Research Article

Nael Abutaha, Lamya Ahmed Al-Keridis, Rania Ali El Hadi Mohamed*, Fahd A. AL-mekhlafi

Potency and selectivity indices of *Myristica fragrans* Houtt. mace chloroform extract against non-clinical and clinical human pathogens

<https://doi.org/10.1515/chem-2021-0097>

received February 21, 2021; accepted October 11, 2021

Abstract: This study assessed the antimicrobial, toxicity, and phytochemical profiles of *Myristica fragrans* extracts. Different solvent extracts were tested for antimicrobial activity against clinical and reference microbial strains, using disc and well diffusion assays and microdilution techniques. Antioxidant potential was investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Cytotoxicity assay was conducted against human umbilical vein endothelial cells (HUVECs) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Acute toxicity was assessed in laboratory Swiss albino mice at a single dose of 2,000 mg/kg body weight for 14 days. To assess the phytochemical constituents, spectrophotometric and gas chromatography-mass spectrometry (GC-MS) methods were used. The chloroform extract revealed antimicrobial potencies against the Gram-positive bacteria and *C. albicans* with minimum inhibitory concentrations. In the DPPH assay, the IC₅₀ value of the chloroform extract was determined to be 1.49 mg/mL. The phenolic and flavonoid contents were 26.64 ± 0.1 mg of gallic acid equivalents/g and 8.28 ± 0.1 mg quercetin equivalents/g, respectively. The IC₅₀ value was determined to be 49 µg/mL against the HUVEC line. No mortality or morbidity was observed. GC-MS analysis

indicated the presence of 2-cyclopenten-1-one (44.72%) as a major compound. The current results provide scientific support for the use of *M. fragrans* in folk medicine.

Keywords: antioxidant, antimicrobial, gas chromatography-mass spectrometry

1 Introduction

The current attention paid to medicinal herbs is attributed to their worldwide availability and use in traditional therapies [1]. Medicinal herbs have been studied for their bioactive secondary metabolites to find alternative sources of antibiotics for pharmaceutical industries and to promote health through the consumption of products containing natural antioxidants. Natural antioxidants have the potential to scavenge free radicals and are effective in preventing diseases caused by oxidative stress [2,3], such as cancer, and neurological and cardiovascular disorders [4].

There is an urgent need for natural therapeutic products with antibacterial and antioxidant activities capable of curing microbial infection and reducing the damaging effects of free radicals [5]. Although the toxicity of most medicinal herbs has not been investigated, it is often believed that extracts derived from natural sources are safer than synthetic compounds [6–8]. Among these phytochemicals, phenols and flavonoids display diverse and potent biological activities [9].

Myristica fragrans Houtt., which belongs to the Myristicaceae family, is more commonly known as nutmeg [10]. The fruit, once mature, is separated into the mace (crimson-colored), which surrounds the seed [11]. Nutmeg has been used for many purposes, such as antimicrobial, psychostimulant, antioxidant, antithrombotic [12], insecticidal, or fungicidal agent [13], as well as to aid in poor digestion, insomnia, urinary incontinence [14], arthritis, and muscle spasm [11]. Some reports have highlighted its anticonvulsant, analgesic, anti-inflammatory [15–17], antidepressant, and anticancer activities, whereas the

* **Corresponding author: Rania Ali El Hadi Mohamed**, Biology Department, Faculty of Science, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia; Epidemiology Department, Scientific Researcher and Research Coordinator, Federal Ministry of Health, Khartoum, Sudan, e-mail: Rasaddig@pnu.edu.sa, tel: +00966594000487

Nael Abutaha: Bioproducts Research Chair Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

Lamya Ahmed Al-Keridis: Biology Department, Faculty of Science, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia

Fahd A. AL-mekhlafi: Bioproducts Research Chair Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia; Department of Agricultural Production, College of Agriculture and Veterinary Medicine, Tamar University, Dhamar, Yemen

seed is reported to control vomiting [13] and have anti-inflammatory effects [15]. The seed is also used to treat diarrhea, dysentery, vomiting, and abdominal distension, toothache, and rheumatic pain and is also used in fragrance industries [18].

However, to our knowledge, *M. fragrans* mace has never been extracted using a sequential solvent of increasing polarity, cytotoxicity on normal cells, and selectivity indices of the promising fraction. Finding new sources of bioactive molecules is of the highest importance, especially for drug-resistant microbes.

In the present study, we aimed to further investigate the antimicrobial activity, phytochemical constituents, acute toxicity, and selectivity index (SI) of different solvent extracts of *M. fragrans* on noncancerous cell lines under *in vitro* conditions.

2 Materials and methods

2.1 Plant material

M. fragrans mace was obtained from Reef al Yamen Co., an herbal shop in Al Morooj District, Riyadh, Saudi Arabia. The plant was authenticated (voucher no. KSU-BRC-136-0) at King Saud University, Riyadh, Saudi Arabia. The mace was ground using a commercial machine. About 20 g of the *M. fragrans* mace powder was extracted with 450 mL of different solvents (hexane, chloroform, ethyl acetate, and methanol) for 24 h in a Soxhlet extractor. The extracts were centrifuged at 10,000×g, evaporated using a rotary evaporator (Heidolph, Germany), and stored at −80°C.

