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SUSCEPTIBILITY OF EXTENDED SPECTRUM B-LACTAMASE PRODUCER METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* TO ESSENTIAL OIL OF AERIAL PARTS OF *ARTEMISIA HERBA ALBA* ASSO

Salwa Ibraheim Eltawaty*[1], Sanaa Osman Yagoub[2] and Idrees Hamad Attitalla[3], Raja M. Momen[4]

[1*]Department of Microbiology and Immunology, Faculty of Pharmacy, Omar Al Mukhtar University, Al-Bayda, Libya.
[2]Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al Neelain University, Khartoum, Sudan.
[3]Department of Microbiology, Faculty of Science, Omar Al Mukhtar University, Al-Bayda, Libya.
[4]Department of Microbiology and Immunology, Faculty of Pharmacy, Triploi University, Libya.

ABSTRACT

Some bacteria has acquired resistance to antibiotics, the antibacterial activity of plant products have gained special interest in the recent decades. *Artemisia herba-alba* Asso is medicinal plant used in Libyan traditional medicine. The methicillin resistant *Staphylococcus aureus* (MRSA) is multi-drug resistant and exhibited high level of resistance to common β-lactam antibiotics. In this study the in vitro antibacterial activity of the plant essential oil against standard *Staphylococcus aureus* ATCC 25923 (SSA) and extended spectrum β-lactamase producer methicillin resistant *Staphylococcus aureus* (ESBP-MRSA) was investigated. Hydro-distillation with Clevenger-type apparatus was used to extract the essential oil of aerial parts of *Artemisia herba alba* and Disc diffusion and Agar dilution methods were used to assess the antibacterial activity and the minimum inhibitory concentration (MIC), respectively. The essential oil the plant showed high activity as antibacterial agent against both standard and clinical bacteria with inhibition zones of 31.3mm and 29.5mm, respectively. The MIC was shown as 3.125mg/ml against tested clinical ESBP-MRSA. Also the essential oil showed higher anti ESBP-MRSA than antibiotics references used. The results of this study showed an important promising antibacterial activity of essential oil of *Artemisia herba-alba* against ESBP-MRSA, and suggest that the essential oil could be used as alternative to Gentamicin and Vancomycin.

KEYWORDS

Anti-Extended spectrum β-lactamase producer Methicillin resistant *Staphylococcus aureus*, Essential oil and *Artemisia herba alba*.

Author for Correspondence:
Salwa Ibraheim Eltawaty,
Department of Microbiology and Immunology,
Faculty of Pharmacy, Omar Al Mukhtar University,
Al-Bayda, Libya.
Email: salwa.eltawaty@omu.edu.ly

INTRODUCTON

Currently due to the broadly miss use of antibiotics that result in the increase of the rate of multi drug resistant pathogens, the rate of occurrence of infectious disease caused by bacteria species are becoming a serious problem (Prabuseenivasan *et al*, 2006[1], Sokovic *et al*, 2007[2], Sbayou *et al*, 2014)[3]. This annoying bacterial resistance to most common

used antibiotics in addition to the popular use of natural plants for treatment prompted researchers to examine new antibacterial agents such as plants extracts (Alim *et al*, 2009)[4] and essential oils (Rahman *et al*, 2011)[5].

Extended spectrum β-lactams producer methicillin-resistant *Staphylococcus aureus* (ESBP-MRSA) are genetically differ from any other strains of *Staphylococcus aureus*. MRSA is developed when a cell of *S.aureus* subjected to horizontal gene transfer and resist β-lactams antibiotic; broad spectrum group includes penicillin derivatives such as oxacillin, methicillin and cephems such as the cephalosporin generations. The resistance of *S.aureus* to methicillin is mediated by the penicillin binding protein (PBP-2a) encoded by the mecA gene which carried on a mobile genetic chromosome named staphylococcal chromosome cassette (SCC *mec*), (Oliveira *et al*, 2001)[6] and this cassette is responsible for horizontal gene transfer of this resistance gene. This *mecA* gene in turn enhances the organism to grow in the presence of beta-lactam antibiotics (Archer *et al*, 1994)[7]. MRSA has spread worldwide shortly after the introduction of methicillin antibiotic where an outbreaks of MRSA were reported early of 1960s after which MRSA has been described in 1961 (Barber, 1961[8], Benner *et al*, 1968)[9]. Simply, MRSA is a description to a *Staphylococcus* bacteria exhibited resistance to methicillin antibiotic either alone or in addition to other antibiotics usually used to treat infection caused by the organism.

