ORIGINAL

Antibiotic resistance pattern of methicillin-resistant *Staphylococcus aureus* isolates from powdered packaged medicinal plants and bottle herbal distillates

Patrón de resistencia a los antibióticos de los Staphylococcus aureus aislados resistentes a la meticilina procedentes de plantas medicinales envasadas en polvo y destilados de hierbas en botella

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Abstract

Background: Human involvement in the medicinal plant's and herbal distillate's processing caused a potential risk of microbial contamination, particularly with Staphylococcus aureus. The present research was performed to assess the prevalence and antibiotic resistance of methicillin-resistant S. aureus bacteria isolated from diverse kinds of powdered packaged medicinal plant and bottle herbal distillate samples.

Methods: Three-hundred different powdered packaged medicinal plant and bottle herbal distillate samples were collected and examined by the culture method. MRSA strains were identified using oxacillin and cefoxitin disk diffusion. The pattern of antibiotic resistance of MRSA isolates was examined using disk diffusion.

Results: Eighteen out of 300 (6.00%) examined samples were contaminated with MRSA. *Rosa damascene* medicinal plant (13.33%) and *Alhagi maurorum* bottle distillate (10%) had the highest MRSA bacteria contamination rate. Bottle herbal distillate samples had a higher contamination rate of MRSA (*P* <0.05). MRSA isolates harbored the highest prevalence of resistance toward trimethoprim-sulfamethoxazole (100%), cefixime (100%), ampicillin (100%), tetracycline (88.88%), erythromycin (88.88%), and rifampin (83.33%) antibiotic agents.

Conclusion: Medicinal plants and herbal distillates were potential sources of antibiotic-resistant-MRSA bacteria.

Keywords: Medicinal plants, herbal distillates, methicillin-resistant Staphylococcus aureus, antibiotic resistance.

Resumen

Antecedentes: La participación del hombre en el procesamiento de plantas medicinales y destilados de hierbas provocó un riesgo potencial de contaminación microbiana, en particular con Staphylococcus aureus. La presente investigación se llevó a cabo para evaluar la prevalencia y la resistencia a los antibióticos de las bacterias S. aureus resistentes a la meticilina aisladas de diversos tipos de muestras de plantas medicinales envasadas en polvo y de destilados de hierbas en botella.

Métodos: Se recogieron 300 muestras diferentes de plantas medicinales envasadas en polvo y de destilados de hierbas en botella y se examinaron mediante el método de cultivo. Las cepas de SARM se identificaron mediante difusión en disco de oxacilina y cefoxitina. El patrón de resistencia a los antibióticos de los aislados de SARM se examinó mediante difusión en disco.

Resultados: Dieciocho de las 300 (6,00%) muestras examinadas estaban contaminadas con SARM. La planta medicinal Rosa damascena (13,33%) y el destilado de botella Alhagi maurorum (10%) presentaron el mayor índice de contaminación por bacterias SARM. Las muestras de destilado de botella de hierbas tenían un mayor índice de contaminación por SARM (P <0,05). Los aislados de SARM presentaron la mayor prevalencia de resistencia a los agentes antibióticos trimetoprim-sulfametoxazol (100%), cefixima (100%), ampicilina (100%), tetraciclina (88,88%), eritromicina (88,88%) y rifampicina (83,33%).

Conclusión: Las plantas medicinales y los destilados de hierbas fueron fuentes potenciales de bacterias SARM resistentes a los antibióticos.

Palabras clave: Plantas medicinales, destilados de hierbas, Staphylococcus aureus resistente a la meticilina, resistencia a los antibióticos.

Introduction

Medicinal plants and herbal distillates are rich sources of therapeutic agents with high beneficial effects on human health. They have a comprehensive role in Iranian folk medicine[1, 2]. Diverse kinds of medicinal plants including *Zataria multiflora (Z. multiflora), Satureja bachtiarica (S. bachtiarica), Aloysia citrodora (A. citrodora), Rosa damascene (R. damascene), Lavandula angustifolia (L. angustifolia), Alhagi maurorum (A. maurorum), Cichorium intybus (C. intybus), Melissa officinalis (M. officinalis), Mentha piperita (M. piperita), and Fumaria officinalis (F. officinalis)* are extensively used as antimicrobial, antioxidant, anticancer, anti-neoplasia, food additive, anti-inflammation, wound healing, antiseptic, anti-diabetic, diuretic, expectorant, stimulating the central nervous system, digestive, anti-mutagenic, sedative, analgesic, etc. agents among people al-around the world[3-10]. They also have an export aspect. Thus, it is essential to ensure the quality and safety of these products.