2.2 Total polyphenol content

Total phenol content was assessed using the Folin–Ciocalteu (FC) reagent [19]. Two microliters of the *M. fragrans* chloroform (MFC) extract was added to 20 µL of the FC reagent (10%) in a 96-well plate. The solution was then pipetted up and down several times and incubated for 10 min at 25°C. Eighty microliters of sodium carbonate (7.5%) were added to the solution and mixed by pipetting up and down several times. The plate was incubated at 25°C for 2 h for color development. The absorbance at 765 nm was measured using a microplate reader (Thermo Scientific Multiskan, China). The phenolic content was calculated as mg/g gallic acid equivalent (GAE) based on the standard curve using the following equation:

$$Y = 0.0036x + 0.0584, \quad (1)$$

with an R^2 value of 0.9951.

2.3 Total flavonoid content

The total flavonoid concentration was evaluated using aluminum chloride (AlCl_3) colorimetric assay [19]. Two microliters of the MFC extract was mixed with 60 µL of methanol, 4 µL of AlCl_3 (10%), 4 µL of potassium acetate, and 112 µL of distilled water in a 96-well plate. The solutions were pipetted up and down several times and then incubated at room temperature for 30 min. The absorbance of the solution was measured at 420 nm. The total flavonoid content was presented as mg/g quercetin equivalent (QuE) based on the standard curve using the following equation:

$$Y = 0.0053x + 0.0566, \quad (2)$$

with an R^2 value of 0.994.

2.4 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The MFC extract radical scavenging potential was assessed *in vitro* following the method described by Abutaha [19]. The reaction mixture (200 µL) consisted of 198 µL of DPPH (0.008% w/v in methanol) in a 96-well plate with 2 µL of various concentrations (0.125–4 mg/mL) of the MFC extract and gallic acid (10–90 µg/mL) as a standard. The mixture was pipetted up and down several times and then kept at 25°C for 30 min. The absorbance was determined at 517 nm. The percentage of scavenging activity was calculated using the following formula:

$$\begin{aligned} &\text{DPPH radical scavenging activity} \\ &= \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100. \end{aligned} \quad (3)$$

2.5 Antimicrobial assays

2.5.1 Microbial strains

A total of nine Gram-negative and Gram-positive bacterial strains and a yeast strain (*Candida albicans*) were obtained from the Department of Microbiology of King Saud University. Microbial strains used included clinical strains *Salmonella typhimurium*, *Escherichia coli*, *Acinetobacter*

baumannii, *Enterococcus faecalis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* and reference strains methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC-33591) and *C. albicans* (ATCC-90028) (Table 1). Methanol (MeOH) was used as a negative control for the antibacterial and antifungal assays.

2.5.2 Culture media

Luria-Bertani agar and potato dextrose agar were employed for the disc and well diffusion assays, whereas Luria-Bertani broth was used to determine the minimum inhibitory concentration (MIC).

2.5.3 Disc and well diffusion assays

The test bacteria and yeast were grown on the Luria-Bertani agar and potato dextrose agar plates, respectively, for 18 h at $36 \pm 1^\circ\text{C}$. Some of the isolated colonies were suspended in 0.9% saline solution, and the optical density at 600 nm was adjusted to 0.01. Fifty microliters of inoculum were swabbed onto agar plates using a sterile cotton swab. For the disc diffusion method, blank discs (6 mm diameter) were loaded with 20 μL of the MFC extract (1 mg/mL, stock solution), for a total dry weight concentration of 20 $\mu\text{g}/\text{disc}$. For the well diffusion method,

a sterilized cork borer (6 mm diameter) was employed to form wells that were loaded with 20 μL of the MFC extract. Discs and wells loaded with MeOH served as a negative control. The plates were incubated for 24 h at $36 \pm 1^\circ\text{C}$. The zones of inhibition resulting from treatment with the MFC extract were measured in millimeters. Tests were carried out in triplicate, and the mean values were calculated.

2.5.4 Broth microdilution method

The microdilution method was employed to assess the MICs of the MFC extract using 96-well plates. Briefly, 100 μL of the Luria-Bertani broth was added into each well. The MFC extract (100 μL) was then added to the first column (wells A1 to H1), and a twofold serial dilution was carried out by pipetting the contents up and down in each well of the first column and transferring 100 μL to the next well of the same row, and so on through to the 10th well. The last 100 μL from the 10th well was discarded. Rows 11 and 12 were maintained for positive control (chloramphenicol) and negative control (MeOH), respectively. Then, 100 μL of different microorganism suspensions was added and the plate was incubated overnight at 37°C . Next day, the results were read using a multi-well microplate reader. The lowest concentration of the MFC extract capable of inhibiting visible growth was considered as the MIC.

Table 1: Antimicrobial activity of the MFC extract

Microorganism	Origin	Resistance phenotype	Well assay zone (mm) 20 μL	Disc assay zone (mm) 20 μL	MIC ($\mu\text{g}/\text{mL}$)	SI
Gram-negative bacteria						
<i>S. Typhimurium</i>	14,028		NA	NA	—	
<i>E. coli</i>	25,922		NA	NA	—	
<i>A. baumannii</i>	Clinical	S	NA	NA		
<i>A. baumannii</i>	Clinical	Clavulanic acid, ampicillin, cefuroxime, cefixime, ceftriaxone, amoxicillin, amoxicillin/clavulanic acid	NA	NA		
Gram-positive bacteria						
<i>E. faecalis</i>	29,212		10	10	3.12	0.06
<i>S. epidermidis</i>	Wild strain R				1.56	0.03
<i>S. epidermidis</i>	Wild strain S				1.56	0.03
<i>S. aureus</i>	29,213		15	14	3.12	0.06
MRSA	ATCC-33591	Met	12	9	3.12	0.06
<i>C. albicans</i>	ATCC-90028	—	14	11	25	0.5
Imidazole	—	—	—	—	12.5 mg/mL	

MIC refers to the concentration necessary to achieve 100% growth inhibition. NA, not active (activity not detected in the range assayed).

