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Chemical Composition and Antimicrobial Effects of *Calendula officinalis* Grown Under Chemical and Biological Conditions on the Methicillin-resistant *Staphylococcus aureus* Isolated from Hospital Infections

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Application of various types of fertilizers can effect on biological activities of *Calendula officinalis*. The present investigation was aimed to study the chemical components and antimicrobial effects of *C. officinalis* grown under chemical and biological conditions on methicillin-resistant *Staphylococcus aureus*. Four hundred samples of hospital infections were collected and cultured. MRSA strains were subjected to the disk diffusion and GC-Mass. One-hundred out of 400 samples of hospital infections were positive for MRSA (25%). All isolates were also positive for *mecA* gene. Forty different chemical components were detected in the *C. officinalis*. The most variable components were found in the control group (1,8-cineole (30.456%), ã-terpinene (25.547%), terpinolene (4.584%), á-terpineol (4.490%) and trans-â-ocinene (4.153%)). Application of biologic and chemical fertilizers caused significant increase in the levels of some chemical components (P <0.05). MRSA strains harbored the highest levels of resistance against tetracycline (95%), ampicillin (92%), penicillin (90%), gentamycin (88%) and ciprofloxacin (77%). Control group had the highest antimicrobial effects but essential oil of the *C. officinalis* enriched with both fertilizers were effective on resistant MRSA. Use of *C. officinalis* growth under both chemical and biologic fertilizers has been recommended as a primary approach for synthesis of effective antibiotic.

Keywords: *Calendula officinalis*, Chemical components, Antimicrobial effects, Methicillin resistant *Staphylococcus aureus*, Biologic fertilizer, Chemical fertilizer.

Staphylococcus aureus (*S. aureus*) is a gram-positive coccal bacterium and a one of the most important causative agents of various types of hospital infections including systemic infections (such as urinary, respiratory and blood infections, pneumonia, sinusitis and food poisoning), as well as skin and soft-tissue infections (such as wounds, abscess, burns, furunclosis and impetigo),[1-3]. Both community-associated and hospital-acquired infections with *Staphylococcus aureus* (*S. aureus*) have increased in the past 20 years, and the rise in incidence has been accompanied by a rise in antibiotic-resistant strains—in particular, methicillin-resistant *S aureus* (MRSA)[4, 5]. MRSA strains have global significance regarding the high prevalence of infections in the cases hospitalized in hospitals[4, 5]. The gene for methicillin resistance, *mecA*, is carried on a 21- to 67-kb element which has an active presence in all infective strains of bacterium[6]. MRSA strains of clinical infections harbored the high levels of resistance against the extensive

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ranges of antibiotics including beta-lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins and other types of antimicrobial agents including erythromycin, cotrimoxazole, tetracycline, penicillin, gentamicin, cefexim and clindamycin[7-10]. According to the high prevalence of MRSA resistance against commonly used antibiotics in hospitals, the need for application and prescription of novel antimicrobial agents is essential.

In recent years, much attentions have been done to the prescription of medicinal plants for treatment of various types of infectious diseases[11]. Medicinal plants are a suitable sources of antimicrobial agents. *Calendula officinalis* (*C. officinalis*) is one of the most commonly used medicinal plants among Iranian people which is native to the Mediterranean regions[12]. *C. officinalis*, commonly known as pot marigold, is an annual herb and belongs to Asteraceae family. Flowers are monoecious (individual flowers are either male or female, but both sexes can be found on the same plant) and are pollinated by Bees. It is noted for attracting wildlife. *C. officinalis* can be broadly applied as an antiseptic, anti-inflammatory and cicatrizing as well as a light antibacterial and antiviral agent[11, 13-15]. The plant contains esquiterpenes glycosides, saponins, xanthophylls, triol triterpenes, flavonoids, volatiles, δ- cadinene, α-cadinol, 1,3,5-cadinatriene and α-muurolol which show anti-oxidative and antimicrobial effects[11, 13-16].

According to the high beneficial aspects of planting of *C. officinalis*, farmers have moved to use from various types of agricultural fertilizers to increasing the quality and quantity, controlling the pests and accelerate the harvesting time of plant. Application of these agricultural fertilizers and especially biologic and chemical (urea) manures can effects on the chemical composition and also therapeutic activities of essential oil extracted from *C. officinalis*. In some cases, significant changes have been occurred in the composition and performance of medicinal plants[17, 18].

