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Research Article
In vitro and *in vivo* Antibacterial Activity of *Cheilocostus speciosus* Rhizome Extract on Resistant Bacteria

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Abstract

Background and Objective: *Cheilocostus speciosus* has traditionally been used in alternative medicine for different therapeutic purposes in different countries, including as an anti-infective agent. Antimicrobial properties of *C. speciosus* against standard bacterial strains have been reported before, however, complementary *in vivo* investigations, as well as, its antimicrobial activity against multi drug-resistant (MDR) bacteria are insufficient, if not lacking. In our study, *in vitro* and *in vivo* methods were used to test its antimicrobial activities on prominent clinical MDR isolates. **Materials and Methods:** *Cheilocostus speciosus* rhizome extracts (CSRE) were prepared using different solvents; methanol, hexane, petroleum ether, ethylene glycol and water. The bactericidal activity of CSRE was tested on methicillin resistant *S. aureus* (MRSA), *A. baumannii*, *K. pneumoniae* serotype K2 (Kp K2), MDR *P. aeruginosa*, *S. typhimurium* and MDR *E. coli*. The standard disc diffusion and the broth micro-dilution methods were used to confirm the efficacy of CSRE against the tested microorganisms. *In vitro* results proved a potent bactericidal effect on MRSA and Kp K2 and a pronounced bacteriostatic effect against *E. coli*. **Results:** We further confirmed the antibacterial activity of CSRE using BALB/c mice, animals were infected with Kp K2 or MRSA along with their standard strains; the extract was found to significantly reduce the bacterial load in mice lungs, liver and spleen. In addition, extracts were found to be more effective on the MDRs than the standard strains. **Conclusion:** It is concluded that CSRE could be a potential source for new antibiotics, further investigations are required to identify the bioactive components.

Key words: *Cheilocostus speciosus*, multidrug-resistance, methicillin-resistant *S. aureus*, *K. pneumoniae* serotype K2, *E. coli*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bacterial resistance is becoming a global burden. The emergence and dissemination of MDR strains in pathogenic bacteria have become a major public health threat since there are fewer effective antimicrobial agents available[1]. In addition, the rapid development of bacterial resistance to new antibiotics brings up concerns that even new families of antimicrobial agents will have a short life span[2,3]. Accordingly, the need to find new antimicrobial agents is of vital importance.

In point of fact, infections caused by MDRs are among the main factors influencing morbidity and mortality in patients undergoing procedures such as chemotherapy for cancer treatment, organ transplantation, intensive care for pre-term newborns and many others[4].

Many medicinal plants have been evaluated for its antimicrobial properties, which could be considered as an important source of natural antimicrobial agents for the challenging and emerging bacterial resistance[5], due to phytochemicals produced during secondary metabolite synthesis in the plants[6,7].

Cheilocostus speciosus is an important medicinal plant used to treat different diseases. Traditionally, its rhizomes have been used as a tonic, astringent[8,9], expectorant, cooling, anthelminthic[10,11], purgative and depurative[12], antituberculosis[13] and antioxidant agent[14]. Leaves were also used to treat mental disorders among others[15].

Here, we report the first study to investigate *in vitro* and *in vivo* antimicrobial effect of CSRE on several MDR bacterial isolates which have been identified by the Infectious Disease Society of America as among the currently more challenging strains in clinical management.

MATERIALS AND METHODS

Plant material: *Cheilocostus speciosus* rhizomes were washed, shade-dried at room temperature and then grinded to a fine powder using electrical grinder. Costus powder was stored in an air-tight container throughout the study. This research project was conducted from September, 2017 to October, 2019 at the Biology and Biotechnology Department at the American University of Madaba, Madaba, Jordan.

Preparation of extracts: Three hundred grams of costus powder was extracted with 900 mL (1:3 w/v) for 72 h with intermittent stirring in the following solvents: water; methanol; hexane; petroleum ether, or ethylene glycol (Sigma-Aldrich, St. Louis, MO, USA). Extracts were then filtered through a Buchner funnel with Whatman number 1 filter paper (Fisher Scientific, Hampton, New Hampshire, USA). Filtrates were evaporated under reduced pressure using rotary evaporator at 40°C (Yamato RE202-A/RE212-A, Yamato Scientific America Inc. Santa Clara, CA, USA), with the exception of ethylene glycol extract.