Human involvement in the packaging, powdering, and further procedures of medicinal plants and preparation of bottle herbal distillates caused their unintentional microbial contamination, particularly with foodborne pathogens[11-17]. *Staphylococcus aureus (S. aureus)* is a foodborne pathogen that originated from the skin and upper respiratory tract. The bacterium is responsible for severe nosocomial and community-acquired infections, foodborne diseases, and food poisoning. Food-related diseases and disorders caused by *S. aureus* are mainly known by abdominal cramps, nausea, vomiting, weakness, diarrhea, and toxic shock syndrome (TSS)[18]. Methicillin-resistant *S. aureus* (MRSA) is an antibiotic-resistant strain with simultaneous resistance toward cephalosporins and penicillins groups of antibiotics[10]. MRSA bacteria mainly harbored the *mecA* gene responsible for the occurrence of resistance toward penicillins[19]. MRSA bacteria harbored higher resistance toward commonly used antibiotic agents, particularly penicillins, cephems, glycopeptides, aminoglycosides, macrolides, and tetracyclines fluoroquinolones, nitrofurantoin, lincosamides, folate pathway antagonists, phenols, ansamycins, and even streptogramins[20-23].

According to MRSA's uncertain role as a foodborne bacterium in medicinal plants and herbal distillates, the present survey was performed to assess MRSA isolates' prevalence and antibiotic resistance from powdered packaged medicinal plants and bottle herbal distillates.

Materials and methods

Samples
From May 2019 to January 2020, a total of 300 diverse kinds of powdered packaged medicinal plants including *Z. multiflora* (n= 30), *S. bachtiarica* (n= 30), *A. citrodora* (n= 30) and *R. damascene* (n= 30) and bottle herbal distillates including *L. angustifolia* (n= 30), *A. maurorum* (n= 30), *C. intybus* (n= 30), *M. officinalis* (n= 30), *M. piperita* (n= 30) and *F. officinalis* (n= 30) were randomly collected from shopping centers, Tehran, Iran. A total of 50 g samples were collected from each powdered packaged medicinal plant and bottle herbal distillate sample using a sterile laboratory tube. All bottle herbal distillates were produced conventionally in small traditional producing units. Additionally, all collected powdered packaged medicinal plants were dried, powdered, and packed conventionally in traditional production units. Specifications about samples were recorded according to their labels. All samples were directly transferred to the laboratory at 4°C.

Isolation and identification of S. aureus
Twenty-five grams of each collected powdered packaged medicinal plant and bottle herbal distillate samples were blended with 225 mL of buffered peptone water (Merck, Germany). At that time, solutions were homogenized using Stomacher (Interscience, Saint-Nom, France). At that point, five milliliters of the achieved solution was transferred into 50 mL Trypticase Soy Broth (TSB, Merck, Germany) supplemented with 10% NaCl and 1% sodium pyruvate and incubated for 18 h at 35°C. At that moment, a loopful of the culture was transferred into Baird-Parker agar supplemented with egg yolk tellurite emulsion (Merck, Germany) and incubated at 37°C for about 24 h. Black shiny colonies enclosed with significant zones identified using biochemical tests including Gram staining, oxidase test, catalase activity, resistance to bacitracin (0.04 U), coagulated test (rabbit plasma), urease activity, glucose O/F test, Voges-Proskauer (Merck, Germany) test, nitrate reduction, phosphatase, deoxyribonuclease (DNase, Merck, Germany) test, mannitol fermentation, hemolysis activity on blood agar (Merck, Germany) and carbohydrate (xylose, sucrose, trehalose and maltose, fructose, lactose, mannose) fermentation tests[23].

Identification of MRSA isolates
MRSA bacteria were recognized using cefoxitin (30 µg) and oxacillin (1 µg) susceptibility test[24]. Principles of the Clinical and Laboratory Standards Institute (CLSI) was applied[24].