According to the high prevalence of MRSA strains in Iranian cases of hospital infections, economic, cosmetic, and pharmaceutical values of *C. officinalis* and lack of published data on the influence of chemical and biologic fertilizers on components and antimicrobial activities of *C. officinalis*, the present study was carried out to evaluate the chemical components and antimicrobial effects of various treatments of *C. officinalis* on the MRSA strains isolated from various types of hospital infections in Iran.

MATERIALS AND METHODS

Ethical issues

The present study was accepted by the ethical committees of the Baqiyatallah University of Medical Sciences and Hajar Hospital, Iran. Written informed consent was obtained from all of the study patients or their parents. Written consent was also signed by the Research Adjutancy of the Islamic Azad University of Shahrekord (IAUSHK 110224) and ethical committees of the educational Hospitals, Tehran, Iran.

Samples collection and MRSA identification

From March to October 2015, overall 500 clinical samples from various types of infections were collected from hospitalized patients of Iranian hospitals and were immediately transferred to the laboratory in cooler with ice-packs.

Twenty-five microliters of each samples were inoculated on Mueller–Hinton broth (MHB, Merck, Germany) supplemented with 6.5% NaCl and homogenized. The suspension was incubated for 16–20 h at 37 °C. One milliliter of the enriched MHB media was added to 9 ml of phenol red mannitol broth containing ceftizoxime (5 ìg/ml) and aztreonam (75 ìg/ml) (PHMB) and incubated for 16–20 h at 37 °C. The surface of the selective isolation medium MRSA ID was inoculated with a sterile loop. The plates were incubated for 24 h at 37 °C (when the colonies were difficult to identify the incubation was protracted for another 24 h). Typical green colonies were primary known as MRSA. Five selected typical colonies per plate were subcultured on Tryptone Soya Agar (TSA, Merck, Germany). Typical colonies were tested with the Staphytect Plus test (Oxoid), a latex agglutination test for the detection of clumping factor, Protein A and certain polysaccharides found in MRSA.

PCR confirmation of MRSA

All of the MRSA strains were cultured on Tripticase Soy Broth (TSB) and were incubated at

37 °C for 18-24 h. Genomic DNA was extracted from bacterial colonies using the DNA extraction and purification kit (Cinagen, Iran) according to the manufacture instruction. Presence of MRSA strains were confirmed using the PCR-based amplification of *mecA* gene[19]. Reaction was performed in a final volume of 50 µL containing 5 µL 10 × buffer + $MgCl_2$, 2 mM dNTP, 2 unit Taq DNA polymerase, 100 ng genomic DNA as a template, and 25 picomole of each primer (5'-AAATCAGATGGTAAAGGTTGGC-3' and 5'-AGTTCTGCAGTACCGGATTTGC-3') (533 bp). PCR was performed using a thermal cycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) under the following conditions: an initial denaturation for 1 minutes at 94°C and 40 cycles including 95°C for 30 s, 55 °C for 30 s and 72 °C 1 min, and a final extension at 72°C for 5 minutes. *S. aureus* ATCC 6538 was used as a positive control and distilled water was used as a negative control. Fifteen microliters of PCR products in all reactions were resolved on a 1.5% agarose gel containing 0.5 mg/ml of SYBR Green in Tris–borate–EDTA buffer at 90 V for 1 h, also using suitable molecular weight markers. The products were examined under ultraviolet illumination.

Treatments of *Calendula officinalis*

Various treatments of *C. officinalis* were produced on the farm. After planting plants in the same conditions, the rebellion began to different treatments. The first treatment of *C. officinalis* was treated with the chemical fertilizer which contain urea. The second treatment was treated using biologic fertilizer. The third treatment was treated with both urea and biologic fertilizers. The control group was growth in a routine condition without any fertilizer. All other conditions including process of irrigation, lighting, soil type, temperature and humidity were similar between treatments. After growing, the *C. officinalis* flowers were collected and immediately transferred to the Medicinal and Aromatic Research Center of the Islamic Azad University of Shahrekord.

Extraction of essential oil

Five-hundred grams of fresh flowers of each treatment were hydro distilled separately for 3 h in an all-glass Clevenger apparatus in accordance with the British pharmacopoeia method[20].