Tested bacteria: Clinical isolates of bacteria were used along with their standard strains. The standard strains of *E. coli* ATCC 8739, *S. typhimurium* ATCC 14028, *P. aeruginosa* ATCC 15442, *K. pneumoniae* ATCC 10031 and *S. aureus* ATCC 6538 were obtained from the American Type Culture Collection (Manassas, VA, USA), while the MDR strains of *E. coli*, *A. baumannii*, *K. pneumoniae* and MRSA were obtained from clinical isolates kindly provided by the Al-Khalidi Hospital (Amman, Jordan). MRSA was isolated from a sore throat patient and was resistant to methicillin, gentamicin, penicillin, oxacillin and cefixime (Sigma-Aldrich-Fluka, St Louis, MO, USA). While Kp K2 was isolated from a patient's blood and was resistant to ampicillin, carbenicillin, amoxicillin and ceftazidime (Sigma-Aldrich, St. Louis, MO, USA).

Bacterial inoculums: The bacterial stock cultures were streaked on Nutrient Agar medium to obtain isolated colonies. Colonies were transferred to Mueller-Hinton (MH) broth (Fisher Scientific, Hampton, New Hampshire, USA) then incubated for 16 h at 37°C. The bacterial suspension was equal to the 0.5 McFarland standards. For disc-diffusion assay, cell suspensions were diluted with MH broth to provide final cell counts of about 10^4 CFU mL^{-1}.

Disc diffusion assay: Antibacterial activity was carried out for crude extracts using disc-diffusion method[16]. The test cultures were swabbed on the top of the sterile Luria broth (LB) Agar (Fisher Scientific, Hampton, New Hampshire, USA) and allowed to dry for 10 min. The tests were conducted at 5 different concentrations of the crude extract (0.5, 1, 1.5, 2 and 2.5 mg per disc). Loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative controls

were prepared using the respective solvents. Plates were incubated for 16 h at 37°C. Inhibition zones (IZ) were recorded in millimeters and each experiment was performed in triplicate.

Broth microdilution assay: The minimum inhibitory concentrations (MIC) of the plant extracts were determined using the broth microdilution method based on[17] Clinical Laboratory Standard Institute M07-A8. In brief, testing was carried out in MH broth pH 7.4 and the twofold serial dilution technique was applied. The final inoculums size was 10^5 CFU mL^{-1} in a 24-well plate. A set of wells containing only inoculated or plain broth were used as controls, plates were incubated at 37°C for 24 h. The wells with no growth were recorded to represent the MIC expressed in 1 mg mL^{-1}. Every treatment was performed in triplicate.

Animal experiments: Male BALB/c mice (Applied Science Private University, Amman, Jordan) aged 6-8 weeks old were used throughout this study. All mice were housed in the animal facility at the American University of Madaba and were treated according to the NIH guidelines. Mice were kept under controlled conditions at 30°C with 12 h variation of light/dark period. Water and feed were provided ad libitum and mice were left to acclimatize for 5 days prior to the experiment.

Twenty four hours before infecting mice with the test strains, three random mice were sacrificed and screened for the presence of *S. aureus* and *K. pneumoniae* in the lungs, spleen and liver, by streaking the tissue homogenates directly on Mannitol salt agar plates (*S. aureus*) or MacConkey plates (*K. pneumoniae*) (Fisher Scientific, Hampton, New Hampshire, USA). Plates were incubated at 37°C for 24 h to check for the presence of both strains.

Inoculum preparation: The test strains, *S. aureus* (ATCC 6538), the clinical isolate MRSA, *K. pneumoniae* (ATCC 10031) and the clinical isolate Kp K2 were refreshed on LB broth at 37°C for 16 h. For inoculum preparation, culture was centrifuged at 3380×g for 15 min (Sorvall ST8 refrigerated centrifuge, Thermo Fisher Scientific, St. Louis, MO, USA) and pellet was washed twice with PBS. The optical density was adjusted at 1±0.02 (at 600 nm) analogous to 10^{11} CFU mL^{-1} and tenfold dilutions of this suspension were used in mice to establish infection dose through a "pilot experiment". The corresponding CFU mL^{-1} was confirmed by plating 100 µL of serial dilution of the inoculum on Mannitol salt agar plates (*S. aureus*) or MacConkey plates (*K. pneumoniae*).