2.6 Synergistic testing

The fractional inhibitory concentration (FIC) index was employed to calculate the synergistic potential between the MFC extract and chloramphenicol against resistance to *Staphylococcus epidermidis* and MRSA. Antimicrobial activity was measured using the checkerboard assay [20] with chloramphenicol in combination with the MFC extract. Both the *Staphylococcus epidermidis* and MRSA were plated in the presence of the MFC extract (4× MIC, 2× MIC, 1× MIC, 0.5× MIC, 0.25× MIC, and 0.125× MIC) in combination with chloramphenicol (4× MIC–0.125× MIC). Experiments were carried out in triplicate, similar to the MIC assessment in the susceptibility testing. The FIC index was calculated with the following formulas:

$$\text{FIC antibiotic} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}, \quad (4)$$

$$\text{FIC antibiotic} = \frac{\text{MIC of extract in combination}}{\text{MIC of extract alone}}, \quad (5)$$

$$\text{FIC index} = \text{FIC}_{\text{chloramphenicol}} + \text{FIC}_{\text{plant extract}}. \quad (6)$$

FIC index values <0.5 suggested synergy, those between 0.5 and 0.75 suggested partial synergy, those between 0.76 and 1 suggested an additive effect, and those >2 suggested antagonism.

2.7 In vitro cytotoxicity

Compounds were assayed at concentrations of 1,111–0.006 µg/mL against a human umbilical vein endothelial cell (HUVEC) line (ATCC CRL-1730™) to assess potential toxicity *in vitro*. Cells were grown in Dulbecco's modified Eagle's medium (UFC Biotech, Saudi Arabia) with 10% fetal bovine serum (Gibco, UK) in 5% CO₂ at 37°C. Controls were treated with methanol alone (0.01%). The cells were incubated with the MFC extract in a 96-well plate for 48 h, and then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added (MTT, Invitrogen). Absorbance was read at 540 nm using a multi-well microplate reader (Thermo Scientific Multiskan, China). The number of viable cells were expressed as a percentage of the methanol control.

2.8 Selectivity index

SI values of the extract were calculated by dividing the IC₅₀ (µg/mL) by the MIC (µg/mL). SI values <1 imply that

the extracts are safe for use in mammalian cells and toxic to bacteria.

2.9 In vivo study

This study was carried out using male Swiss albino mice weighing 28 ± 2 g. The mice were maintained in poly-acrylic cages under standard environmental conditions and provided with free access to water and a standard diet. For the acute toxicity test, ten mice were distributed into two groups, each consisting of five mice. Group I (controls) received only phosphate-buffered saline (PBS). Group II received a single oral dose of 2,000 mg/kg body weight (BW) extract. All procedures were performed according to the guidelines of the National Institutes of Health. The protocol was approved by the Ethics Committee of King Saud University, Saudi Arabia. All procedures were performed with the approval of the Animal Ethics Committee of the King Saud University Zoology Department.

2.10 Hemolytic activity (HC)

The HC of the MFC extract was assessed on mouse and human erythrocytes following the modified method of a previous study [21]. A 3% (v/v) suspension of erythrocytes was incubated with various concentrations of the MFC extract in a 96-well plate for 30 min at 37°C. The plates were centrifuged at 3,000 rpm, and the supernatant was aspirated and used to calculate the released hemoglobin at 540 nm. The percentage of hemolysis was expressed compared to the positive control (0.1% Triton X-100); PBS was used as a negative control. Experiments were carried out in triplicate. The hemolysis percentage was calculated using the following equation:

$$\begin{aligned} \text{Hemolysis percentage} \\ = \frac{\text{Absorbance of sample}}{\text{Absorbance of positive control}} \times 100. \end{aligned} \quad (7)$$

2.11 Gas chromatography-mass spectrometry (GC-MS) analysis

The MFC extract was analyzed via GC-MS (Agilent Technologies, USA) using helium as the carrier gas. The flow rate was 1 mL/min, and the program was as follows: Maintaining

at 50°C for 10 min and then heating from 50 to 220°C at a rate of 4°C/min, hold at 220°C for 10 min and then increase to 240°C at a rate of 1°C/min, followed by a final hold at 240°C for 10 min. Identification of compounds was carried out by comparing their mass spectra to the available literature (NIST11).

3 Results

3.1 Total phenol and flavonoid contents

Total phenolic and flavonoid contents were assessed using gallic acid and quercetin standards, respectively. The MFC extract contained a considerable amount of phenols (26.64 ± 0.1 mg GAE/g extract) and flavonoids (8.28 ± 0.1 mg QuE/g extract).

3.2 DPPH radical scavenging activity

The scavenging activity of the MFC extract is presented in Figure 1. The MFC extract showed concentration-dependent scavenging activity with an IC_{50} value of 1.49 mg/mL. Although the extract showed lower IC_{50} value than gallic acid ($IC_{50} = 81.2 \mu\text{g/mL}$), we revealed that the extract does contain compounds that can donate protons to the free radicals.

