Article

Antibacterial Efficacy of Some Medicinal Plants on Multidrug Resistance Bacteria and Their Toxicity on Eukaryotic Cells

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Abstract: The aim of this study was to evaluate the *in vitro* antimicrobial activity of medicinal methanolic plant extracts against multidrug-resistant bacteria to determine the cytotoxicity of these extracts on eukaryotic cells, and to confirm their efficacy against Methicillin-Resistant *Staphylococcus aureus* (MRSA) in experimental animals. The effects of the methanol extract of sixty folk plants were investigated on; MRSA, Extended Spectrum Beta-Lactamase *E. coli* and MDR *Pseudomonas aeruginosa* by disc diffusion and MIC assay. Cytotoxicity was determined using MTT and hemolysis of human erythrocytes. Three plant extracts with the highest antimicrobial activities were tested using a challenge experiment on mice. Systemic infection was performed by intraperitoneal inoculation of (5×10^6 CFU/mL) of MRSA isolate. Then mice received 300 mg/kg body weight of the plant extracts daily for seven days. The efficacy of plant extracts was evaluated by general health, mortality rate, gross lesion, and histopathology study of inoculated mice. Only ten plants showed activities against different MDR bacteria with inhibitory zones ranging from (8 to 22 mm) in diameter. Of the ten medicinal plant extracts, *Camellia sinensis* and *Aloysia citrodora* showed the highest activities against MRSA and MSSA isolates, with MIC value ranging from 0.5 to 1.5 mg/ml, followed by *Hibiscus sabdariffa*, *Thymus vulgaris*, and *Glycyrrhiza glabra*. Furthermore, the extract of the effective plants showed low toxicity against Vero and fibroblasts cell lines, along with inhibitory activities to erythrocytes membrane disruption. The *in vivo* study demonstrated that *Camellia sinensis* showed significant activity against MRSA infections in mice. The results validate that these plants are effective and safe antibacterial agents against multidrug-resistance bacteria, and have the potential to be utilized as an alternative to antibiotics for the treatment of bacterial infections.

Keywords: medicinal plants; antibacterial efficacy; MRSA; ESBLEC; MDR *P. aeruginosa*

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1. Introduction

The alarming increase in infectious diseases has become a serious global health problem, with a significant increase in morbidity and mortality due to extensive use and misuse of antibiotics [1,2]. Recently, more bacteria have become resistant to available antibiotics [3,4]. The Infectious Diseases Society of America recognizes antimicrobial resistance as “one of the most important threats to human health worldwide” [5]. The most famous resistance patterns are; Methicillin-Resistant *Staphylococcus aureus* (MRSA), Extended Spectrum Beta-Lactamase *Escherichia coli* (ESBLEC) and *Pseudomonas aeruginosa*. For these reasons, it is necessary to search for the possibility of finding an alternative novel and effective antibacterial agent against multidrug-resistant bacteria.

The spread of antibiotic resistance is continuously occurring, and it is a nonstop process, but it can be slowed [6]. The best solution is to invest in the development of new antimicrobial compounds from plant sources [7]. Chemical compounds isolated from

plants that are characterized as antimicrobial agents are significant and differ in structures and activity against bacterial pathogens [8,9]. Plant-derived products are an attractive source of antimicrobial agents considering they are natural and affordable, nontoxic to host and active at low concentrations [10–12]. Numerous recent studies have reported plant components that have antimicrobial properties and are effective against a wide spectrum of Gram positive and Gram negative bacteria, fungi, and viruses [13]. Methanol plant extracts were used to evaluate their antimicrobial effects. A comparative study performed by [14], showed that methanol extraction yield high content of phenolic, flavonoid, alkaloid and terpenoids. They also revealed high antioxidant capacity and promising anti-inflammatory activity when compared with other extracts such as ethanol, chloroform, dichloromethane, acetone, gallic acid and ascorbic acid. The Arab world is blessed with diverse climate conditions which enable the growth of a wide variety of plants [15]. Many folk herbal plants used in traditional remedies are still unexplored, and many of them need studies to ensure that they can be used as drugs for different infectious diseases caused by multidrug-resistant bacteria. According to the world health organization, medicinal plants would be the best source for getting a diversity of drugs [16].

Traditional plants may contain compounds that have adverse effects on humans [17]. Contrarily, plants associated microorganisms referred to as phytomicrobiome have the potential to secrete metabolites important industrially, in medicine and for environmental preservation [18]. Toxicity profiling is crucial to evaluate the use of traditional plants or their bioactive compound in human consumption [19]. MTT assay enzyme-based method is the best-known method, which refers to the normal function of mitochondria in a living cell through cellular reduction reaction involves the pyridine nucleotide cofactors NADH/NADPH [20]. The erythrocyte hemolysis assay has been widely used to evaluate membrane stabilizing activities as a general indication of membrane toxicity [21].