Infection and treatment with *S. aureus*: After the hexane plant extract was evaporated to dryness at 40°C, it was dissolved in water at 200 mg mL^{-1} prior to this experiment. Twenty animals were divided into 4 groups (1-4). Infection with *S. aureus* (ATCC 6538) was established in groups 1 and 3 with 200 µL of 1×10^8 CFU mL^{-1} by intraperitoneal injection. While infection in groups 2 and 4 was established using MRSA at the amount of 2×10^7 CFU mL^{-1} using the same volume and route. One hour after bacterial inoculation, groups 3 and 4 were force fed hexane extract at the amount of 200 mg mL^{-1} per kg of body weight through feeding needles, hexane extract was fed once daily throughout the duration of this experiment. Animals were observed daily for 7 days.

Infection and treatment with *K. pneumoniae*: Mice groups 5-8 were inoculated intraperitoneally with 200 mg kg^{-1} of the immunosuppressant cyclophosphamide (CP) (Sigma-Aldrich, St. Louis, MO, USA) 4 days prior to inoculation with *K. pneumoniae*. Animals were monitored for 4 days after CP injection before bacterial inoculation. Kp K2 was provided by Al-Khalidi Hospital (Amman, Jordan) was isolated from human blood and was shown to be generally virulent in mice[18].

Twenty immunocompetent mice were divided to 4 groups (5-8), groups 6 and 7 were force fed hexane extract at the amount of 200 mg mL^{-1} per kg of body weight through feeding needles once daily throughout the duration of this experiment. Groups 5 and 7 were infected intraperitoneally with 200 µL of 1×10^7 CFU mL^{-1} of standard *K. pneumoniae* (ATCC 10031) or 1×10^4 CFU mL^{-1} of Kp K2 (groups 6 and 8). Animals were observed for 72 h post infection.

Bacteriological examination in tissues

Pilot experiment: qualitative examination (re-isolation of test strains from tissues): The presence of test strain in lung, liver and spleen was determined by streaking tissue homogenate in the pilot experiment directly on the appropriate selective media. The plates were incubated at 37°C for 24 h and checked for the presence of the test strain.

Main experiment

Bacterial load determination in mouse lungs, liver and spleen: Mice were sacrificed by cervical dislocation 7 days after *S. aureus* inoculation or 72 h after *K. pneumonia* inoculation. Lungs, liver and spleen were aseptically removed, weighed and homogenized (Fisherbrand™ 150 Handheld Homogenizer Motor, Fisher Scientific, Hampton, New Hampshire, USA) for up to 30 sec in sterile glass tubes with 1 mL of sterile saline. The tissue homogenate was vigorously agitated with a Vortex mixer (VWR® Fixed Speed Vortex Mixer, Suwanee, GA, USA) to disrupt bacterial aggregates, homogenate was plated for CFU counting. Serial 1:10 dilutions were spread on the appropriate selective media (100 µL/plate) and incubated for 18-20 h at 37°C, then the numbers of viable bacterial CFU were determined. The data were expressed as CFU g^{-1} in the lung, liver, or spleen (mean±standard deviations).

RESULTS

Antimicrobial activity of CSRE against MDR bacteria: Disc diffusion and MIC assays. In order to initially test the antimicrobial effectivity of CSRE against the selected standard and MDR bacterial strains, 5 extraction solvents were tested using disc diffusion assay. All the negative control discs containing only the vehicle did not produce any inhibition with any of the solvents. Inhibition zone (mm) is shown in Table 1-6, the extent of the antibacterial activity was solvent and dose dependent. All extracts excluding water produced antimicrobial activities against all the bacterial strains tested to different extents, with the exception of *A. baumannii* that was resistant to all extracts at the tested dosages. Methanol, hexane and petroleum ether extracts (Table 1-3) were the most effective against all bacterial strains besides *A. baumannii*.

Table 1: Bactericidal effect of CSRE in methanol

	Inhibition zone (mm) concentration of CSRE extract (mg)					
Tested bacteria	0.5	1.0	1.5	2.0	2.5	MIC (mg mL^{-1})
Klebsiella pneumoniae	-	6.8±0.02	8.1±0.12	9.7±0.07	11.1±0.08	1.25
Kp K2	7.3±0.11	9.5±0.00	10.1±0.15	11.3±0.06	12.0±0.03	0.312
Staphylococcus aureus	6.0±0.01	8.0±0.02	10.0±0.03	11.0±0.01	12.3±0.00	0.625
MRSA	9.0±0.03	10.0±0.02	11.5±0.07	12.0±0.15	12.5±0.12	0.156
Pseudomonas aeruginosa	6.1±0.04	6.5±0.02	7.0±0.11	7.8±0.01	8.1±0.04	1.25
MDR *Pseudomonas aeruginosa*	-	6.3±0.12	6.5±0.02	7.0±0.01	8.0±0.03	2.5
Salmonella typhimurium	-	-	6.0±0.00	7.0±0.03	7.0±0.02	>5
Acinetobacter baumannii	-					NA