3.3 *In vitro* susceptibility and synergistic studies

In *in vitro* susceptibility assays, none of the tested extracts (hexane, ethyl acetate, and methanol) showed antimicrobial activity against tested organisms except MFC extract. Figure 2 revealed that the MFC extract had antimicrobial effects against Gram-positive bacteria and *C. albicans* (Table 1) as evaluated by the disc and well diffusion methods, as well as by MIC using the microdilution assay. The inhibition zones measured against the Gram-positive bacteria and *C. albicans* ranged from 9 to 15 mm at 20 μg extract treatment per disc/well. The MIC results confirmed the results obtained using the disc and well diffusion methods. The MFC extract showed antibacterial activity against Gram-positive strains, with values ranging from 1.56 to 3.12 $\mu\text{g/mL}$. However, the MIC of the MFC extract against *C. albicans* was 25 $\mu\text{g/mL}$. Only resistant bacteria were selected, namely, MRSA and *S. epidermidis*. The MFC

Figure 1: DPPH scavenging activity (%) of different concentrations (4–0.125 mg/mL) of the crude MFC extract.

extract resulted in an FIC index value of 1.49 and 1.24 against MRSA and *S. epidermidis*, respectively, which demonstrated no effect in combination with chloramphenicol (Table 2).

3.4 Cytotoxicity on HUVECs

There was a concentration-dependent decrease in cell survival with almost complete inhibition of proliferation at 1,111 $\mu\text{g/mL}$; the IC_{50} values of the MFC extract are presented in Figure 3. To assess the effect of the extract

Figure 2: Petri dishes revealing zones of inhibition produced by MFC extract on methicillin-resistant *Staphylococcus aureus* (MRSA) using disc and well diffusion assay.

Table 2: Results of MFC extract combination against chloramphenicol

	MRSA	Staph
MIC ($\mu\text{g/mL}$)		
Chloramphenicol	6.25	6.25
MFC extract	3.12	1.5
Control (Methanol)	0.0	0.0
Plant extract in combination with chloramphenicol		
FIC index	1.49	1.24
Outcome	No effect	No effect

on HUVECs, the morphologies were observed with inverted light and fluorescent microscopes after staining with Hoechst. As shown in Figure 3, after treatment with $123.4 \mu\text{g/mL}$ of MFC for 48 h, the morphology of the cells was altered, and the density of cells was reduced. Cells were found floating, as well as rounded, fragmented, and shrunken. The treated cells showed a decrease in cell count with an increase in the concentration of the extract.

3.5 Acute toxicity study

The oral toxicity of the MFC extract was assessed as per OECD guideline 423 [22], where the highest test dose of $2,000 \text{ mg/kg BW}$ was administered. No mortality was observed, and toxicity symptoms of the extract-treated mice and control groups were observed first for 6 h and then for 14 days. Treated mice showed no sedation, lethargy, drowsiness, or changes in breathing. Therefore, the extract seems to be safe at a dose of $2,000 \text{ mg/kg}$, and the LD_{50} was higher than $2,000 \text{ mg/kg}$.

3.6 Hemolytic activity

The HC assay is valuable to assess whether the cytotoxicity is attributed to direct cell membrane damage. Figure 4 shows the toxicity results for mouse and human erythrocytes treated with the chloroform extract. A significant ($p \leq 0.05$) variation was detected among the treatments; the positive control (Triton) displayed 100% lysis, whereas PBS displayed no RBC lytic activity. The chloroform extract showed low HC (under 10%) below $3.1 \mu\text{g/mL}$. Then, from 6.2 to $50 \mu\text{g/mL}$, the HC increased considerably, demonstrating the toxicity of the MFC extract toward mouse erythrocytes. In contrast, the chloroform extract showed HC_{50} equal to $220 \mu\text{g/mL}$ demonstrating lower toxicity of the MFC extract toward human erythrocytes.

Figure 3: Cytotoxicity of MFC extract on HUVEC lines. (a) Values are mean values of three experiments. (b) Light and fluorescent photomicrographs (20 $\times$) of (1) control and (2) MFC extract ($123.4 \mu\text{g/mL}$)-treated cells for 24 h. (3) Untreated and (4) treated cells stained with Hoechst 33,342 to show condensed DNA. Scale bars: XX μm .

3.7 GC-MS analysis

The analysis of the MFC extract demonstrated the presence of 22 compounds (Table 3). The major phyto-constituents were 2-cyclopenten-1-one (44.7%), 3-methyl-2-phenyl-pyridine (13.7%), *Z,Z*-4,15-octadecadien-1-ol acetate (11.7%), and 1-(1,5-dimethyl-4-hexenyl)-benzene (5.2%).

4 Discussion

Many antibiotics have lost their efficiency because of the development of bacterial resistance and the expression of resistance genes [23]. Antibiotics can also be responsible for allergic reactions, immune suppression, and hypersensitivity [24]. Therefore, it is necessary to identify

Figure 4: The hemolytic effect of the MFC extract on (a) human (HC₅₀: 220 µg/mL) and (b) mouse blood cells (HC₅₀: 16.11 µg/mL) after 1 h of incubation. Result were considered significant at (*) $p < 0.05$.

promising antimicrobial agents for the treatment of infectious diseases.