Antibiotic resistance of MRSA isolates
The phenotypic pattern of antibiotic resistance of MRSA bacteria isolated from powdered packaged medicinal plant and bottle herbal distillate samples was assessed using the disk diffusion method using the Mueller-Hinton agar (Merck, Germany) medium. A 0.5 McFarland standard concentration of MRSA was prepared and used for the disk diffusion. Ideologies of Clinical and Laboratory Standards Institute (CLSI) were used for this goal[24]. Diverse kinds of antibiotic groups

including tetracycline (30 µg/disk), doxycycline (30 µg/disk), trimethoprim-sulfamethoxazole (1.25/23.75 µg/disk), erythromycin (15 µg/disk), kanamycin (30 µg/disk), cefixime (30 µg/disk), chloramphenicol (30 µg/disk), rifampin (5 µg/disk), imipenem (10 µg/disk), and ampicillin (10 µg/disk) were applied for this goal (Oxoid, UK)[25]. The method was performed using the way designated previously[23.] Media were incubated aerobically at 37°C for about 24 h. Afterward, the diameter of the bacteria's growth inhibition zone was measured and compared with those recorded by the CLSI. MRSA BAA 2313 was prepared from the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, and used as control.

DNA extraction and quality examination

MRSA isolates were sub-cultured on TSB media (Merck, Germany) and incubated for 48 h at 37ºC. Genomic DNA was extracted from MRSA colonies using the DNA extraction kit (Thermo Fisher Scientific, St. Leon-Rot, Germany). Guidelines of the producing company were performed for this purpose. The purity (A260/A280) of extracted DNA was examined by the NanoDrop device (NanoDrop, Thermo Scientific, Waltham, MA, USA)[26-28]. The extracted DNA quality was examined using electrophoresis on 2% agarose gel[29-31.]

Detection of the methicillin-resistance encoding gene

The *mecA* gene in the MRSA isolates was examined using polymerase chain reaction (PCR). **Table I** shows the PCR conditions used for the detection of the *mecA* gene[32]. All PCR and electrophoresis ingredients were prepared commercially (Thermo Fisher Scientific, Germany). Eppendorf Mastercycler (Hamburg, Germany) device was applied in PCR. All runs included a negative DNA control of sterile PCR grade water (Thermo Fisher Scientific, Germany) and positive DNA control consisting of positive DNA of each target gene. Ten microliters of PCR products were examined by electrophoresis in a 2% agarose gel in 1× TBE buffer at 90 V for 30-40 min, stained with SYBR Green (Thermo Fisher Scientific, Germany). MRSA (BAA 2313) (Faculty of Veterinary Medicine, University of Tehran) and PCR-grade water (Thermo Fisher Scientific, Germany) were used as positive and negative controls, respectively.

Statistical analysis

Statistical analysis was done using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact two-tailed test were used to assess any significant relationship between the data achieved in this survey. P-value <0.05 was considered a significant statistical level[33, 34].

Results

The presence of the *mecA* gene was confirmed in all of the MRSA isolates (**Figure 1**).

Figure 1: PCR gel electrophoresis of the mecA gene in the MRSA isolates. M: Marker (100 bp, Thermo Fisher Scientific, Germany), 1: Negative control (PCR-grade water, Thermo Fisher Scientific, Germany), 2: Negative sample, 3-7: Positive samples for the mecA gene (532 bp), 8: Positive control (MRSA ATCC BAA2313).
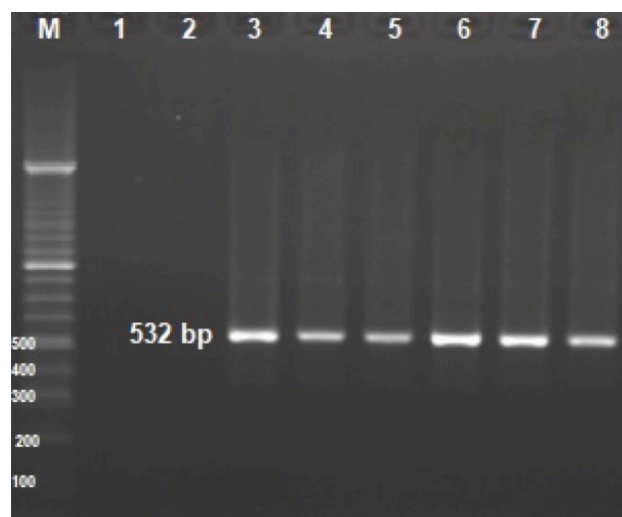

Table II shows MRSA bacteria's prevalence in diverse kinds of powdered packaged medicinal plant and bottle herbal distillate samples. Eighteen out of 300 (6.00%) powdered packaged medicinal plant and bottle herbal distillate samples were contaminated with MRSA. R. damascene powdered packaged medicinal plant (13.33%) and A. maurorum bottle herbal distillate (10%) had the highest prevalence of MRSA bacteria. Statistically significant difference was obtained for the prevalence of MRSA between powdered packaged medicinal plants and bottle herbal distillates (P <0.05).