Values are expressed as mean±standard deviation (n = 3) for each sample, MIC: Minimium inhibition concentration, NA: Not applicable

Table 2: Bactericidal effect of CSRE in hexane

	Inhibition zone (mm) concentration of CSRE extract (mg)					
Tested bacteria	0.5	1.0	1.5	2.0	2.5	MIC (mg mL^{-1})
Klebsiella pneumoniae	7.0±0.06	8.2±0.02	9.4±0.07	9.9±0.03	11.0±0.1	0.312
Kp K2	6.5±0.01	7.8±0.01	9.0±0.01	11.0±0.03	13.0±0.05	0.625
Staphylococcus aureus	8.0±0.01	9.0±0.07	12.1±0.09	13.4±0.1	14.0±0.15	0.312
MRSA	8.0±0.03	11.0±0.01	12.0±0.1	13.0±0.07	15.0±0.1	0.156
Pseudomonas aeruginosa	6.0±0.04	6.6±0.12	7.1±0.01	7.4±0.11	7.7±0.04	2.5
MDR *Pseudomonas aeruginosa*	-	6.3±0.12	6.5±0.02	7.0±0.01	7.3±0.03	2.5
Salmonella typhimurium	-	-	6.1±0.00	7.3±0.03	7.8±0.02	>5
Acinetobacter baumannii	-	-	-	-	-	NA

Values are expressed as mean±standard deviation (n = 3) for each sample, MIC: Minimium inhibition concentration, NA: Not applicable

Table 3: Bactericidal effect of CSRE in petroleum ether

	Inhibition zone (mm) concentration of CSRE (mg)					
Tested bacteria	0.5	1.0	1.5	2.0	2.5	MIC (mg mL^{-1})
Klebsiella pneumoniae	-	-	7.0±0.01	8.0±0.01	9.0±0.03	1.25
Kp K2	-	-	-	-	-	NA
Staphylococcus aureus	7.0±0.01	8.0±0.02	9.0±0.01	10.0±0.03	12.0±0.05	0.625
MRSA	6.0±0.01	8.0±0.03	9.0±0.02	10.0±0.01	10.5±0.04	1.25
Pseudomonas aeruginosa	-	-	-	-	-	NA
MDR *Pseudomonas aeruginosa*	-	-	-	-	-	NA
Salmonella typhimurium	-	-	-	-	-	NA
Acinetobacter baumannii	-	-	-	-	-	NA

Values are expressed as mean±standard deviation (n = 3) for each sample, MIC: Minimium inhibition concentration, NA: Not applicable

Table 4: Bactericidal effect of CSRE in ethylene glycol

	Inhibition zone (mm) concentration of CSRE (mg)					
Tested bacteria	0.5	1.0	1.5	2.0	2.5	MIC (mg mL^{-1})
Klebsiella pneumoniae	-	-	-	-	7±0.02	NA
Kp K2	-	-	-	-	6±0.01	NA
Staphylococcus aureus	-	-	-	-	6±0.02	NA
MRSA	-	-	-	-	7±0.03	NA
Pseudomonas aeruginosa	-	-	-	-	-	NA
MDR *Pseudomonas aeruginosa*	-	-	-	-	-	NA
Salmonella typhimurium	-	-	-	-	-	NA
Acinetobacter baumannii	-	-	-	-	-	NA

Values are expressed as Mean±standard deviation (n = 3) for each sample, MIC: Minimium inhibition concentration, NA: Not applicable

Table 5: Bacteriostatic activity of different extracts of *C. speciosus* rhizome against *E. coli* and its resistant form

		Inhibition zone (mm)														
		Hexane (mg)					Petroleum ether (mg)					Water (mg)				
Tested organisms	State	0.5	1	1.5	2	2.5	0.5	1	1.5	2	2.5	0.5	1	1.5	2	2.5
Escherichia coli	Bacteriostatic	12.0	12	18	20	20	12	17	18	20	21	-	-	-	-	-
MDR *E. coli*		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6: Bactericidal activity of different CSRE against *E. coli* and its resistant form

		Inhibition zone (mm) at 2.5 mg of *C. speciosus*		
Tested organisms	State	Methanol	Ethylene glycol	Water
Escherichia coli	Bactericidal	10±0.02	-	-
MDR *E. coli*		-	9±0.03	-

Values are expressed as mean±standard deviation (n = 3) for each sample

The MIC values for the different extracts are represented in their corresponding tables. MIC was determined as the minimum concentration of the extract that didn't produce visible growth. Ampicillin and ciprofloxacin, where applicable, or just plain media were used as references, the MIC values ranged between 0.156-2.5 mg mL^{-1} for *K. pneumoniae*, MDR *K. pneumoniae*, *S. aureus*, MRSA, *P. aeruginosa* and MDR *P. aeruginosa*. On the other hand, MIC values of *S. typhimurium* exceeded 5 mg mL^{-1}.