It is very important to associate *in vitro* antimicrobial and cytotoxicity assay with *in vivo* toxicity using animal models. The systemic toxicity test was evaluated toxicity in tested animals following a given oral, dermal, or inhalation exposure, according to routes of human exposure. Challenge test focused on *in vivo* validation of the antimicrobial potential of plants is in need. Therefore, the aims of this study were to evaluate the *in vitro* antimicrobial characteristics of sixty folks medicinal methanolic plant extracts used in Jordan against multidrug resistance bacteria; MRSA, MSSA, ESBL *E. coli*, and MDR *P. aeruginosa*, to determine the cytotoxicity of these plant extracts on eukaryotic cells, and to confirm the efficacy of these plants against MRSA in mice.

2. Materials and Methods

2.1. Bacterial Strains

Ten isolates of ESBLs *E. coli*, 10 MRSA, 10 MSSA, and 5 MDR *P. aeruginosa* were used in this study. All of these bacterial strains were isolated from clinical and animal specimens and characterized by multi-drug resistant profiles. The isolates were obtained from the stock culture of Microbiology Research laboratory, Faculty of Veterinary Medicine, Jordan University of Science and Technology. Reference strains of *E. coli* (NTCC O157), *S. aureus* (ATCC 25923), MRSA (ATCC 93300), and *P. aeruginosa* (ATCC 9027) were also used.

2.2. Plant Collection and Preparation

Sixty plants were collected from local market at Amman and from the Faculty of Pharmacy (JUST). One hundred grams of each air-dried plant sample were extracted using 500 mL of absolute methanol (Waltham, MA, USA) with continuous shaking using a rotary shaker machine (Micra Technologies, Haryana, India) for 72 h at room temperature. The whole mixture was filtered and evaporated at 40 °C under reduced pressure using rotary evaporator (Buchi R-215, Schwabach, Switzerland); the total volume was reduced to 10 mL and dried using water bath (Mettler, Schwabach, Germany) and vacuum desiccator (Sartorius AG, Gottingen, Germany). Out of the sixty screened plants ten were selected after a rapid examination to determine their anti-bacterial effect (Table 1).

Table 1. Scientific and common names of the medicinal plants that have potential antibacterial activity used in this study.

Plant Number	Scientific Name	Family	Common Name	Arabic Name	Plant Form
1	<i>Camellia sinensis</i>	Theaceae	Green tea	Shay Akhdur	Herb
2	<i>Hibiscus sabdariffa</i>	Malvaceae	Roselle	Karkadeh	Flower
3	<i>Aloysia citrodora</i>	Verbenaceae	lemon verbena	Lemon Aloysia	Herb
4	<i>Glycyrrhiza glabra</i>	Fabaceae	Liquorice	Erqsus	Root
5	<i>Thymus vulgaris</i>	Lamiaceae	Thyme	Za'atar	Herb
6	<i>Urtica pilulifera</i>	Urticaceae	Nettles	Qarass	Herb
7	<i>Phlomis brachyodon</i>	Lamiaceae	Phlomis	Jerusalem Sage	Shrub
8	<i>Plantago lanceolata</i>	Plantaginaceae	Plantain	Lamb's Tongue	Shrub
9	<i>Anchusa azurea</i>	Boraginaceae	Bugloss	Blue Bull Tongue	Shrub
10	<i>Pallenis spinosa</i>	Asteraceae	Spiny golden star	Al najmeiah	Flower

2.3. In Vitro Antibacterial Activities Assay

2.3.1. Disc Diffusion Assay

One hundred mg of the dried plant extracts were dissolved in 10% dimethylsulfoxide (DMSO) (Sigma, Hamburg, Germany) to a final concentration of 100 mg/mL and sterilized by filtration through 0.22 µm cellulose membrane filter (Indiamart, Vadodara, India). The modified disc diffusion assay was performed using 6 mm filter paper disc (Oxoid, UK) that was impregnated with 50 µL plant extract (10 mg/disc) at a concentration of 200 mg/mL and kept at room temperature for one hour [13]. The bacterial broth culture was adjusted to (0.5) McFarland turbidity, and then streaked evenly onto Müller Hinton agar. The plant extract discs and antibiotic discs were applied on the surface of Mueller Hinton Agar MHA (Oxoid, UK) plates and incubated under aerobic conditions at 37 °C for 24 h. The tests were carried out in triplicate. Zone of inhibition ≥ 11 mm indicates susceptibility of the tested bacteria and consider as high antibacterial activity [22]. The inhibition of bacterial growth was evaluated by measuring the diameter (mm) of the clear zone around each disc [23]. Vancomycin (10 µg/disc) (Oxoid, UK) and gentamycin (10 µg/disc) (Oxoid, UK) were used as a positive control, while DMSO was used as a negative control [13].