GC-mass analysis

In order to study the chemical compositions of 4 different treatments of *C. officinalis* flowers, the GC-mass analysis method was used using an Agilent 6890 Series II gas chromatograph (Palo Alto, USA) coupled to an Agilent 5973 quadrupole mass spectrometer with electron ionization mode (EI) generated at 70 eV (ion source at 230 °C and transfer line at 280 °C). The GC was performed using a J&W DB-5 (5% diphenyl- 95% dimethyl silicone) capillary column (30 m x 0.25 mm i.d. x 0.25 µm film), and helium was used as a carrier gas (1 mL min-1). The initial temperature was programmed from 35 °C to 60 °C (at 1 °C min-1), to 170 °C (3 °C min-1), to 200 °C (8 °C min-1), and to 280 °C (15 °C min-1), and maintained at 280 °C for 5 min. The injector port (splitless mode, 0.5 min) was at 250 °C. Retention indexes were calculated with reference to nalkanes. All compounds were identified by comparison of both the mass spectra (Wiley 275 library) and the retention index data found in the literature[21].

Antimicrobial effects of various treatments of the Calendula officinalis

Agar disc diffusion method was used for screening of antibacterial activity of *C. officinalis* flowers[14]. MRSA isolates of human hospital infections were spread on to Nutrient Agar (NA, Merck, Germany) medium. Paper discs were separately impregnated with 25µl of the 0.5 mg/mL plant essential oil and placed on the inoculated agar plates. All the plates were allowed to stay at room temperature for 30 min to allow diffusion of the essential oil then incubated at 37 ºC for 24 hrs. Susceptibility of MRSA isolates against the essential oil of *C. officinalis* flowers were also compared with the commercially antimicrobial disks. Susceptibility of MRSA isolates were tested against ampicillin (10 u/disk), gentamycin (10 µg/disk), penicillin (10 u/disk), cotrimoxazole (30 µg/disk), lincomycin (2 µg/disk), ciprofloxacin (5 µg/disk), clindamycin (2 µg/disk), imipenem (30 u/disk), tetracycline (30 µg/disk), cefexime (5 µg/disk) and azithromycin (15 µg/disk) antibiotic agents (Oxoid, UK). The diameter of the zone of inhibition produced by essential oil and also each antibiotic disc was measured and interpreted based on the protocol of the Clinical and Laboratory Standards Institute (CLSI)[22]. *S. aureus* ATCC 25923 was used as quality control organism in antimicrobial

susceptibility determination.

Statistical analysis

Antimicrobial effects of the *C. officinalis* flowers and each antimicrobial agent were tested 3 times. Results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. Statistical analysis was performed using SPSS/19.0 software (SPSS Inc., Chicago, IL) for significant relationship between the antibiotic susceptibility of MRSA strains of hospital infections. The chi-square test and Fisher's exact 2-tailed test analysis were performed in this study. Statistical significance was regarded at a P value < 0.05.

RESULTS AND DISCUSSION

Of 400 samples of hospital infections collected from various types of hospital infection samples of Iranian health centers and hospitals, 100 samples (25%) were positive for presence of MRSA. Presence of the *mecA* gene was identified in all of the MRSA isolates of the hospital infections using the PCR method. Figure 1 shows the results of the gel electrophoresis for amplification of the *mecA* gene.

Frequency of chemical components in various treatments of *C. officinalis* essential oil is shown in table 1. Totally, 40 different chemical components were detected in the essential oil of the *C. officinalis* flowers. Control group of *C. officinalis* harbored the most variable chemical components. Totally, 1,8-cineole (30.456%), γ-Terpinene (25.547%), Terpinolene (4.584%), α-Terpineol (4.490%) and Trans-β-ocinene (4.153%) were the most commonly detected components in the control group. Increase in the levels of 1,8-cineole, trans-β-ocinene, α-copaene, α-bourbonene, α-Terpinyl acetate, α-Bisabolene, spathulenol, α-cadinol, γ-Cadinene, α-cadinene and α-Bisabolol in the *C. officinalis* group treated with urea manure, 1,8-cineole, trans-β-ocinene, α-copaene, α-Terpinyl acetate, α-muurolene, spathulenol, α-Cadinene, α-cadinene and T-muurolol in the group treated with biologic manure and finally 1,8-cineole, Trans-β-ocinene, α-copaene, α-Terpinyl acetate, â-Bisabolene, spathulenol, α-Eudesmol, α-Cadinol, ã-cadinene, γ-cadinene and α-Bisabolol in the group treated with both urea and biologic manures were significant ($P < 0.05$).

