extracts exhibited a superior inhibitory profile against MRSA ATCC 43300 compared to mupirocin and ciprofloxacin, as determined through antimicrobial susceptibility testing. It is possible that the combined action of the antimicrobial substances present in the *E. alte* extract works synergistically to overcome MRSA's defense mechanisms against antibiotics. Further research is necessary to elucidate the individual mechanisms of action of each pure compound and explore their synergistic interactions to enhance their overall efficacy.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Haya Ayyal Salman: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. **Amira Suriaty Yaakop:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Conceptualization. **Fuad Al-Rimawi:** Writing – review & editing, Methodology, Formal analysis. **Ana Masara Ahmad Makhtar:** Writing – review & editing, Validation. **Muath Mousa:** Formal analysis. **Mohammad H. Semreen:** Formal analysis. **Naiyf S. Alharbi:** Writing – review & editing, Methodology, Funding acquisition.

Declaration of competing interest

The authors have no conflicting interests to declare.

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Abbreviations

C. albicans	Candida albicans
CDC	Centers for Disease Control and Prevention
E. alte	Ephedra alte
E. coli	*Escherichia coli*
GC-MS	Gas chromatography-mass spectrometry
GERD	Gastroesophageal reflux disease
HPLC	High-performance liquid chromatography
IZ	Inhibition zone
K. oxytoca	Klebsiella oxytoca
MBC	minimum bactericidal concentration
MIC	Minimal inhibitory concentration
MDRB	Multidrug-resistant bacteria
MRSA	Methicillin-resistant *Staphylococcus aureus*

PBP2a Penicillin-binding protein
S. aureus *Staphylococcus aureus*
S. typhimurium *Salmonella typhimurium*
TIC Total ion chromatograms

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