2.3.2. Minimum Inhibitory Concentration Assay (MIC) and Minimum Bactericidal Concentration Assay (MBC)

The plants that showed antibacterial activity by disc diffusion assay were further tested by microdilution assay. The initial plant extracts concentration (100 mg/mL) was diluted by two-fold dilution manner in MHB in 96 well plates (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.19 mg/mL). One hundred µL bacterial inoculum size of 1×10^6 CFU/mL was added to each well except the negative control, so the final inoculum size become 5×10^5 CFU/mL. The positive control was vancomycin for Gram positive bacteria and gentamycin for Gram negative bacteria. Wells containing the bacterial inoculum were used as positive control and wells containing plant extract as a negative control. Each plant extract was run in triplicate. The MIC considered as the lowest concentration of plant extract dilution that inhibited any visible growth of the tested bacteria. Bacterial growth in each sample was confirmed by adding 40 µL indicator solution (0.2 mg/mL) p-iodonitrotetrazolium chloride (INT) (ZellBio, Lonsee, Deutschland, Germany) to every well and incubated at 37 °C for 30 min. The microbial activity was indicated by the formation of a pink-red color while inhibition of the growth was observed by the continuation of purple color, and the color changes were assessed visually. The lowest concentration that prevented the purple color change was considered as the MIC [19,24]. The MBC was determined by adding 50 µL aliquots of the extract preparations that did not show any growth after incubation during MIC assays, to 150 µL broth in the 96-well plate (Sigma, Hamburg, Germany). These preparations were incubated at 37 °C for 48 h. The MBC was regarded as the lowest concentration of extract, which did not produce a color change after addition of INT as mentioned above. Bacterial growth in each sample was confirmed by adding 40 µL indicator solution (0.2 mg/mL) p-iodonitrotetrazolium chloride (INT)

to every well and incubated at 37 °C for 30 min. The microbial activity was indicated by the formation of a pink-red color while inhibition of the growth was observed by the continuation of purple color, and the color changes were assessed visually. The lowest concentration that prevented the purple color change was considered as the MIC and the MBC was considered as the lowest concentration of plant extract that did not produce a color change after addition of INT [19,24].

2.4. Cytotoxicity Assay

2.4.1. The Erythrocyte Hemolysis

Erythrocytes were obtained from human blood by removing the plasma and buffy coat by centrifugation at 5000 rpm for five minutes, then was suspended in phosphate-buffered saline (10 Mm phosphate, 150 Mm sodium chloride (Sigma, Hamburg, Germany)) making a final concentration of 2% erythrocytes. The concentration of plant extracts was prepared at (100 mg/mL) and mixed with erythrocytes keeping the final volume up to 1 mL. The cells were incubated at 37 °C for 1 h, then centrifuged at 1500 rpm for 10 min and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm using the spectrophotometer (Bio Rad Laboratories, Kidlington, UK). Two controls were prepared without plant extracts; positive control, the cells were suspended in 0.1% Triton X-100 (complete hemolytic), and negative control, cells were suspended in normal saline [21]. The mixture was centrifuged at 1500 rpm/a minute, and the free hemoglobin in the supernatant was measured at 540 nm. The hemolysis percentage was calculated by the following formula [24]:

$$\% \text{ hemolysis} = \frac{A_t - A_n}{A_c - A_n} \times 100\%$$

A_t : absorbance of test sample

A_n : absorbance of the negative control (normal saline)

A_c : absorbance of the positive control (Triton X-100)