***Staphylococcus aureus*:** Methanol, hexane and petroleum ether exhibited the best bactericidal effect against *S. aureus* at 2.5 mg of extract concentration with IZ of 12.3, 14 and 12 mm, respectively. The same extracts also showed the best bactericidal effect against MRSA at 2.5 mg concentration with IZ of 12.5, 15 and 10.5 mm, respectively (Table 1-3).

***Klebsiella pneumoniae*:** Methanol, hexane and petroleum ether inhibited the growth of *K. pneumoniae* at the highest concentration of 2.5 mg, showing an IZ of 11.1, 11 and 9 mm, respectively (Table 1-3). While diameters of 12, 13 mm were recorded for Kp K2 with methanol and hexane extracts, in that order and a minimum inhibition with ethylene glycol (6 mm). No inhibition was observed with petroleum ether (Table 3).

***Pseudomonas aeruginosa*:** Only methanol and hexane extracts were effective against *P. aeruginosa* and its resistant form, with trivial IZ of 7.3-8.1 mm at the maximum dosage of 2.5 mg (Table 1-2).

***Salmonella typhimurium*:** *Salmonella typhimurium* also showed some inhibition with methanol and hexane extracts, recorded diameters were 7 and 7.8 mm, respectively at 2.5 mg extract concentration (Table 1-2).

Water and glycol extracts: *C. speciosus* glycol extract showed a minimum inhibition with *K. pneumoniae* and *S. aureus* including their MDR strains, with IZ of 6-7 mm (Table 4). Water extract didn't have antibacterial effect on any of the bacterial strains that we have tested, including at the highest concentration of 2.5 mg of extract. In a separate experiment, we tested if water and glycol extracts would be more effective at higher concentrations (5 or 7.5 mg), results obtained didn't show enhanced antimicrobial activity.

***Escherichia coli*:** The best bacteriostatic effect was at 2.5 mg extract with a diameter of 20 mm with hexane and 21 mm

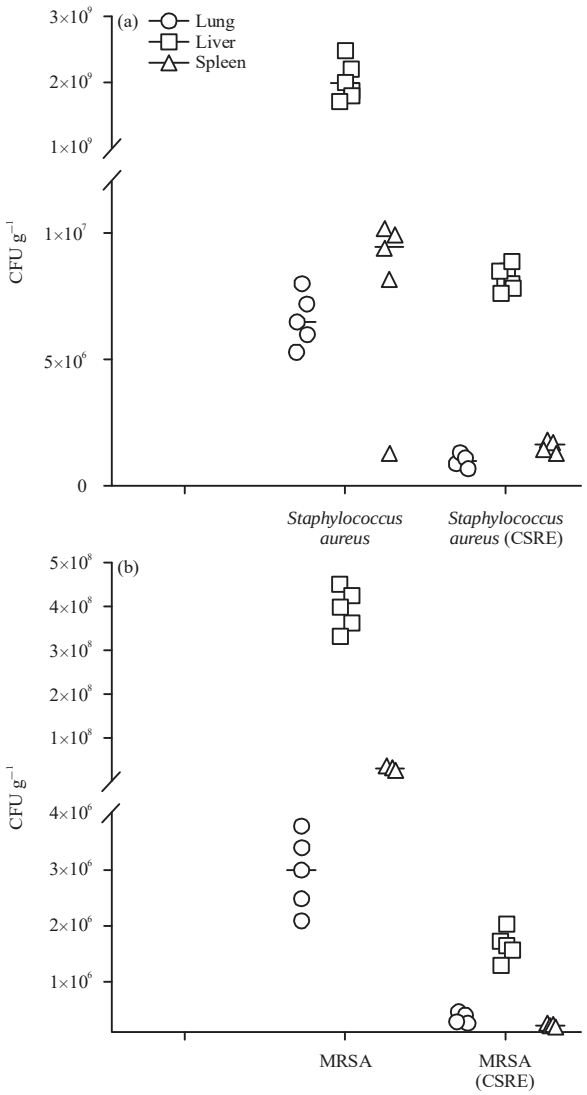

Fig. 1(a-b): Effects of intraperitoneal injection of CSRE in hexane on (a) *S. aureus* and (b) MRSA bacterial load in different tissues

with petroleum ether extracts (Table 5). On the other hand, a moderate bactericidal effect against *E. coli* and its MDR was observed with methanol (10 mm) and ethylene glycol extracts (9 mm) (Table 6).