Table III shows MRSA bacteria's antibiotic resistance pattern isolated from diverse powdered packaged medicinal plant and bottle herbal distillate samples. MRSA isolates showed the highest prevalence of resistance against trimethoprim-sulfamethoxazole (100%), cefixime (100%), ampicillin (100%), tetracycline (88.88%), erythromycin (88.88%), and rifampin (83.33%) antibiotic agents. The prevalence of resistance toward imipenem (27.77%) and doxycycline (44.44%) was lower than other examined antibiotic agents. A statistically significant difference was obtained between the type of samples and the prevalence of antibiotic resistance (*P* <0.05).

Table I: PCR conditions used for the detection of the mecA gene[32].

Target gene	Primer sequence (5'-3')	PCR product (bp)	PCR programs	PCR volume (50μL)
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	532	1 cycle: 94 °C ----------- 2 min. 30 cycles: 94 °C ----------- 30 s 55 °C ----------- 30 s 72 °C ----------- 30 s 1 cycle: 72 °C ----------- 4 min	5 μL PCR buffer 10X 2 mM MgCl$_2$ 150 μM dNTP 0.75 μM of each primers F & R 1.5 U Taq DNA polymerase 3 μL DNA template

Table II: Prevalence of MRSA bacteria in examined samples.

Types of samples		N. samples collected	N. samples contaminated with MRSA (%)	Distribution of mecA gene in MRSA isolates (%)
Medicinal plants	M. piperita	30	1 (3.33)	1 (100)
	Z. multiflora	30	1 (3.33)	1 (100)
	S. bachtiarica	30	1 (3.33)	1 (100)
	A. citrodora	30	1 (3.33)	1 (100)
	R. damascene	30	4 (13.33)	4 (100)
	Total	**150**	**8 (5.33)**	**8 (100)**
Herbal Distillates	L. angustifolia	30	2 (6.66)	2 (100)
	A. maurorum	30	3 (10)	3 (100)
	C. intybus	30	2 (6.66)	2 (100)
	M. officinalis	30	1 (3.33)	1 (100)
	F. officinalis	30	2 (6.66)	2 (100)
	Total	**150**	**10 (6.66)**	**10 (100)**
Total		**300**	**18 (6.00)**	**18 (100)**

Table II: Antibiotic resistance pattern of MRSA bacteria isolated from examined samples.

Types of samples (N. MRSA)		N. samples contaminated with MRSA (%)									
		Tet*	Dox	Tr-sol	Ert	Kan	Cef	C30	Rif	Imp	Am
Medicinal plants	M. piperita (1)	1 (100)	-	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	1 (100)
	Z. multiflora (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	S. bachtiarica (1)	1 (100)	-	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	1 (100)
	A. citrodora (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	1 (100)
	R. damascene (4)	3 (75)	2 (50)	4 (100)	3 (75)	3 (75)	4 (100)	2 (50)	2 (50)	2 (50)	4 (100)
	Total (8)	7 (85.50)	4 (50)	8 (100)	7 (85.50)	7 (85.50)	8 (100)	6 (75)	6 (75)	3 (37.50)	8 (100)
Herbal Distillates	L. angustifolia (2)	2 (100)	1 (50)	2 (100)	2 (100)	-	2 (100)	1 (50)	2 (100)	1 (50)	2 (100)
	A. maurorum (3)	2 (66.66)	1 (33.33)	3 (100)	2 (66.66)	2 (66.66)	3 (100)	1 (33.33)	2 (66.66)	1 (33.33)	3 (100)
	C. intybus (2)	2 (100)	1 (50)	2 (100)	2 (100)	-	2 (100)	1 (50)	2 (100)	-	2 (100)
	M. officinalis (1)	1 (100)	-	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	1 (100)
	F. officinalis (2)	2 (100)	1 (50)	2 (100)	2 (100)	-	2 (100)	1 (50)	2 (100)	1 (50)	2 (100)
	Total (10)	9 (90)	4 (40)	10 (100)	9 (90)	3 (30)	10 (100)	5 (50)	9 (90)	3 (30)	10 (100)
Total (18)		16 (88.88)	8 (44.44)	18 (100)	16 (88.88)	10 (55.55)	18 (100)	11 (61.11)	15 (83.33)	5 (27.77)	18 (100)

*Tet: tetracycline (30 μg/disk), Dox: doxycycline (30 μg/disk), Tr-sol: trimethoprim-sulfamethoxazole (1.25/23.75 μg/disk), Ert: erythromycin (15 μg/disk), Kan: kanamycin (30 μg/disk), Cef: cefixime (30 μg/disk), C30: chloramphenicol (30 μg/disk), Rif: rifampin (5 μg/disk), Imp: imipenem (10 μg/disk), Am: ampicillin (10 μg/disk).