2.4.2. MTT (Cell Proliferation) Assay

Preparation of Cell Lines for MTT Assay and Culture Condition

Vero cell line (ATCC CCL-81) and human fibroblast (CO135C) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Hamburg, Germany) with high glucose contents, supplemented with 10% Fetal bovine serum (FBS) (Sigma, Hamburg, Germany), glutamine (2 mM), penicillin (100 units/mL) (Euroclone, Figino, Pero, Italy) and streptomycin (100 µg/mL) (Euroclone, Figino, Pero, Italy) and were cultured at 37 °C in a humidified 5% CO₂ and incubated for a week. The cells were diluted to a density of 1.5×10^4 cells/well for Vero cell and 1×10^4 cells/well for human fibroblast. The cells were treated with *Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Glycyrrhiza glabra*, and *Thymus vulgaris* according to manufacturer's instructions CellTiter-Glo. Eight concentrations (500, 400, 300, 200, 100, 50, 25, and 12.5 µg/mL) were prepared from each selected plant extract. Exponentially growing cells were seeded (in 100 µL of growth medium) in 96 well microtiter plates (Sigma, Hamburg, Germany) and incubated for 24-h incubation to form a partial monolayer. After that, the media was removed, added 100 µL of the DMEM medium containing the plant extract (initially dissolved in DMSO), and reincubated for 24 h. Then 15 µL of the MTT solution (Promega, Madison, WI, USA) was added to each well and incubated for 1 to 4 h. Blue crystals were formed after 4 h of cell contact with the MTT solution. Solubilization/stop solution (100 µL) was added and incubated for another 1 h. The absorbance can be recorded in 1 h or up to several days later. Untreated cells were used as negative control; doxorubicin (Indiamart, Vadodara, India) was used as a positive control and prepared in the same manner. Each treatment was repeated in triplicate. The optical density (OD) was measured at 590 nm using a microplate reader (BioTek, Winooski,

VT, Uruguay) [19]. The percentage of the cell viability was calculated according to the following equation:

$$\% \text{ of the cell viability} = \frac{1 - \text{OD of treated cells}}{\text{OD of untreated cells}} \times 100\%$$

2.5. In Vivo Assay

2.5.1. Animals and Conditions

A total of 140, seven-to-eight-week-old healthy mice with a bodyweight of 25–30 g were used in this study and maintained in the animal house at JUST. The mice were distributed randomly in isolate metallic cages. Mice were acclimated for a week under controlled conditions at 22 °C with relative humidity between 30% and 70% and 12 h variation of light and dark cycle. Treatment and the maintenance of the animals were in accordance with the Jordan University of Science and Technology Animal Care and Use Committee (project identification code, JUST-ACUC, Project # 123/2018) which follows the international animal care and use guidelines (ILAR, 1996).

2.5.2. Acute Toxicity Test

The fixed-dose procedure for acute toxicity was carried out according to protocol OECD [25] for the evaluation of safety and efficacy of tested plants. Forty mice weighing around 25 ± 2 g were randomly separated into four groups with 10 mice in each group (Group I: *Camellia sinensis*; group II: *Aloysia citrodora*; group III: *Hibiscus sabdariffa*; and group IV: water negative control). The mice were administered with a plant dose of 300 mg/kg of body weight by gavage-feeding once daily for two weeks. The negative control group received water only and animals were observed daily for 14 days. The clinical signs were observed including changes in skin and fur; changes in the eyes and mucous membranes, abnormal changes in behavior and activity, convulsions, salivation, diarrhea, sleep, and death.

2.5.3. Mouse Systemic Infection Assay (Challenge Test)

Bacterial Strains and Culture Conditions

The MRSA strain was selected from the other four tested strains after mortality assay was performed, which caused the highest morbidity and mortality at bacterial concentration of 5.0×10^6 CFU/mL [26]. Furthermore, the challenge strain was resistant to methicillin (Oxoid, UK), cefixime (Oxoid, UK), oxacillin (Oxoid, UK), gentamycin (Oxoid, UK), and penicillin (Oxoid, UK). The pure culture was kept at -80 °C, refreshed on MHB (Oxoid, UK) and subcultured on MHA media before use.

Inoculum Preparation

A single colony was inoculated in a 10 mL TSB and incubated in a shaker incubator (220 rpm) at 37 °C overnight then diluted to reach the OD 0.132 at 600 nm. The bacterial suspension was pelleted by centrifugation at $3500 \times g$ at 4 °C for 10 min. The cell pellet was washed in 1Xpbs (phosphate buffer saline), centrifuged again under the same conditions, and finally resuspended in 2 mL saline to acquire the concentration of 5×10^6 CFU/mL.

Challenge Procedure

Mice were divided into 9 groups (negative not infected or treated; positive infected not treated, 4 groups infected and treated with selected plants or vancomycin, and 3 groups infected and treated with combination of selected plant extracts), each group was 10 mice except the positive control was 20 mice and each mouse was inoculated intraperitoneally (ip) with 0.2 mL of bacterial suspension. The negative control group was inoculated with 0.2 mL TSB. Then mice received plant extracts or vancomycin at the dose of 300 mg/kg body weight by gavage-feeding once daily and continued for 7 days after infection. Mice were monitored daily and observed with a survival status. Mice that remained alive were sacrificed at the end of the eighth day of the challenge experiment. Visceral organs (heart,

lung, liver, kidney, and spleen) were examined for pathological lesions. Visceral organs with lesions were subjected to histopathological examination. The severity of the damage on lung and liver were scored as follows: lung: normal (0), mild lesion: edema and fibrin (1), moderate lesion: small blood patches and moderate suppurative bronchitis (2), and severe lesion: large blood patches with severe suppurative bronchitis (3). Liver: normal (0) and abnormal lesion: suppurative hepatitis (1) [26].

