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Research Article

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Alternative treatment of methicillin-resistant *Staphylococcus aureus* and extended spectrum beta-lactamases producing multiresistant gram-negative bacteria from nosocomial infection by *Marrubium vulgare* methanolic compounds

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ABSTRACT

The emergence of the multiresistant bacteria responsible of nosocomial infections (NI) represented a real public health problem. These germs are the pathogenic opportunist ones such as the Methicillin-Resistant Staphylococcus aureus (MRSA) and Extended Spectrum Beta-Lactamases producing Gram-negative bacteria(ESBLpGN). The aim is to determine the effect of methanolic extract from the leaves of Marrubium vulgare against two Methicillin-Resistant Staphylococcus aureus (MRSA) and five Extended Spectrum Beta-Lactamases producing Gram-negative bacteria(ESBLpGN) (E.coli, P.vulgaris, k. pneumonia, Ps. aeruginosa and Ac baumanii) isolated from Urinary Nosocomial Infection and Sirgucal Site Infection, which was tested by both agar disc diffusion and microdilution methods. Marrubium vulgare extract more significantly inhibited the growth of Gram-positive (MRSA) from SSI than Extended Spectrum Beta-Lactamases producing Gram-negative bacteria (ESBL) from UIN. This study showed that Marrubium vulgare extract has a potent antibacterial activity against multiresistant pathogenic bacteria MRSA and ESBL producing Gram-negative responsible of NI. Further studies will be required to evaluate the practical values of therapeutic applications.

Keywords: Antibioresistance, ESBLpGN, *Marrubium vulgare*, Methanolic extract, MRSA

INTRODUCTION

The multiresistance among prevalent pathogens is increasing at an alarming rate, leading to greater patient morbidity and mortality from nosocomial infections. Among gram-positive organisms, a prevalent most resistant pathogens are methicillin – resistant *Staphylococcus aureus*, its pathogenicity , ubiquitous characteristic, and absence of nutritional requirements qualify this bacterium as an example of adaptation and dissemination[1] , especially when the skin barrier is ruptured. This adaptation also affects its aptitude to resist to several antibiotic agents, such as methicillin, which extended to resist against most β-lactams and currently reach vancomycin considered until now as one of the last available antibiotic in this case. As regards bacteria Gram-negative, the important causes of resistance include extended-spectrum β-lactamases (ESBLs) in *Klebsiella pneumonia*, *E.coli*, *Proteus vulgaris* and multi-drug resistance genes observed in *Pseudomonas aeruginosa*, *Acinobacter baumanii*. Herbal medicine is considered a soft medicine. The interest in plants with antimicrobial properties has been revived because of the current problems associated with the use of antibiotics [2]. Recently a large number of extracts of plant have been investigated for their biological activity, notably antibacterial properties, they gain increasing interest as a natural alternative to synthetic drugs particularly against microbial agent and for their potential use as alternative remedies for the treatment of many infections. *Marrubium vulgare* is an aromatic plant, which is commonly known as "Marriouet" in Algeria, or "horehound" in Europe, it's naturalized in North and South America, the Mediterranean district and

Western Asia. In Algeria herbal medicine, *Marrubium vulgare* has been known as a traditional therapy as tonic, aromatic, stimulant, expectorant, diaphoretic and diuretic properties. It was formerly much esteemed in various uterine, visceral and hepatic affections and in phthisis[3]. The plant is reported to possess hypoglycemic [4], antihypertensive [5], antiinflamatory [6], antioxidant activity [7] and many other reported biological activities. The aim of the present study was carried out to evaluate the antibacterial activity of the methanolic extract of *Marrubium vulgare*, against multiresistant bacteria, methicillin –resistant *Staphylococcus aureus* and Extended Spectrum Beta-Lactamases producing Gram-negative, the causative agents of Nosocomial Infection in the surgical ward.

EXPERIMENTAL SECTION

2.1. Plant materiel

Fresh *Marrubium vulgare* leaves were collected during the florescence phase from March to April 2009 in the region of Mascara. This plant was identified according to the African Flowering Plants Database. The plant material was identified by a local expert and a voucher specimen (0309) was deposited in the herbarium center of the department of Biology, Mascara University for future reference. Fresh aerial parts (leaves) were washed and dried at room temperature for 2 weeks according to the standard procedures. They were then ground into powder in a ball mill.

2.2. Preparation of methanolic extract

The extraction was done by cold maceration of fine powder (20 g) in 200 ml of methanol. The mixture was agitated for 30 min [8], and then maintained at rest for 24 h. The resulting extract was sterilized by filtration, the solvent completely removed using a rotary evaporator and the extract stored at +4°C until further use. Before testing, the methanolic extract was freshly reconstituted in methanol at a final concentration of 120 mg/ml which was used to further preparation of serial dilutions from 200 mg/ml to 25 mg/ml. preliminary studies were undertaken in our laboratory on the determination of the choice of different concentrations.