Table 2 represents the antibiotic susceptibility pattern of MRSA strains isolated from hospital infections against various treatments of the *C. officinalis* essential oil. We found that the control group had the highest antimicrobial effects on the MRSA strains of human clinical samples, followed by treatments No 3 and 2. Treatment No 1 had the lowest antimicrobial effects. In the other hand, the prevalence of susceptibility of MRSA against the control group, treatments No 3, 2 and 1 were 45%, 30%, 24% and 18%, respectively ($P <0.05$). MRSA strains harbored the highest levels of resistance against treatment No 1 (41%), followed by treatments No 2 (40%) and 1 (36%). Statistically significant difference was seen between the types of *C. officinalis* and prevalence of resistance ($P <0.05$).

Table 3 shows the antibiotic susceptibility pattern of MRSA strains isolated from hospital infections against commonly used antibiotics. We found that the MRSA strains harbored the highest levels of resistance against tetracycline (95%), ampicillin (92%), penicillin (90%), gentamycin (88%) and ciprofloxacin (77%). MRSA strains harbored the highest levels of susceptibility against imipenem (95%), azithromycin (76%), cotrimoxazole (66%), clindamycin (65%) and lincomycin (59%). Significant difference was also seen between the types of antibiotic disk and pattern of antibiotic resistance ($P <0.05$). Antibiotic susceptibility of the treatment No 3 of the *C. officinalis* was entirely higher than tetracycline, ampicillin, penicillin, gentamycin, cefexime and ciprofloxacin ($P <0.05$).

As far as we know, the present investigation is the first prevalence report of chenmical composition and antimicrobial effects of various treatments of the *C. officinalis* on the MRSA strains isolates from various type of hospital infections. As said, 25% of samples were positive for MRSA which was substantially high. Extraordinary, lopsided and indiscriminate prescription of methicillin and also other types of antimicrobial agents are the main reasons for the high prevalence of MRSA strains in clinical infection samples. Contamination of the hospital environment and lack of personal hygiene especially in patients who were carrier of MRSA strains is another reason for the high prevalence of MRSA. Deficiency of appropriate sanitizer

maybe the other reason.

MRSA strains of our research harbored the highest levels of resistance against tetracycline, ampicillin, penicillin, gentamycin, cefexime and ciprofloxacin antibiotics which was similar to the results of various previously published articles[23-29]. All of these researches have suggested the synthesis, formulation and application of novel antimicrobial agents to overcome occurrence of high antibiotic resistance in the MRSA strain of human and even animal clinical samples.

We found that enrichment of the agricultural soils with chemical and biologic

Table 1. Frequency of chemical composition of various treatments of *Calendula officinalis* essences

Number	Chemical components	Distribution in various treatments (%)			
		Control*	Treatment 1	Treatment 2	Treatment 3
1	α-Thujene	0.459	-	-	-
2	α-Pinene	3.032	-	0.553	
3	Camphene	0.256	-	-	-
4	Sabinene	0.293	-	-	-
5	P-Pinene	0.490	0.784	-	-
6	1-Octen-3-ol	0.291	-	-	-
7	1,8-cineole	30.456	53.755	43.512	50.431
8	p-cymene	2.495	-	-	-
9	α-Terpinene	0.283	-	-	-
10	β-caryophyllene	1.289	-	-	-
11	Trans-β-ocinene	4.153	7.61	11.562	15.834
12	Benzene acetaldehyde	1.354	-	-	-
13	γ-Terpinene	25.547	-	-	-
14	cis-Sabinene hydrate	2.928	3.319	2.319	1.863
15	Terpinolene	4.584	1.044	0.954	0.753
16	α-phellandrene	0.390	-	-	-
17	α-terpineol	0.676	0.403	1.345	0.945
18	Carvacrol	3.146	1.343	1.060	0.877
19	Terpinene-4-ol	0.369	-	-	-
20	α-Terpineol	4.490	1.493	1.263	0.967
21	n-Dodecane	0.279	-	-	-
22	Carvacrol methy ether	0.301	-	0.412	0.287
23	α-copaene	0.318	3.425	5.759	6.996
24	α-bourbonene	0.510	1.598	1.645	2.035
25	α-Terpinyl acetate	0.520	1.899	2.321	3.413
26	Eugenol	0.676	0.392	0.216	0.02
27	n-Tetradecane	3.146	1.343	1.060	0.895
28	)E-(Caryophyllene	0.369	-	-	-
29	α-muurolene	2.805	2.327	4.359	3.125
30	β-Bisabolene	0.622	2.880	2.985	5.612
31	)E-(γ-Bisabolene	0.301	-	0.412	1.534
32	Spathulenol	0.318	1.529	5.579	4.525
33	β-Eudesmol	0.510	1.598	1.765	3.456
34	α-Cadinol	0.520	1.899	2.078	2.684
35	γ-Cadinene	3.319	5.652	12.345	15.675
36	Cadina 1,4-diene	0.950	1.044	1.887	1.345
37	α-cadinene	1.434	3.714	7.342	9.823
38	α-Bisabolol	0.024	0.452	0.793	1.372
39	α-cadinol	0.616	0.845	2.564	1.231
40	T-muurolol	0.324	1.178	2.422	4.102