2.6. Statistical Analysis

In vitro, experimental data were expressed as the mean $\pm$ SD. The results of the histopathological scoring system were analyzed using Kruskal–Wallis test and Mann–Whitney test. Only *p*-values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. In Vitro Antibacterial Effect of Plant Extracts

3.1.1. Disc-Diffusion Assay

Results revealed that 10 methanol plant extracts displayed different potent activities against an array of Gram positive and Gram negative bacteria (Table 2), of which, *Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Thymus vulgaris*, *Phlomis brachyodon*, *Anchusa azurea* and *Glycyrrhiza glabra* showed the highest antibacterial activity against MRSA isolates with an inhibition zone of 19.9, 19.3, 14.7, 14.5, 14.5, 13.9, and 13.1 mm, respectively. *Aloysia citrodora*, *Camellia sinensis*, *Glycyrrhiza glabra*, *Hibiscus sabdariffa*, *Thymus vulgaris*, *Phlomis brachyodon*, and *Anchusa azurea* showed antibacterial activity against MSSA isolates with an inhibition zone of 20, 19.5, 15.4, 14.6, 14.3, 14.2, and 13.1 mm, respectively. *E. coli* were affected by *Phlomis brachyodon* (16.4 mm), *Pallenis spinosa* (13.9 mm), *Urtica pilulifera* and *Anchusa azurea* (12.4 mm) and *Hibiscus sabdariffa* (11.5 mm) while *P. aeruginosa* was not affected by any of the extracts except *Hibiscus sabdariffa* zone of inhibition (12 mm). Results of the antimicrobial activity of the tested plant extracts showed that *P. aeruginosa* was the most resistant isolate, followed by *E. coli*. *Camellia sinensis* and *Aloysia citrodora* showed the best antibacterial effects against *S. aureus*.

Table 2. Antibacterial activities of plant extracts against the tested bacteria.

Plants (10 mg/disc)	Antibacterial Activity (Zone of Inhibition, mm)							
	MRSA ATCC 43300	MRSA Isolates	MSSA ATCC 25923	MSSA Isolates	P.A. ATCC 9027	P.A. Isolates	<i>E. coli</i> NTCC O157	<i>E. coli</i> Isolates
<i>Camellia sinensis</i>	22	19.9	23	19.5	9	8.5	0	0
<i>Aloysia citrodora</i>	21	19.3	18	20	9	9.3	10	8.9
<i>Hibiscus sabdariffa</i>	15	14.7	15	14.6	12	12	11	11.5
<i>Glycyrrhiza glabra</i>	13	13.1	15	15.4	0	0	0	0
<i>Phlomis brachyodon</i>	13	14.5	14	14.2	10	9.6	16	15.4
<i>Thymus vulgaris</i>	15	14.5	15	14.3	0	0	0	0
<i>Urtica pilulifera</i>	8	8.7	8	8.2	9	9.7	13	12.4
<i>Anchusa azurea</i>	13.4	13.9	14	13.1	0	0	12	12.4
<i>Plantago lanceolata</i>	12	12.3	12	12.5	0	0	0	0
<i>Pallenis spinosa</i>	0	0	0	0	8	8.4	13	13.9
Vancomycin (30)	20 (S)	18 (S)	21 (S)	20 (S)	NT	NT	NT	NT
Gentamycin (10)	26 (S)	25 (S)	28 (S)	24 (S)	20 (S)	18 (S)	25 (S)	22 (S)

Values of zones of inhibition are the mean of 10 tested MRSA, MSSA and *E. coli* isolates and five *P. aeruginosa* isolates. NT: Not tested; P.A: *P. aeruginosa*; R: resistant; S: susceptible.