2.3. Total Flavonoid Content

The total of flavonoid content was determined by using the colorimetric assay according to [9]. A calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CE)/100 g dried plant. Briefly, an aliquot of 1 ml of sample was added to an equal volume of solution of 2% $AlCl_3$, 6 H_2O, mixed evenly and allowed to stand at room temperature for 10 min. The absorbance was then read at 430 nm.

2.4. Determination of the antimicrobial activity

2.4.1. Microbial strains

Multiresistant microbial strains isolated from UI and SSI were selected for the antimicrobial activity. Seven strains were used: two Gram- positive bacteria, *Staphylococcus aureus* methicillin-resistant (MRSA). Five Extended Spectrum Beta-Lactamase producing Gram-negative bacteria namely, *Escherichia coli* , *Klebsiella pneumoniae* , *Proteus vulgaris* (Multiresistant Enterobacteriace), *Pseudomonas aeruginosa* and *Acinetobacter baumanii*.

2.4.2. Antimicrobial activity assay

Antimicrobial activity was determined by the agar disc diffusion assay [10]. Inoculm for the assays were prepared by diluting scraped cell mass in 0.85% NaCl sterile solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometric reading at 580 nm. Cell suspensions were finally diluted to 10^6 CFU /ml. The extracts were dissolved in dimethyl sulfoxide (DMSO) or distilled water. Petri plates were prepared with 20 ml of sterile Mueller Hinton agar (Sigma, Paris, France) surface inoculate by suspension of cell (200 µl). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10min. The tests were conducted at different concentrations of the sterile methanolic extract of *Marrubium vulgare*. (200, 100, 50 and 25 mg/ml) in Sterile filter paper discs (6 mm). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated at 37C for 24 h. Gentamicin (15µg) and cefotaxim (30µg) were used as positive controls. Negative controls were performed using paper discs loaded with 20 µl of the aqueous DMSO. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. After that, the inhibition zones were measured in millimeters by Vernier calipers. All tests were repeated two times to minimize test error. An inhibition zone of 14 mm or greater (Including diameter of the disc) was considered as high antibacterial activity [11].

2.4.3. Determination of Minimum Inhibitory Concentration (MIC) by microdilution

The minimal inhibitory concentration (MIC) values for bacterial growth were determined by a serial dilution technique using 96-well microtiter plates [12]. Amount of substance used in MIC determination was calculated after evaporating the solvent of 1 ml of extract and then solubilizing the dry extract in 20% v/v DMSO. The solution was subsequently diluted for 10-fold with Mueller Hinton broth. One hundred microliter from broth bacterium or yeast solutions and dilutions were transferred into microtitration plates and incubated for 24 h at 37°C. The positive control contained 100 µl of bacterium solution plus 100 µl Mueller Hinton broth. Negative control contained only 100 µl dilute plus 100 µl of extract without bacteria was evaluated according to turbidity occurred after 24 h by comparing to the control well. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, which is a well clear. All experiments were made in duplicates.

2.4.4. Statistical analysis

Values are given as arithmetic means ± standard error of the mean. Data were statistically analyzed by using one-way analysis of variance (STAVIEW version 5.0, Abacus Concepts, Berkeley, CA), for in vitro antimicrobial activity, we consider Log CFU≤ Log1 as significant. P values <0.05 were considered significant.

RESULTS AND DISCUSSION

3.1. Total flavonoid content

The yield of methanolic extract of *Marrubium vulgare* was about 26.196 % ± 1.31. The total flavonoid content from the methanolic extract of *Marrubium vulgare* was of 27.69 mg CE/100gr. Different studies were undertaken on the determination of the content of total flavonoid compounds. Study of Bouterfes et al., (2014) revealed that content of total extractable flavonoid (TEF) from *Marrubium vulgare* leaves collected from Tessala region of Algeria was about 32.5 % [13] .

3.3. Antimicrobial activities

3.3.1. Susceptibility test

The results of the antimicrobial activity of methanolic extracts of *Marrubium vulgare* leaves are given in Table 1.