Animal experiments

Establishing an infection dose of *S. aureus* and *K. pneumoniae* (pilot experiment): The mice that were inoculated with 200 µL of 1-3×10^7 CFU mL^{-1} *S. aureus* or mice inoculated with up to 0.5-1×10^7 CFU mL^{-1} MRSA, remained clinically healthy up to 6 days post infection. Bacteria could not be isolated from lungs, liver and spleen after 3 days post infection onward. No significant variation in body weight could be observed in the treated and controlled group. While mice inoculated with 200 µL of 1×10^8 CFU mL^{-1} of *S. aureus* or 2×10^7 MRSA showed signs of pathological condition with watery nose and lethargic condition. On day seven post infection re-isolation of the *S. aureus* was also recorded from lungs, liver and spleen. The loss in body weight of the mice was also noticed at the end of the experiment. When animals were inoculated with higher doses, all animals showed adverse clinical signs characterized by apathy, lethargy, loss of body weight, ruffled coat and around 50% mice were dead by 4th day post infection.

Similar procedure was used to establish the infection dose of *K. pneumoniae* and Kp K2, mice that were inoculated with 2 and 5×10^6 CFU mL^{-1} of *K. pneumoniae* or inoculated with 3 and 5×10^4 CFU mL^{-1} of Kp K2 didn't present any symptoms for up to 72 h post infection. Also bacteria could not be isolated from lungs or spleen after 48 h post infection. The mice inoculated with 200 µL of 1×10^7 CFU mL^{-1} of *K. pneumoniae* or 1×10^4 CFU mL^{-1} Kp K2 showed signs of pathological and lethargic conditions, bacteria was recovered from lungs and spleen 72 h post infection. Higher doses of inoculation lead to 90% death approximately 48 h post infection.

Main experiment: The dose of 200 µL of 1×10^8 CFU mL^{-1} of *S. aureus*, 2×10^7 MRSA, or 200 µL of 1×10^7 CFU mL^{-1} of *K. pneumoniae* or 1×10^4 CFU mL^{-1} Kp K2 at which no mortality and re-isolation from all the organs were achieved was selected for the main study. In animals that were treated with CSRE, a reduction in bacterial load was recorded in several tissues, the lungs exhibited a decrease of 84.4% in *S. aureus* and 88.2% in MRSA, a 99.6% of both *S. aureus* and MRSA in the liver and a 79.4% reduction in *S. aureus* 99.3% of MRSA was measured in the spleen (Fig. 1a-b).

The CSRE was able to reduce lungs bacterial load of *K. pneumonia* and Kp K2 at 62.8 and 67.6%, respectively and to about 100% in the spleen for both strains (Fig. 2a-b).

Body weight: Body weight of the animals at the end of the experiment was significantly less in infected mice compared with the control group that maintained or had a slight increase in their weight. On day 8 of the experiment, a percentage of 5.36% weight loss was observed in mice infected with *S. aureus* and a 5.85% with MRSA, nevertheless,