3.1.2. Minimum Inhibitory Concentration Assay (MIC) and Minimum Bactericidal Concentration Assay (MBC)

Five of the most effective plant extracts; *Camellia sinensis*; *Aloysia citrodora*; *Hibiscus sabdariffa*; *Glycyrrhiza glabra*, and *Thymus vulgaris* were tested by MIC and MBC against *S. aureus*, *E. coli*, and *P. aeruginosa*. Plant extracts that showed a zone of inhibition ≥ 14 mm in disc diffusion test were tested by microdilution assay. Gentamycin and vancomycin were used as positive controls. Ten isolates for each of MRSA, MSSA and *E. coli* and five isolates of *P. aeruginosa* were tested. Results are presented in Table 3. Among the three bacterial species, the *Camellia sinensis* extract showed the highest antibacterial activity

against *S. aureus* (MRSA and MSSA) with a MIC value (0.5 mg/mL). Extracts of *Camellia sinensis* and *Aloysia citrodora* displayed wide spectra of antibacterial activity in comparison to other plants. *E. coli* and *P. aeruginosa* showed the highest resistance profile in comparison to *S. aureus*.

Table 3. MIC and MBC results of plant extracts against selected bacterial species.

Plant Extracts	Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (mg/mL)							
	MRSA		MSSA		<i>P. aeruginosa</i>		<i>E. coli</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Aloysia citrodora</i>	1.3 ± 0.7	2.5 ± 1.4	1.5 ± 1.0	2.8 ± 1.7	5.3 ± 1.9	9.4 ± 4.0	7.5 ± 3.5	15.0 ± 7.0
<i>Camellia sinensis</i>	0.5 ± 0.2	0.8 ± 0.4	0.5 ± 0.5	1.1 ± 0.9	3.1 ± 1.7	5.6 ± 3.6	NT	NT
<i>Hibiscus sabdariffa</i>	4.0 ± 2.0	9.2 ± 4.1	5.2 ± 1.5	9.9 ± 3.1	3.1 ± 0	6.3 ± 0	9.4 ± 3.1	19.9 ± 6.9
<i>Glycyrrhiza glabra</i>	0.9 ± 0.5	1.8 ± 1.0	0.7 ± 0.2	1.4 ± 0.3	NT ¹	NT	NT	NT
<i>Thymus vulgaris</i>	5.5 ± 4.9	10.9 ± 5.2	4.0 ± 1.6	8.1 ± 6.25	NT	NT	NT	NT
Vancomycin µg/mL	1.56 ± 0.50	2.2 ± 0.63	1.9 ± 0.93	2.6 ± 1.11	NT	NT	NT	NT
Gentamycin µg/mL	NT	NT	NT	NT	5.6 ± 1.96	6.4 ± 1.96	4.6 ± 0.92	4.2 ± 0.86

Values are mean ± SD; ¹ NT: not tested because zone of inhibition was <14 mm in disc diffusion.

3.2. Cytotoxicity Assay

3.2.1. The Erythrocyte Hemolysis

None of the selected plant extracts (*Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Thymus vulgaris*, and *Glycyrrhiza glabra*) possess any hemolytic activity against human erythrocytes at 100 mg/mL (Figure 1). The highest hemolytic activity was found by methanolic extract of the *Hibiscus sabdariffa*, and the minimum activity was by *Glycyrrhiza glabra*.

Figure 1. Toxicity effects of selected plant extracts against human erythrocytes.

3.2.2. MTT Assay

To explore the possible biological activity of the plant extracts, it was further evaluated by *in vitro* antiproliferative characteristics on two normal cell lines; human fibroblast cell lines and monkey Vero cell lines. This study showed no toxicity at the tested concentrations on both Vero and fibroblast cell lines (Figure 2A,B).

Figure 2. (A) Toxicity of selected plant extracts against Vero cell lines; (B) Toxicity of selected plant extracts against human fibroblast cell lines.

3.3. In Vivo Assay

3.3.1. Acute Cytotoxicity

Mice were given 300 mg/kg body weight dose of the plant extracts (*Camellia sinensis*, *Aloysia citrodora*, and *Hibiscus sabdariffa*). Administered plant extracts did not cause any toxicity symptoms, adverse effects, or mortality up to two-week post-induction in any of the plant-treated groups. All mice in the acute toxicity study showed similar visual characteristics to that of the negative control group. There were no changes in the skin, fur, eyes, mucous membranes, respiration, locomotion, convulsion, salivation, or sleep in any of the mice during the days of the study. After two weeks of the acute toxicity experiment, there were no significant differences observed in gross lesions among different groups. The vital organs (heart, lung, liver, kidneys, and spleen) from treated mice revealed no significant difference when compared with the untreated negative control group.