Table1: Antibacterial activity profile of methanolic extracts of *Marrubium vulgare* leaves (expressed as diameter of inhibition zone in mm)

Strain	Inhibition zone (mm)						
	Methanolic extract (mg/ml)					PC	
	NC	200	100	50	25	GM (15µg)	CTX (30µg)
SARM1	0,0±0,0	15±0,01	13±0,06	8,5±0,01	7,5±0,04	26±0,1	----
SARM2	0,0±0,0	14±0,2	12±0,04	9,0±0,02	4,0±0,1	20±0,2	----
Pseudomonas aeruginosa	0,0±0,0	13±0,05	9,0±0,02	7,5±0,4	7,0±0,2	----	15±0,01
Acinetobacter baumanii	0,0±0,0	14±0,04	11±0,01	9,5±0,3	8,5±0,3	----	18±0,3
Escherichia coli	0,0±0,0	12±0,1	9,0±0,05	7,5±0,02	6,0±0,01	----	13±0,1
Proteus vulgaris	0,0±0,0	12±0,06	9,0±0,03	8,0±0,01	4,0±0,05	----	15±0,2
Klebsiella pneumonia	0,0±0,0	11±0,07	8,0±0,08	7,0±0,2	6,5±0,02	----	16±0,1

Abbr.-NC-negative control, PC-positive control, GM- gentamycin, CTX- cefotaxim, -----not determined

The methanolic extract showed a varying degree of antimicrobial activity against bacteria. The 100mg/ml of methanolic extract of *Marrubium vulgare* was the most effective on *Staphylococcus aureus, Acinetobacter baumanii.* The 200mg/ml of methanolic extract of *Marrubium vulgare* had an inhibitory effect on the growth of *Staphylococcus aureus* methicillin-resistant(MRSA), *Klebsiella pneumonia, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Acinetobacter baumanii.* Overall, the methanolic extract exhibited a stronger antibacterial activity against *Staphylococcus aureus* methicillin-resistant (MRSA) (7.5–15 mm), *Acinetobacter baumanii* (8.5–14mm) and *Pseudomonas aeruginosa* (07–13 mm) than Multiresistant Enterobacteriaceae Extended Spectrum Beta-Lactamase producing (04–10 mm) and . This difference is probably due to a difference in the chemical composition of the bacterial cell-wall. Al-Bayati and Sulaiman (2008) specify that the antibacterial effect can also be due to various chemical substances contained in the extract [14]. Well-standardized studies are needed to identify which components of the extract exert an antibacterial effect against Gram positive and Gram negative bacteria. According to Kanyonga et al., (2011) methanolic extract of *M.vulgare* was very much effective against *B.subtilis, S.epidermidis and S.aureus* (Gram-positive bacteria) and a moderate affective against *P.vulgaris* and *E.coli* while ineffective in case of *P.aeruginosa* [15].

3.3.2. Minimum inhibitory concentration (MIC)

The methanolic extract either inhibited or enhanced the growth depending on which test strain was used (Figure 1). Generally, the methanolic extract was active against the total nosocomial bacteria isolated and identified from SSI and UNI.