Table 2. Antibiotic susceptibility pattern of MRSA strains isolated from hospital infections against various treatments of the *Calendula officinalis* methanol extracts

Study groups	Susceptibility pattern of 100 isolated strains of MRSA (%)		
	Susceptible	Intermediate	Resistant
Control	45 (45)	30 (30)	25 (25)
Treatment 1	18 (18)	41 (41)	41 (41)
Treatment 2	24 (24)	36 (36)	40 (40)
Treatment 3	30 (30)	34 (34)	36 (36)

Table 3. Antibiotic susceptibility pattern of MRSA strains isolated from hospital infections against commonly used antibiotics

Antibiotic agents	Susceptibility pattern of 100 isolated strains of MRSA (%)		
	Susceptible	Intermediate	Resistant
Ampicillin	3 (3)	5 (5)	92 (92)
Gentamycin	3 (3)	9 (9)	88 (88)
Penicillin	4 (4)	6 (6)	90 (90)
Cotrimoxazole	66 (66)	22 (22)	12 (12)
Lincomycin	59 (59)	26 (26)	15 (15)
Ciprofloxacin	10 (10)	13 (13)	77 (77)
Clindamycin	65 (65)	23 (23)	12 (12)
Imipenem	95 (95)	4 (4)	1 (1)
Tetracycline	1 (1)	4 (4)	95 (95)
Cefexime	12 (12)	16 (16)	62 (62)
Azithromycin	76 (76)	14 (14)	10 (10)

fertilizers can change chemical components of essential oil extracted from them. However, the variety of chemical components and even percent of some of them in the control group which was growth in the normal condition were higher than those of chemical, biological and both groups but the levels of some specific components were increased. Totally, 1,8-cineole, â-bisabolene, trans-â-ocinene, á-copaene, spathulenol, á-bourbonene, á-terpinyl acetate, T-muurolol, á-muurolene, á-cadinol, ã-cadinene, á-cadinene, â-eudesmol, and á-bisabolol chemical components had been increased in the essential oils of treatment groups compared with control. However, the antimicrobial effects of the control group were higher than other treatments but we found that antibiotic resistant strains of MRSA had the higher susceptibility to treatment No 3 than control group (P <0.05). In keeping with this, the antimicrobial effects of control group were higher than treatments No 2 and 1 (P <0.05). It seems that application of both biologic and chemical fertilizers can improve the antimicrobial effects of *C. officinalis* against MRSA.

In a study which was conducted by Davary Panah and Farahvash (2014)[30] the effects of biological and chemical fertilizers were significant (P <0.01 and P <0.05) on all of parameters of *C. officinalis* (plant height, stem diameter, essential oil, total fresh weight and total dry weight). The y showed the highest amount of essential oil was achieved in the group enriched with both nitroxine fertilizer and urea fertilizers which was similar to our findings. Rafiee *et al.* (2013)[31], Rahmani et al. (2009)[32], Bieski et al. (2013)[33], Jevdovic et al. (2013)[34] and Arab et al. (2015)[35] reported similar results. Unfortunately, there were no published data on the effects of fertilizers on the chemical components of *C. officinalis* essential oil.