3.3.2. Systemic Infection and Treatment Experiment (Challenge Test)

In the negative control group, no significant gross pathological changes were seen in the examined mice. All organs were normal. While in the positive control group, there were remarkable gross changes within different organs. These changes were exhibited as kidney congestion with multifocal to coalescing abscesses, diffuse blood patches in lungs with severe bronchopneumonia, and congested hearts and livers. Upon histopathological study, the positive control was affected by chronic suppurative bronchitis; accompanied by severing lymphocytic perivascular infiltrate with multifocal suppurative hepatitis.

The effect of plant extracts in infected mice and mortality rate are presented in Table 4. Two of the ten mice treated with *Camellia sinensis* had mild suppurative bronchopneumonia. Two infected mice in *Aloysia citrodora* treated group had visible multifocal blood patches in the lung, and histopathological changes ranged from moderate to severe suppurative bronchitis with mild hepatitis. Mice in *Hibiscus sabdariffa* treatment group showed moderate to severe suppurative bronchitis and moderate hepatitis, the entire liver also exhibited accentuated lobular pattern. The combination of the tested plant extracts demonstrated that the *Camellia sinensis* had a synergistic effect and caused a reduction of infection when used with *Hibiscus sabdariffa* or *Aloysia citrodora*.

Table 4. Effects of plant extract treatment on clinical outcome of MRSA infected mice at 8 days post-infection following treatment with tested plants and vancomycin; mortality, gross lesions of lung and liver, and percentage of histopathological score for lung and liver.

Groups	Mortality %	Gross Lesion of 10 Mice		Lungs Lesion Scores %				Liver Lesion Scores %	
		Total No. of Abnormal Lungs	Total No. of Abnormal Liver	Normal	Mild	Moderate	Severe	Normal	Abnormal
G1: Negative Control	0	00	00	100	0	0	0	100	0
G2: Positive Control	15	20	20	0	0	0	100	15	85
G3: <i>Camellia sinensis</i>	0	2	00	80	10	10	0 *	100	0 *
G4: <i>Aloysia citrodora</i>	0	5	1	50	30	20	0 *	90	10 *
G5: <i>Hibiscus sabdariffa</i>	0	6	3	40	40	20	0 *	70	30 *
G6: <i>Camellia sinensis</i> and <i>Aloysia citrodora</i>	0	4	0	60	30	10	0 *	100	0 *
G7: <i>Camellia sinensis</i> and <i>Hibiscus sabdariffa</i>	0	6	2	40	40	20	0 *	80	20 *
G8: <i>Hibiscus sabdariffa</i> and <i>Aloysia citrodora</i>	0	6	2	40	30	30	0 *	80	20 *
G9: Vancomycin control	0	7	2	30	30	40	0 *	80	20 *

Number of mice in each group was 10 mice except the positive control was 20 mice, * Significant: $p \leq 0.05$.

All treated plant groups showed a significant reduction of lesions compared with the infected untreated group ($p < 0.05$). *Camellia sinensis* treated group was highly similar to that of the negative control. Moreover, the liver lesion was a common feature of mice subjected to *Hibiscus sabdariffa*.

4. Discussion

Antibiotic resistance is one of the major threats to global health and bacterial resistance is steadily increasing [27]. Misuse of antibiotics has accelerated the antibiotic resistance process which has become harder to treat [6]. The present study has focused on using plants to combat bacterial infections. Plants are considered the richest bioactive secondary metabolites which have become essential to screen potential as sources of antimicrobial agents against a wide range of MDR bacteria [10,19,28]. An increasing interest in studying herbal remedies worldwide was observed because it is safe and effective [23,26,29].

In the present investigation, *in vitro* antibacterial activities of 60 plant extracts were evaluated for their antibacterial activity against MRSA, MSSA, ESBL *E. coli* and MDR *P. aeruginosa* isolated from human and animals. Ten of them (*Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Glycyrrhiza glabra*, *Phlomis brachyodon*, *Thymus vulgaris*, *Urtica pilulifera*, *Anchusa azurea*, *Plantago*, and *Pallenis spinosa*) exhibited the highest antibacterial activity. The highest inhibitory zone was for extracts of *Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Thymus vulgaris*, *Glycyrrhiza glabra*, *Anchusa azurea*, and *Phlomis brachy-*

odon against MRSA and MSSA; this is similar to [30], Gram positive bacteria were more sensitive than Gram negative bacteria. ESBL *E. coli* were affected by *Phlomis brachyodon*, *Pallenis spinosa*, *Urtica pilulifera*, *Anchusa azurea*, *Aloysia citrodora*, and *Hibiscus sabdariffa*. *Camellia sinensis* showed the greatest antimicrobial effect when compared to the other plants, this is in agreement with the results of [22]. In contrast, *P. aeruginosa* was not affected by any of the extracts except *Hibiscus sabdariffa* and not in agreement with the results of [22] results that *Hibiscus sabdariffa* extract had no antimicrobial activity against Gram positive and Gram negative bacteria.