To survive, plants have developed defense mechanisms against microbes, herbivores, and other plants competing for nutrients, space, and light [25] by producing secondary metabolites that also function in physiology and structural maintenance [25]. As presented in Table 1, the antimicrobial potential of the MFC extract was investigated against Gram-negative and Gram-positive bacteria. In this investigation, the inhibitory effect of the MFC extract was higher than that determined by Shafiei et al. [26] who found lower antibacterial activity of the ethyl acetate and ethanol extracts of mace with MICs

ranging from 20 to 40 mg/mL against different oral pathogens. In another study, the MIC values of nutmeg essential oil showed inhibitory effect against *S. typhimurium* (MIC 1.5 µg/mL), *S. kentucky*, *B. subtilis*, *E. faecalis*, *E. durans*, *E. aerogenes*, *E. faecium*, *E. coli*, *K. pneumoniae*, *L. innocua*, *S. enteritidis*, *P. aeruginosa*, *S. infantis*, and *C. albicans*, with MIC values ranging from 3.1 to 12.5 µg/mL. However, the extract showed weak activity against *S. epidermidis* (MIC 100 µg/mL) and no antibacterial activity against *S. aureus* [27]. In another report, no inhibition was observed for nutmeg seed extract against MRSA, vancomycin-resistant Enterococci, or *P. aeruginosa* or *K. pneumoniae*; however, it was active against *C. albicans* and *E. coli* at 200 µg/mL [14].

The higher antibacterial activity of the MFC extract appeared to be primarily due to its higher content of bioactive compounds. Phenols have received considerable attention because of their scavenging ability [28], antioxidant potential [29], antiviral, antibacterial, anticancer, anti-inflammatory, and anti-allergic activities [30,31]. Plants rich in phenolic compounds are used in the food industry because they lower the risk of health disorders, prolong shelf life, and preserve the nutritional quality of food [32]. The DPPH assay is commonly employed for the investigation of antioxidant potential, and antioxidant extracts that exhibit scavenging activity may contribute to the inhibition of oxidative stress [33]. In our experimental setup, we found a concentration-dependent reduction in DPPH because of the scavenging activities of the MFC extract (Figure 1). However, the extract was not more effective than the positive control, gallic acid (IC₅₀ = 1.49 and 81.2 µg/mL, respectively). Results of this research suggested that the radical scavenging capacity of the MFC extract may be due to its content of phenolic acids and flavonoids capable of donating hydrogen atoms. Thus, the therapeutic properties of *M. fragrans* mace could be attributed to the phenolic content present. Flavonoids constitute a special class of phenolic compounds and are known to scavenge or delay oxidation by shifting a single electron to OH⁻ and O₂⁻ radicals [34]. Several reports confirmed the antioxidant activity of different parts of *M. fragrans* [35,36].

Researchers have used HUVEC lines to assess the cytotoxicity of test samples against normal cells [37,38]. Similar methods have also been utilized in high-throughput tests for drug discovery [39]. The MFC extract showed concentration-dependent inhibition against a non-cancer HUVEC line (IC₅₀ = 49.18 µg/mL). The United States National Cancer Institute designated that plant crude extracts are safe if the LC₅₀ is ≥20 µg/mL and cytotoxic if the LC₅₀ is ≤20 µg/mL [40]. As the MFC extract had an LC₅₀ of >20 µg/mL, it may be regarded as safe for use.

The SI was assessed for the MFC extract using the MIC for the selected microbes (Table 2). Generally, lower

Table 3: Phytochemical constituents identified in the chloroform mace extract of *Myristica fragrans*

Peak	Chemical name	Formula	Molecular weight	Retention time (min)	Percentage of total	Bioactivity
1	2-Cyclopenten-1-one	C ₅ H ₆ O	82.1	12.415	44.72	Anti-inflammatory [46] and anticancer [47]
2	3-Methyl-2-phenyl-pyridine	C ₁₂ H ₁₁ N	169.2	13.210	13.79	
3	1,1'-Ethylidenebis-benzene	C ₁₄ H ₁₄	182.2	13.719	0.24	
4	5,6,7,8-Tetra 1,4-anthracenedione			19.960		
5	dl-3-Acetamido-perydro-3-methyl-2-oxofuran			20.380	2.86	
6	1-(1,5-Dimethyl-4-hexenyl)-benzene	C ₁₅ H ₂	202.3	21.143	5.29	Antioxidant and anti-inflammatory [48]
7	1,5,9-Cyclododecatriene	C ₁₂ H ₁₈	162.2	21.608	0.32	
8	6-Octadecenoic acid, methyl ester, (Z)	C ₁₉ H ₃₆ O ₂	296.4	21.652	1	Anticancer [51]
9	Z,Z-4,15-Octadecadien-1-ol acetate	C ₂₀ H ₃₆ O	308.5	22.091	11.75	
10	9,12-Octadecadienoic acid	C ₁₈ H ₃₂ O ₂	280.4	22.524	1.24	Anticancer [49]
11	Z-9-Hexadecen-1-ol acetate	C ₁₈ H ₃₄ O ₂	282.4	22.715	3.45	
12	1H-Indene, 1-hexadecyl-2,3-dihydro-	C ₂₅ H ₄₂	342.6	22.912	1.43	
13	Anthracene, tetradecahydro-	C ₁₄ H ₂₄	192.3	23.090	0.49	
14	Cyclohexanamine, N-cyclohexyl	C ₁₂ H ₂₃ N	181.3	23.777	0.26	Antimicrobial [50]
15	Oleic acid	C ₁₈ H ₃₄ O ₂	282.4	23.892	0.67	Anticancer [51]
16	N-(2-Hydroxy-2-phenyl-ethyl)-4-methyl-benzenesulfonamide, trimethylsilyl ether	C ₁₈ H ₂₅ NO ₃ SSi	363.5	24.700	0.3	
17	Pentanoic acid (octahydroquinolizin-1-yl)methyl ester	C ₇ H ₁₄ O ₂	130.1	25.291	0.6	Anticancer [52] and antimicrobial [53]
19	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,4b,5,9,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1a,4aβ,4ba,10aα)]-	C ₂₁ H ₃₂ O ₂	316.4	25.533	0.62	
21	Benzene, 1-methoxy-4-(2-phenylethenyl)-	C ₁₅ H ₁₄ O	210.2	26.284	3.02	
22	Bicyclo[6.1.0]non-1-ene	C ₉ H ₁₄	122.2	26.564	0.52

Figure 5: Some of the active compounds isolated from *Myristica fragrans* extract and reported in the literature.