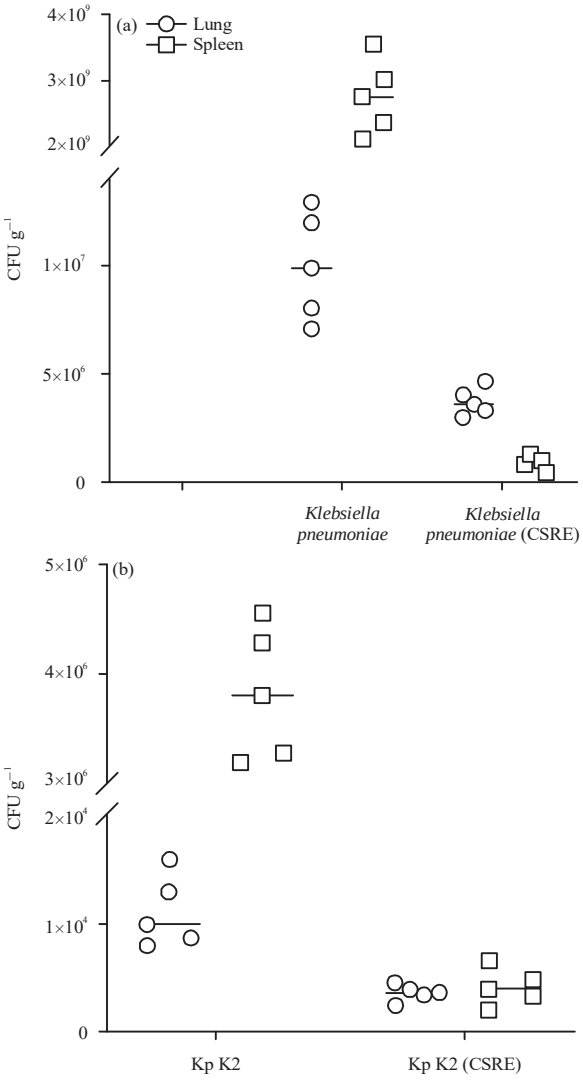

Fig. 2(a-b): Effects of intraperitoneal injection of hexane extract of CSRE on infected mice with (a) *K. pneumoniae* and (b) Kp K2

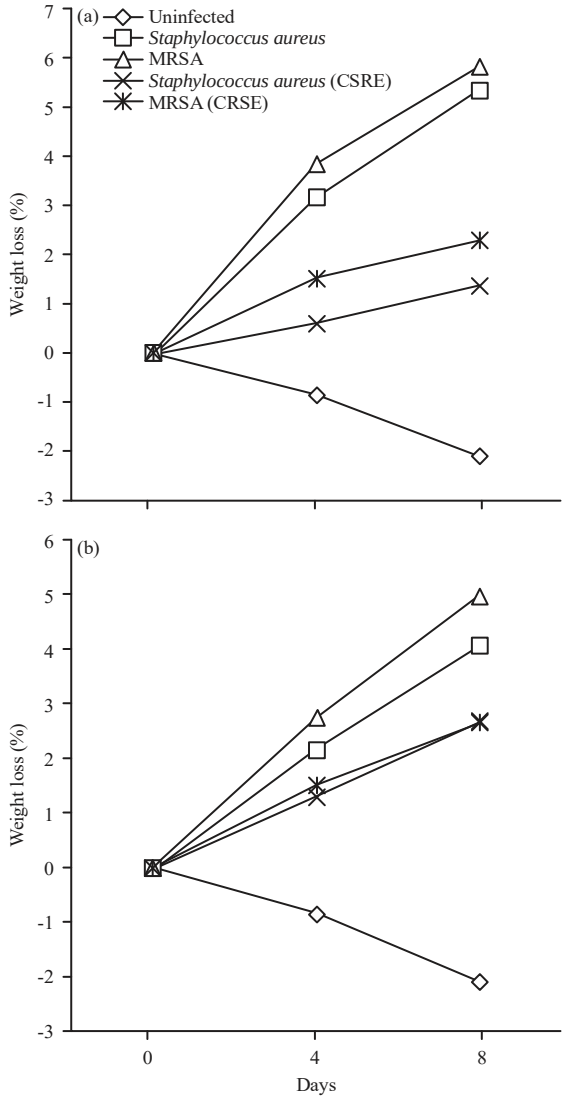

Fig. 3(a-b): Weight loss (%) in mice infected with (a) *S. aureus* and MRSA on days 4 and 8 post infection and (b) *K. pneumoniae* and Kp K2 days 4 and 8 post infection, with or without CSRE hexane treatment

a decrease in 1.3% only was observed in *S. aureus* mice treated with CSRE hexane extract (decreasing the weight loss in the mice by about 75%) and a weight loss of 2.3% in MRSA infected mice treated with CSRE (decreasing the weight loss in the mice by about 60%) (Fig. 3a).

The same trend was observed with mice infected with *K. pneumoniae* and Kp K2; a weight loss of 4.08 and 4.99%, respectively were recorded on the eighth day of the experiment, while treatment of *K. pneumoniae* infected mice with CSRE resulted with a 2.68% weight loss, reducing weight loss by about 35% and a 2.65% in Kp K2 infected animals, reducing it by about 47% (Fig. 3b).

DISCUSSION

A substantial bacteriostatic effect was obtained with hexane and petroleum ether extracts against *E. coli*, consistent with results reported by Sidkey *et al.*[19]. The interest in research on medicinal plants has increased over the last few decades due to the emergence of MDR strains of important pathogens[20].