All plants display (MIC) values less than 8 mg/mL in MIC test are considered as antimicrobial agents [31]. *Camellia sinensis* and *Aloysia citrodora* exhibited wide spectra of antimicrobial activity in comparison to *Hibiscus sabdariffa*, *Glycyrrhiza glabra*, and *Thymus vulgaris* (Table 3). Among the three tested bacterial species, *Camellia sinensis* extract showed the highest antibacterial activity against *S. aureus* (MRSA and MSSA), exhibiting the lowest MIC value (0.5 mg/mL) which is in agreement with the report of [22]. Additionally, *Camellia sinensis* has anti-bacterial activity against MDR *P. aeruginosa*, which is similar to a previous report [32]. *Aloysia citrodora* showed a MIC value of 1.3 mg/mL and 5.3 mg/mL against MRSA and *P. aeruginosa*, respectively, which is in agreement with [33], were essential oils of *Aloysia citrodora* possessed significant antibacterial effect against both Gram negative and Gram positive bacteria, with MICs ranged between 2.84 and 8.37 mg/mL. Furthermore, there were no differences between the effects of plant extracts against MRSA and MSSA that is similar to the report of [34]. The infection severity of MRSA is due to lack of therapeutic options, but not due to its virulence [34]. Gram negative bacteria (*E. coli* and *P. aeruginosa*) were less susceptible to antibacterial plant extracts which may be due to their outer membrane lipopolysaccharides, which is similar to the report of [35]. The antibacterial effects were higher against *S. aureus* and comparatively lower in the case of *Salmonella* and *E. coli* [35]. Results of this study are also consistent with many previous reports, which find a good indication for the potential of some plants to be applied as antimicrobial to treat bacterial infections [13,19].

The toxicity of plant extract needs scientific validation to ensure it is safe to use by humans [19,33]. Our results indicated that *Camellia sinensis*, *Aloysia citrodora*, *Hibiscus sabdariffa*, *Thymus vulgaris*, *Glycyrrhiza glabra* did not have any toxicity at 100 mg/mL using erythrocyte hemolysis assay. This is similar to the report of [24,36]. It is also worth mentioning that our results of the cytotoxicity assay are in agreement with many previous studies that investigated plants toxicity on human fibroblast and monkey Vero cell lines [24,29,33]. The results of the *in vitro* MTT assay showed no cytotoxicity for all tested plants when compared with doxorubicin, which is used as a positive control [37].

Our challenge study demonstrated that MRSA-induced systemic infection after estimated acute cytotoxicity. All mice in the acute toxicity study were within normal limits. According to our results, *Camellia sinensis*, *Aloysia citrodora*, and *Hibiscus sabdariffa* are safe to use as remedy which is in agreement with the results of [38,39]. The previous three plants showed a significant decrease in the severity of the *in vivo* MRSA infection compared to the positive control. *Camellia sinensis* treated group has the most protective ability against MRSA infections among all groups including vancomycin, which is consistent with [35] results of the *Camellia sinensis* seed saponins which possess strong antibacterial activities against infected chickens by *E. coli*, *S. aureus*, and *Salmonella* [35]. In recent years reduced vancomycin susceptibility of MRSA was noted [40] which is consistent with our results. The activity of *Aloysia citrodora* extract was improved when combined with *Camellia sinensis*. Hence, the combination effects indicated synergistic efficacy. However, combinations of *Camellia sinensis* with *Hibiscus sabdariffa* were antagonists. In general, the synergistic effects of the combination of different herbs with various bioactive constituents have shown progressive protective effects [41,42].

5. Conclusions

This study gives important data regarding *in vivo* antimicrobial efficacy of plant extract. *in vitro* results of this study are consistent with the *in vivo* results. *Camellia sinensis*, *Aloysia citrodora*, and *Hibiscus sabdariffa* have significant potent antimicrobial activity against MDR bacteria with no toxicity toward eukaryotic cells. *Camellia sinensis* has also significant potent antimicrobial activity against MRSA. Ongoing phytochemical research is required to investigate new bioactive compounds derived from plants that possess antimicrobial effects and focusing on the synergistic effect of different plant extracts and antibiotics to enhance the effectiveness of antimicrobial drugs against MDR bacteria. Alongside this, further studies are needed to determine the adverse effects, detect LD 50, toxicity for the acute and chronic effect of plant extracts, and study the mode of action of plant extracts *in vivo* before they can be used as therapeutic agents.

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