SI values reflect that extracts are not suitable for use as antimicrobials owing to metabolic toxic effects [41]. When the SI value is >1 , an extract may be considered safer to mammalian cells than to bacteria [41,42]. From the results of this investigation, the MFC extract had an SI value greater than 1 for all selected pathogens, indicating that the antimicrobial extract is not merely composed of by-products of metabolism. The MFC extract can be considered safe if used to treat pathogens when the SI values against those pathogens are greater than 1.

Our results also showed that the MFC extract was safe and that the LD_{50} value was higher than 2,000 mg/kg. Our result is in accordance with the study of a previous report that showed no apparent toxicity of *M. Fragrans* acetone mace extract in rats [43]. In contrast, mice treated orally with mace ethanol extract (0.3 mg/day for 7 days) of *M. Fragrans* showed a significant increase in creatine phosphokinase level, no change in catalase activities, and showed morphological perturbation in mice's liver [44].

Assessing acute toxicity is an initial experiment that provides a basis for classification and labeling [45]. In our acute toxicity assay, the extract up to 2,000 mg/kg caused no toxicity in the treated mice, and the LD_{50} value could not be calculated, as it was larger than the largest dose recommended by OECD guidelines [22] (2,000 mg/kg). No mortality, behavioral, or toxicity symptoms were observed. It was therefore assumed that the LD_{50} value of the extract was higher than 2,000 mg/kg and that the extract can be categorized as unclassified material.

Our result was similar to the report of Ali and his group where the HC of the seed essential oil of *M. fragrans* revealed maximum HC at 10 mg/mL for human erythrocytes [46]. The hemolytic assay is often employed in studies of membrane-active novel xenobiotics and antimicrobial agents. The membrane of the RBC is composed of lipids and proteins. Transmembrane proteins provide the cell structure and mechanical properties and facilitate ion flow, which can have a great impact on the cellular sensitivity to toxic extracts [47]. Plant extracts

interact with different cellular systems and may cause cell damage. The purpose of the hemolytic test was to assess the safety of the extract in blood. The hemolytic assay was performed because the extract may have possessed biological HC and thus may not have been suitable for pharmacological formulation. The lysis effect of the MFC extract may be due to a direct effect on the membranes of the RBCs. The membrane of the human RBC is structurally similar to that of mammals such as mice, rats, rabbits, dogs, monkeys, and horses [48,49]. However, the difference between the HC of human and mouse erythrocytes could be attributed to the variation in the composition between species [50]. Further investigation is required to confirm our results using different animal models.

To investigate the profile of components of the MFC extract, GC-MS analysis was performed. Extract analysis showed some major compounds (2-cyclopenten-1-one [44.7%], 1-(1,5-dimethyl-4-hexenyl)-benzene [5.2%], 6-octadecenoic acid, methyl ester, [Z] [1%]) that were reported previously to have anticancer activity against different cancer cell lines (Table 3). However, some of the minor compounds in the extract have been reported to have antimicrobial activity, such as cyclohexanamine, *N*-cyclohexyl-pentanoic acid, (octahydroquinolizin-1-yl) methyl ester, and oleic acid (Table 3, Figure 5).

5 Conclusion

This study provides information on the antimicrobial and toxicological properties of the MFC extract, which is traditionally prescribed by herbalists. The extract inhibited the growth of all Gram-positive bacteria tested, as well as of *C. albicans*. The SI of the extract was greater than 1. In the assessment of acute toxicity, administration of the MFC extract produced no major toxicological effects after administration of 2,000 mg/kg BW. However, further study is needed to isolate the active compounds, study their biological properties, and assess their safety and efficacy in humans.

Acknowledgements: This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University, through the Research Groups Program Grant no. (RGP-1442 -0037).

Funding information: This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman

University, through the Research Groups Program Grant no. (RGP-1442-0037).

Author contributions: N.A. conceived and designed the experiments; L.A.A.K., R.A.E.H.M., N.A., and F.A.A.M. collected the samples, performed the experiments, and analyzed the data. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: Authors declare no conflict of interest.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: All data generated or analyzed during this study are included in this published article.