In a study which was conducted by Efstratiou et al. (2012)[14], results showed that the *C. officinalis* extracts represented exceptional antibacterial activity against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella aerogenes*, *Enterococcus faecalis* and *Klebsiella pneumonia*. Chakraborthy (2008) [36] reported that the lowest Minimum Inhibitory Concentration (MIC) values of *C. officinalis* were observed for ethanol extract, chloroform extract, water extract and petroleumether extract against the bacteria. They showed that the extracts of *C. officinalis* leaves were significantly effective against both Gram-positive and Gram-negative organisms. Similar studies about the high antimicrobial effects of the *C. officinalis* on pathogenic bacteria have been reported previously by Faria et al. (2011) [37] (Brazil), Bissa and Bohra (2011) [38] (India) and Rigane et al. (2013) [39] (Tunisia). High antimicrobial effects of the *C. officinalis* is due to its antimicrobial chemical components. Recent study revealed that triterpenoid like calendulaglycoside, triterpenoid saponin like faradiol, asorhamnetin3-O-neohesperidoside, quercetin and isorhamnetin are the main chemical components of the *C. officinalis* which are responsible for antioxidative, anti-cancer, antimicrobial, anti-inflammatory and wound healing effects[40]. In fact, application of biologic and chemical fertilizers caused to increase in the levels of these chemical components of *C. officinalis*. Other researchers showed that the mode of antimicrobial action of the *C. officinalis* may be related to its ability to inactivate microbial enzymes, inhibition of DNA gyrase, inhibit cytoplasmic membrane function[41, 42].

Results of the documented reports revealed that the main compounds within Calendula are the triterpenoids[43, 44] which are claimed to be the most important anti-inflammatory and antimicrobial components within the plant. Other constituents identified in Calendula such as the saponins, micronutrients, flavonoids, and polysaccharides, may also be responsible for the antimicrobial, anti-inflammatory, antioxidant, and wound healing effect of the plant[43-45]. The antimicrobial activity of essential oil of C. officinalis is attributed to its main chemical components including citral (aldehyde), geraniol (primary alcohol), eugenol (phenol), menthol (secondary alcohol) and cinnamic aldehyde (aldehyde)[46]. Compounds such as linalool, citral, geraniol, or thymol are more antiseptic agents in the essential oil of the *C. officinalis*[47].

In conclusion, the results of our survey revealed that enrichment of agricultural soil with biologic and chemical fertilizers can improve the medicinal and especially antimicrobial effects of *C. officinalis*. As it showed, MRSA had the high prevalence and also antibiotic resistance in Iran. In addition, enrichment of *C. officinalis* with both biologic and chemical fertilizers had the highest effects on the antimicrobial activities of extracted essential oil on the MRSA. Simultaneous application of both types of fertilizers improved the percent of some important chemical components. MRSA strains which had a high levels of resistance against variety of tested antibiotics (tetracycline, ampicillin, penicillin, gentamycin, ciprofloxacin imipenem, azithromycin, cotrimoxazole, clindamycin and lincomycin), were susceptible against the essential oil extracted from the *C. officinalis* enriched with both types of fertilizers. We recommended the use of *C. officinalis* grown in soil enriched with both chemical and biologic fertilizers as a primary approach for synthesis and formulation of novel antibiotic drug for treatment of resistant strains of MRSA in hospital. In keeping with this, thoughtful

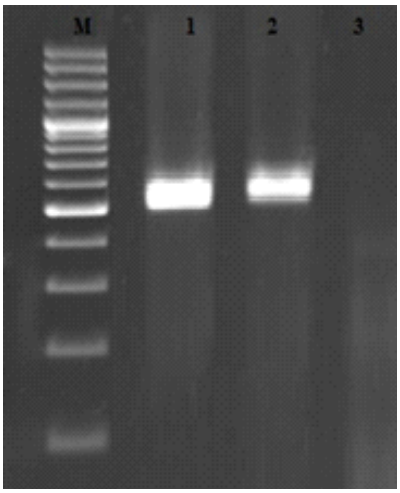

Fig. 1. Gel electrophoresis of PCR products for *mecA* gene of the MRSA strains isolated from various types of hospital infections. M: 100 bp ladder, 2: Positive sample for the *mecA* gene (533 bp), 2: Positive control and 3: Negative control

prescription of antibiotics is another required option to prevent from extension of MRSA strains.

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