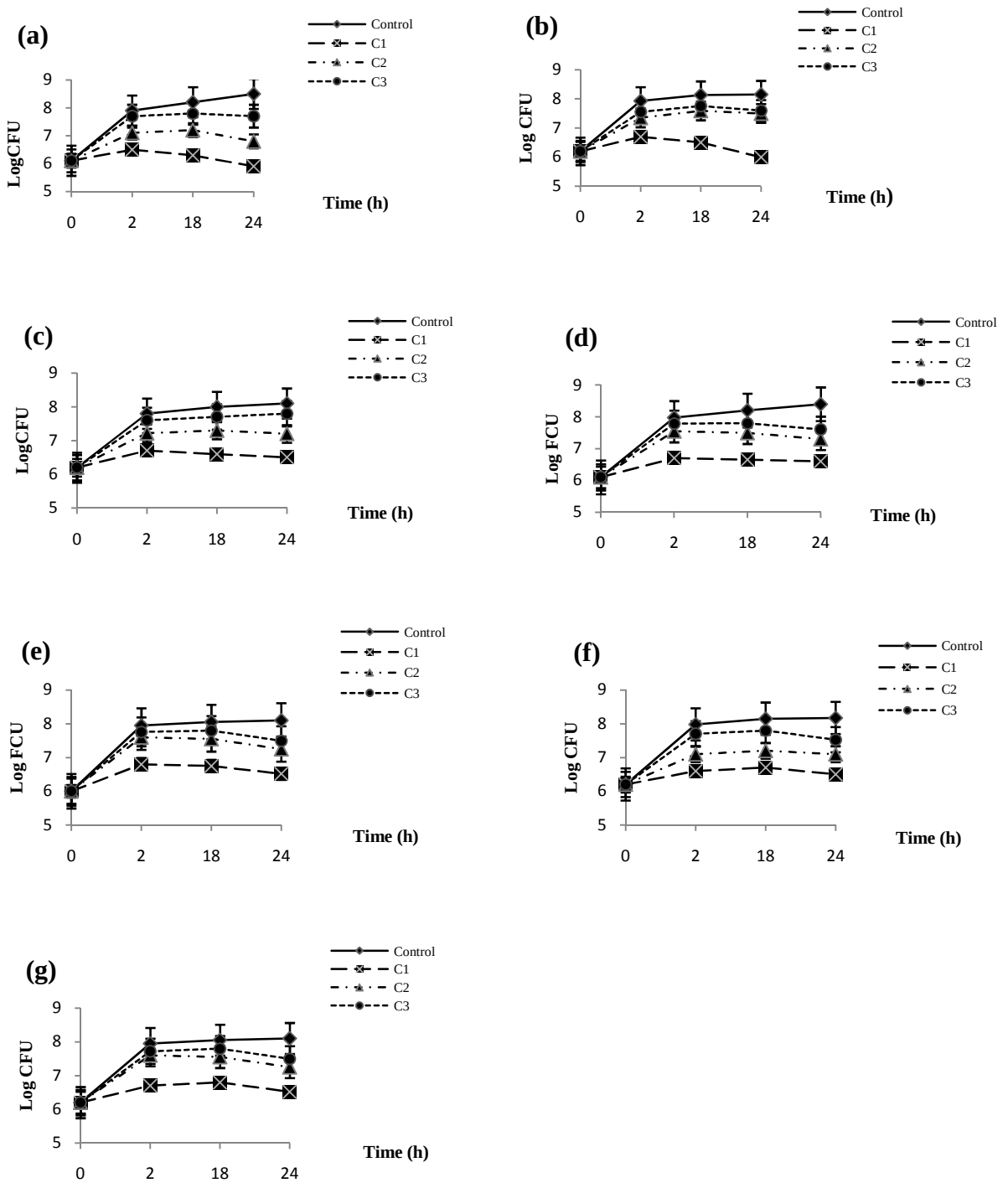

Figure 1: Antibacterial effect of methanolic extract of *Marrubium vulgare* leaves on multiresistants strains from NI.
C1: 200mg/ml; C2:100mg/ml; C3:50mg/ml. (a) MRSA1. (b) MRSA2. (c) *Klebsiella pneumonia.* (d) *Proteus vulgaris.* (e) *Escherichia coli.* (f) *Pseudomonas aeruginosa.* (g) *Acinetobacter baumanii.*

The MIC was 100mg/ml for MRSA (1+2), a significant reduction in growth was revealed after 18 h of incubation. *Acinetobacter baumanii* were also susceptible to this concentration of the extract. However the concentration of (200 mg/ml) methanolic extract was more active than the others against *E.coli, Klebsiella pneumonia, Proteus vulgaris* and *Pseudomonas aeruginosa.* This result disagrees with a previous report, much showed that *Marrubium vulgare* had no antibacterial activity against *Pseudomonas aeruginosa* [16], which may be due to different types between strains, isolation and different methods used. Our results suggest that the antibacterial effect was more remarkably pronounced on Gram positive than Gram negative. Our findings are in accordance with other studies [17,13]. In this case, the activity of the antimicrobial agent depends on the physiological state of bacteria, bacteria in

the exponential phase of growth being more sensitive. In addition, Al-Bayati and Sulaiman (2008) specify that bacterial resistance can be due to a modification of the target site, bypass of pathways, decreased uptake (reduction of intracellular concentration of antimicrobial agents, either through reduction of membrane permeability or by an active efflux pump) [14]. Kahlouche-Riachi et al., (2012) affirm that the methanolic extract of *Marrubium vulgare* exerts at a concentration of 25 mg/ml a significant effect on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris,* and specify that the sensitivity of the flowering tops of *M.vulgare* against *Proteus vulgaris* and *E.coli* can be due to the presence of terpenoids reported from this plant [18]. This observed effect could be attributed to the high content of tannin [19]. In addition Kanyonga et al., (2011) demonstrated that the methanolic extract of *Marrubium vulgare* exhibited significant antibacterial activity at Higher dose: 200, 400 and 600 mg/ml against *Staphylococcus aureus* and moderately effective against *P.vulgaris* and *E.coli.* While ineffective in case of *Ps.aeruginosa* [15].

CONCLUSION

The rather alarming emergence of MRSA and ESBLpGNB raises a therapeutic problem with the absence of new molecules and the diversity of resistance mechanisms of bacteria. The results of the present research demonstrated that *Marrubium vulgare* leaves methanolic extract can affect the growth of MRSA and ESBLpGNB isolated from UNI and SSI. We suggest that the extracts of *M.vulgare* may be a promising alternative for the treatment of NI. However extraction and purification of bioactive compounds produced, essentially, an effect against antibiotic-resistant strains as MRSA and ESBLpGNB.

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