References

- [1] Sharifi-Rad J, Sureda A, Tenore GC, Daglia M, Sharifi-Rad M, Valussi M, et al. Biological activities of essential oils: from plant chemoeology to traditional healing systems. *Molecules*. 2017;22:70.
- [2] Cabre M, Camps J, Paternain J, Ferre N, Joven J. Time-course of changes in hepatic lipid peroxidation and glutathione metabolism in rats with carbon tetrachloride-induced cirrhosis. *Clin Exp Pharmacol Physiol*. 2000;27:694–9.
- [3] Marrelli M, Menichini F, Statti GA, Bonesi M, Duez P, Menichini F, et al. Changes in the phenolic and lipophilic composition, in the enzyme inhibition and antiproliferative activity of *Ficus carica* L. cultivar Dottato fruits during maturation. *Food Chem Toxicol*. 2012;50:726–33.
- [4] Park EJ, Choi KS, Kwon TK. β -Lapachone-induced reactive oxygen species (ROS) generation mediates autophagic cell death in glioma U87 MG cells. *Chem-Biol Interact*. 2011;189:37–44.
- [5] Sofi FR, Raju C, Lakshmisha I, Singh RR. Antioxidant and antimicrobial properties of grape and papaya seed extracts and their application on the preservation of Indian mackerel (*Rastrelliger kanagurta*) during ice storage. *J Food Sci Tech*. 2016;53:104–17.
- [6] Bagheri G, Mirzaei M, Mehrabi R, Sharifi-Rad J. Cytotoxic and antioxidant activities of *Alstonia scholaris*, *Alstonia venenata* and *Moringa oleifera* plants from India. *Jundishapur J Nat Pharm Prod*. 2016;11:e31129.
- [7] Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. Antioxidant activity of some Algerian medicinal plant extracts containing phenolic compounds. *Food Chem*. 2006;97:654–60.

- [8] Wannes WA, Mhamdi B, Sriti J, Jemia MB, Ouchikh O, Hamdaoui G, et al. Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food Chem Toxicol.* 2010;48:1362–70.
- [9] Tungmunthum D, Thongboonyou A, Pholboon A, Yangsabai A. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: an overview. *Medicines.* 2018;5(5):93.
- [10] Stein U, Greyer H, Hentschel H. Nutmeg (myristicin) poisoning —report on a fatal case and a series of cases recorded by a poison information centre. *Forensic Sci Int.* 2001;118:87–90.
- [11] Periasamy G, Karim A, Gibrelibanos M, Gebremedhin G. Nutmeg (*Myristica fragrans* Houtt.) oils. In *Essential oils in food preservation, flavor and safety*. Cambridge, MA: Academic Press; 2016. p. 607–16.
- [12] Saxena R, Patil P. Phytochemical studies on *Myristica fragrans* essential oil. *Biol Forum-An Int J.* 2012;62–4.
- [13] Alibabae, M, Safaralizadeh, MH. Fumigant toxicity of Nutmeg seed essential oil (*Myristica fragrans* Houtt.) (MF, Myristicaceae) on cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Bruchidae). *New horizons in insect science: towards sustainable pest management*. New York City: Springer; 2015. p. 127–33.
- [14] Ibrahim MA, Cantrell CL, Jeliakova EA, Astatkie T, Zheljzkov VD. Utilization of nutmeg (*Myristica fragrans* Houtt.) seed hydrodistillation time to produce essential oil fractions with varied compositions and pharmacological effects. *Molecules.* 2020;25:565.
- [15] Burt SA. *Antibacterial activity of essential oils: potential applications in food*. The Netherlands: Utrecht University; 2007.
- [16] Wardono P, Mujahidin D, Lailiyya N. Analysis of myristica fragrans oil as sleep inducing agents for developing aromatic bio-composite material. *Adv Sci Lett.* 2018;24:2395–9.
- [17] Mahfuz I, Khalequzzaman M. Contact and fumigant toxicity of essential oils against *Callosobruchus maculatus*. *Univ J Zool Rajshahi Univ.* 2007;26:63–6.
- [18] Zhang WK, Tao S-S, Li T-T, Li Y-S, Li X-J, Tang H-B, et al. Nutmeg oil alleviates chronic inflammatory pain through inhibition of COX-2 expression and substance P release in vivo. *Food Nutr Res.* 2016;60(1):30849.
- [19] Abutaha N, Al-zharani M, Al-Doaiss AA, Baabbad A, Al-Malki AM, Dekhil H. Anticancer, antioxidant, and acute toxicity studies of a Saudi polyherbal formulation, PHF5. *Open Chem.* 2020;18(1):472–81.
- [20] Kuok C-F, Hoi S-O, Hoi C-F, Chan C-H, Fong I-H, Ngok C-K, et al. Synergistic antibacterial effects of herbal extracts and antibiotics on methicillin-resistant *Staphylococcus aureus*: A computational and experimental study. *Exp Biol Med.* 2017;242:731–43.
- [21] Lum KY, Tay ST, Le CF, Lee VS, Sabri NH, Velayuthan RD, et al. Activity of novel synthetic peptides against *Candida albicans*. *Sci Rep-UK.* 2015;5:9657.
- [22] OECD. Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing Paris; 2008.
- [23] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *Pharm Therapeutic.* 2015;40(4):277.
- [24] Legendre DP, Muzny CA, Marshall GD, Swiatlo E. Antibiotic hypersensitivity reactions and approaches to desensitization. *Clin Infect Dis.* 2014;58:1140–8.
- [25] Wink M, Botschen F, Gosmann C, Schäfer H, Waterman PG. Chemotaxonomy seen from a phylogenetic perspective and evolution of secondary metabolism. *Annu Rev Plant Biol.* 2018;364–433.
- [26] Shafiei Z, Shuhairi NN, Md Fazly Shah Yap N, Harry Sibungkil C-A, Latip J. Antibacterial activity of *Myristica fragrans* against oral pathogens. *Evid-Based Compl Alternat Med.* 2012;2012.
- [27] Özkan OE, Olgun Ç, Güney B, Mahmut G, Güney K, Saim A. Chemical composition and antimicrobial activity of *Myristica fragrans* & *Elettaria cardamomum* essential oil. *Kastamonu Univ J Forestry Faculty.* 2018;18:225–9.
- [28] Hatano T, Edamatsu R, Hiramatsu M, Mori A, Fujita Y, Yasuhara T, et al. Effects of the interaction of tannins with co-existing substances. VI.: effects of tannins and related polyphenols on superoxide anion radical, and on 1, 1-Diphenyl-2-picrylhydrazyl radical. *Chem Pharm Bull.* 1989;37:2016–21.
- [29] Pan Y, Wang K, Huang S, Wang H, Mu X, He C, et al. Antioxidant activity of microwave-assisted extract of longan (*Dimocarpus longan* Lour.) peel. *Food Chem.* 2008;106:1264–70.
- [30] Nunes XP, Silva FS, Almeida JRGdS, Barbosa JM, de Lima JT, de Araújo LA, et al. Biological oxidations and antioxidant activity of natural products. New York: INTECH Open Access Publisher; 2012.
- [31] Montoro P, Braca A, Pizzi C, De Tommasi N. Structure–antioxidant activity relationships of flavonoids isolated from different plant species. *Food Chem.* 2005;92:349–55.
- [32] Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects—a review. *J Funct Food.* 2015;18:820–97.
- [33] Mahdi-Pour B, Jothy SL, Latha LY, Chen Y, Sasidharan S. Antioxidant activity of methanol extracts of different parts of *Lantana camara*. *Asian Pac J Trop Biomed.* 2012;2:960–5.
- [34] Panche A, Diwan A, Chandra S. Flavonoids: an overview. *J Nutritional Sci.* 2016;5:e47.
- [35] Ullah MF. Antioxidative and xanthine oxidase inhibitory activities and phytochemical screening of the hydro-alcoholic extract of mace, aril of *Myristica fragrans*: Implication as an adjuvant therapy in gout. *Int J Food Prop.* 2017;20:694–703.
- [36] Assa JR, Widjanarko SB, Kusnadi J, Berhimpon S. Antioxidant potential of flesh, seed and mace of nutmeg (*Myristica fragrans* Houtt.). *Int J Chem Tech Res.* 2014;6(4):2460–8.
- [37] Cao Y, Gong Y, Liu L, Zhou Y, Fang X, Zhang C, et al. The use of human umbilical vein endothelial cells (HUVECs) as an *in vitro* model to assess the toxicity of nanoparticles to endothelium: a review. *J Appl Toxicol.* 2017;37:1359–69.
- [38] Ahmed HOA, Syed Abdul Rahman SN, Awang K, Abdul Wahab N, Looi CY, Thomas NF, et al. Cytotoxic constituents from the rhizomes of *Curcuma zedoaria*. *Sci World J.* 2014;2014:321943.
- [39] Vazão H, Rosa S, Barata T, Costa R, Pitrez PR, Honório I, et al. High-throughput identification of small molecules that affect human embryonic vascular development. *Proc Natl Acad Sci.* 2017;114:E3022–31.