With reference to the many studies conducted on the antimicrobial activities of CSRE that we reviewed, this study is the first to demonstrate the antibacterial activities of the

CSRE against various clinical isolates of bacteria with the more novel and alarming transferable multiple drug resistance mechanisms: Kp K2, MRSA, MDR *P. aeruginosa*, *S. typhimurium*, *A. baumannii* and MDR *E. coli*.

Generally, the methanol and hexane extracts proved to be more potent than the petroleum ether and ethylene glycol extracts. The water extract did not show any inhibition, although its preparation mimics the traditional method of use; no antimicrobial activity was observed in it, which might be due to less solubility of active components in water[21,22].

The disc diffusion assay was used as a preliminary method to test if the crude extract had antimicrobial activity against the bacterial strains in question. In disc diffusion assay, methanol and hexane extracts exhibited the most bactericidal effect and MIC values against MRSA>*S. aureus*>Kp K2> *K. pneumoniae*, with a IZ ranging from 11-15.2 mm at CSRE concentration of 2.5 mg and MIC between 0.156-1.25 mg mL^{-1}, indicating that extraction with methanol and hexane resulted in a variety and better quantity of bioactive compounds of the plant rhizomes, such observation has also been reported by other groups[23]. A less pronounced effect was obtained with petroleum ether and a negligible effect was observed with ethylene glycol. However, ethylene glycol and hexane extracts resulted in a notable bacteriostatic effect with *E. coli*, ranging from 21-21 mm (Table 5).

In the *in vivo* part of the study we have used BALB/c mice for their susceptibility to infection with both *K. pneumoniae* and *S. aureus* for the reason that certain hosts are more susceptible to infection more than others. Among the MDR strains of *K. pneumoniae* we have chosen the Kp K2 since it's proven to be virulent in mice. To further ensure that BALB/c mice will be infected with *K. pneumoniae* or Kp K2, we have treated the mice with 200 mg kg^{-1} of the immunosuppressant cyclophosphamide (CP) 4 days prior to inoculation with *K. pneumoniae*. Male mice were chosen over female as they are more effectively infected with *S. aureus* and *K. pneumoniae* than females[18].

The dose of bacteria to establish infection depends on the type and virulence of strain, host species, quantity of pathogen and site of inoculation. Therefore, a pilot study was conducted to confirm pathogenicity and infectious dose of the tested bacteria in mice. The experiment revealed that 200 µL dose of 10^8 CFU mL^{-1} of *S. aureus*, a 2×10^7 CFU mL^{-1} of MRSA, a 10^7 CFU mL^{-1} of *K. pneumoniae* and a 10^4 CFU mL^{-1} of Kp K2 was sufficient to induce infection and this infection persisted till the required time needed to obtain sufficient results.

In the main experiment, the extract revealed that it effectively inhibited the growth of *S. aureus* and MRSA to up to 88.2% in the lungs, up to 99.6% in the liver and up to 99.3% in the spleen. Similar results were obtained with *K. pneumoniae* and Kp K2 infected animals, CSRE reduced the bacterial load in lungs to about 67.6% and almost totally cleared it in the spleens. The potency of CSRE to kill bacteria was more obvious toward the MDR strains of the tested bacteria than the wild type.

This antibacterial activity may be indicative of the presence of several metabolic toxins or broad-spectrum antibiotics. Several metabolites from herb species, including alkaloids, tannins and sterols, have previously been associated with antimicrobial activity[23].

CONCLUSION

To the best of our knowledge, this is the first report investigating *in vivo* antimicrobial efficacy of CSRE. However, there is a need to seek for active constituents, its serum attainability levels, pharmacokinetic properties and diffusion. Current study offered a scientific basis for the medicinal application for CSRE against MRSA and Kp K2-associated infections. The antimicrobial activities could be enhanced if their active components are purified and adequate dosage determined for proper administration in order to prevent the usage of in appropriate concentration, a common practice among many traditional medical practitioners.

SIGNIFICANCE STATEMENT

This study discovers that the antimicrobial property of *C. specious* rhizome extract extends beyond the standard bacterial strains to its resistant clinical isolates, *in vitro* and *in vivo*. Our results will encourage other researchers to uncover the active ingredients of this plant, hoping to be a new source for efficient antibiotics against some of the prominent resistant bacteria. It is worth mentioning that our novel application of testing the antimicrobial effect of a plant extract in animal model might advance other investigations on medicinal plants that were short in *in vivo* confirmation of their results. This study will help the researcher to uncover some aspects of the critical areas of traditional medicine that other researchers were not able to explore.

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