Discussion

S. aureus and MRSA are bacteria that reside on the skin and respiratory tract, contamination of powdered packaged medicinal plant and bottle herbal distillate samples occurs unintentionally. Additionally, manipulating medicinal plants and herbal distillates during their processing in the manufacturing units is crucial for their contamination.

The present survey was performed to assess MRSA bacteria's prevalence and antimicrobial resistance isolated from powdered packaged medicinal plant and bottle herbal distillate samples. The prevalence of MRSA in examined samples was 6.00%. Isolates showed higher resistance toward trimethoprim-sulfamethoxazole, cefixime, ampicillin, tetracycline, erythromycin, and rifampin antibiotic agents. Some of the examined samples, such as *Z. multiflora* and *S. bachtiarica* medicinal plants and *M. officinalis*, and M. piperita herbal distillates, had a lower contamination *S. aureus* rate. One of the probable reasons for this finding is the high antimicrobial effects of *Z. multiflora, S. bachtiarica, M. officinalis, M. piperita*, and *C. intybus* against diverse kinds of bacteria[35-38]. Thus, S. aureus' growth and survival have been decreased and even stopped in these medicinal plants and their derived products.

S. aureus is most expected to originate from herbal product's contact with food handlers throughout harvesting, processing, and storage, and its absence reflects acceptable hygiene practices. Our findings also revealed that herbal distillates had a higher contamination rate with MRSA than medicinal plants. The probable reason

is maybe the extinction of MRSA bacteria during medicinal plants' drying process. Additionally, the processing of herbal distillates requires more human involvement and manipulation. Thus, MRSA bacteria's transmission from the infected staff and workers producing units to the herbal distillates may be another reason for MRSA's high prevalence in these samples. Despite the high importance of the topic, many limited surveys have been conducted in this field. A survey concocted by Sousa Lima et al. (2020)[39] disclosed that the prevalence of *S. aureus* bacteria amongst the homemade and commercial herbal medicine samples (*Lippia alba, Peumus boldus Molina, Cymbopogon citratus, Carapa guianensis, Copaifera langsdorffii, Stryphnodendron adstringens, Costus spicatus, and Arrabidaea chica*) was 88.50% and 23.50%, respectively. Kaume et al. (2012)[40] described that the prevalence of *S. aureus* amongst the medicinal plants marketed to patients who suffered from the HIV infection in Kenya was 71.40%, which was entirely higher than our findings. Esimone et al. (2007)[41] also reported that the prevalence of *S. aureus* amongst the medicinal plants sold in Nigeria was 8.70%. Similarly, a high contamination rate of herbal products with *S. aureus* and other Staphylococcal species has been reported previously from Bangladesh[42], Korea[43], Germany[44], Sudan[45], and Saudi Arabia[46]. Species of examined medicinal plants, methods of examination, the hygienic condition of producing factories, and their staff are essential factors that affected the prevalence of staphylococcal contamination in medicinal plants and herbal distillates.

Irregular and unauthorized prescription of antibiotics is the main reason for the high prevalence of antibiotic resistance in MRSA isolates. Ngemenya et al. (2019)[47] described that the *S. aureus* strains isolated from herbal remedies in Cameroon were resistant against five classes of examined antibiotic agents (amikacin, cefotaxime, cefuroxime, imipenem, trimethoprim, and ceftriaxone). Braide et al. (2013)[48] stated that the *S. aureus* bacteria isolated from herbal remedies were susceptible to ofloxacin, chloramphenicol, gentamicin, pefloxacin, ciprofloxacin, and erythromycin antibiotic agents. Presence of infectious agents other that MRSA has also been reported in different investigations conducted in Iran[49-54].

Conclusion

As it showed, medicinal plants and herbal distillates samples were potential sources of antibiotic-resistant-MRSA bacteria. It seems that their consumption may cause foodborne infections caused by MRSA bacteria, which pose an essential public health threat. The present survey is the first report of the prevalence of antibiotic-resistant-MRSA isolated from *Z. multiflora, S. bachtiarica, A. citrodora*, and *R. damascene* powdered packaged medicinal plants and *L. angustifolia, A. maurorum, C. intybus, M. officinalis, M. piperita* and *F. officinalis* bottle herbal distillates, globally. Bottle herbal distillates harbored a higher prevalence of MRSA isolates and also higher antibiotic resistance.

Acknowledgments

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