- [40] Abdel-Hameed E-SS, Bazaid SA, Shohayeb MM, El-Sayed MM, El-Wakil EA. Phytochemical studies and evaluation of anti-oxidant, anticancer and antimicrobial properties of *Conocarpus erectus* L. growing in Taif, Saudi Arabia. European. J Med Plants. 2012;93–112.
- [41] Elisha IL, Jambalang AR, Botha FS, Buys EM, McGaw LJ, Eloff JN. Potency and selectivity indices of acetone leaf extracts of nine selected South African trees against six opportunistic Enterobacteriaceae isolates from commercial chicken eggs. BMC Compl Altern M. 2017;17(1):90.
- [42] Cho-Ngwa F, Abongwa M, Ngemenya MN, Nyongbela KD. Selective activity of extracts of *Margaritaria discoidea* and *Homalium africanum* on *Onchocerca ochengi*. BMC Compl Altern M. 2010;10:62.
- [43] Gayathri R, Venkataraman A, Vishnupriya V, Jainu M. Acute toxicity studies of acetone mace extract of *Myristica fragrans* houtt on rats. Drug Invent Today. 2018;10(8):1508–10.
- [44] El Malti J, Bourhim N, Amarouch H. Toxicity and antibacterial effect of Mace of *Myristica fragrans* used in Moroccan gastronomy: biochemical and histological impact. J Food Saf. 2008;28:422–41.
- [45] Ukwuani A, Abubakar M, Hassan S, Agaie B. Toxicological studies of hydromethanolic leaves extract of *Grewia crenata*. Int J Pharm Sci Drug Res. 2012;4:245–9.
- [46] Ali A, Yasir M, Jilani MI, Shoaib S, Latif M, Ahmad A, et al. Chemical composition and *in vitro* evaluation of cytotoxicity, antioxidant and antimicrobial activities of essential oil extracted from *Myristica fragrans* Houtt. Pol J Env Stud. 2021;30(2):1585–90.
- [47] Pagano M, Faggio C. The use of erythrocyte fragility to assess xenobiotic cytotoxicity. Cell Biochem Funct. 2015;33:351–5.
- [48] Virtanen JA, Cheng KH, Somerharju P. Phospholipid composition of the mammalian red cell membrane can be rationalized by a superlattice model. Proc Natl Acad Sci. 1998;95:4964–9.
- [49] Zwaal R, Van, Deenen L. Protein patterns of red cell membranes from different mammalian species. Biochim Biophys Acta. 1968;163(1):44–9.
- [50] Namdee K, Carrasco-Teja M, Fish M, Charoenphol P, Eniola-Adefeso O. Effect of variation in hemorheology between human and animal blood on the binding efficacy of vascular-targeted carriers. Sci Rep-UK. 2015;5:11631.