MRSA is responsible for many human infections which are difficult to treat, especially extended-spectrum plasmid-mediated Amp C ß-lactamase - producing strains. It shows rapid evolutionary changes and epidemiology expansion and becomes a major cause of community acquired and nosocomial infections worldwide (Klevens *et al*, 2007)[10]. MRSA infection is classified to either health care-associated MRSA (HA-MRSA) occur in people who have been in hospitals and other health care setting such as dialysis centres or nursing homes or community-associated MRSA (CA-MRSA) which occur in community among healthy people. HA-MRSA infection is typically associated with invasive devices or procedures such as surgeries, artificial joints or intravenous tubing while CA-MRSA infection is associated with skin to skin contact in crowded conditions such as child care workers; high school wrestlers. The rate of prevalence of MRSA has been raised in community and health care settings (Alexander *et al*, 2010)[11]. According to the National Nosocomial Infections Surveillance (2004)[12] 60% of the MRSA prevalence in intensive care units has been recorded in the Unites States (US).

Resistance of *S.aureus* to Vancomycin (the drug of choice for treating most MRSA infections caused by multi-drug resistant strains) has been increased. In addition to this, the unfavourable intravenous route and annoyed side effects of the new discovered synthetic anti *Staphylococcus aureus* drugs such as Daptomycin and Linezolid guide the researchers to study and explore if the medicinal plants could solve this problem and improve human health (Boucher *et al*, 2010)[13].

Artmesia herba-alba is a medicinal and aromatic dwarf plant considered as the largest genus in the tribe *Anthemideae* and one of the larger genera in the family *Asteraceae* (Ali *et al*, 2019)[14] named as Sheeh in Libya and many Arabic countries (Dahmani-Hamzaoui and Baaliouamer, 2010[15], Tilaoui *et al*, 2011)[16]. It is a greenish-silver perennial herb with a height of 20-40 cm and erects rigid stems. It is wild grow in arid areas of the Mediterranean region spreading into India, middle-east and north-western Himalayas (Vernin *et al*, 1995)[17], and also it is found common on the steppes of the North Africa and Middle East (Kwak *et al*, 1997). It is wild grow in North east of Libya. It has been reported that essential oils of plants have many biological activities such as antihypertensive, antidiabetis, antiviral, antibacterial, antioxidant, non-phytotoxic compounds and anticancer activities, (Hudaib and Aburjai, 2006[18], Rahman *et al*, 2011[5], Mizanur-Rahman *et al*, 2013[19], Luciardi *et al*, 2016[20], Ali *et al*, 2019)[14]. In Libya, this plant is traditionally used by native people as anthelmintic via drinking after boiling the aerial parts in water and also is used for prevention of wound infection after surgery via smell of the plant after heating in olive oil. This study aimed to investigate the antibacterial activity of essential oil of aerial parts of *Artemisia herba-alba* against

extended spectrum beta-lactamase methicillin resistant *Staphylococcus aureus* (ESBL-MRSA).

MATERIAL AND METHODS

Plant material

Aerial parts of *Artemisia herba-alba* were collected in August 2018 from Al-Abraq region west of Al-Bayda city, eastern north of Libya. The species was identified by Dr. Hussein Altagori, Department of plant, Faculty of science, Benghazi University, Libya.

Reference Antibiotics Discs

Augmentin 30µg (Amoxicillin 20µg + Clavulanic acid 10µg), Ceftazidime 30µg, Ceftriaxone 30µg, Gentamicin 10µg and Vancomycin 30µg are standard antibiotics discs used as references in this study. They were bought from Bioanalyse@ YSE Tibbi Malzemeler San. Expire dates were 10 - 18 months valid after the date of the assay.

Bacterial strains

The antibacterial activity was studied against standard American Type Culture Collection *Staphylococcus aureus* ATCC 25923 and clinical extended spectrum B-lactamse producer Methicillin resistant *Staphylococcus aureus*. The standard strain obtained from Medicinal and Aromatic Plant and Traditional Medicine Research Institute, National Centre for Research, Khartoum, Sudan and the clinical isolates were obtained from patient admitted to Benghazi Medical Centre, Benghazi, Libya.

Preparation of plant essential oil

300g of dried and ground aerial parts of *A. herba-alba* were hydro-distilled for 3 h in a Clevenger-type apparatus to obtain the essential oil. Each sample was extracted twice. Essential oil was dried over anhydrous sodium sulfate (Na2SO4). To prepare 100mg. 0.5ml of essential oil was mixed with 4.5ml of 40% (v/v) dimethyl-sulfoxide (purchased from BIOCHEM Chemopharma) and stored in sealed vials protected from light at 4°C until antibacterial assay.

Preparation of bacterial suspension

An overnight nutrient agar slant growth of each of the standard strain and the clinical isolates were washed with sterile normal saline 0.9% and brought to a solution of 10^8 C.F.U/ml by calibration with McFarland 0.5 solution and each kept in labelled sterile capped test tubes.

Antibacterial activity

Disc diffusion assay

Antibacterial activity was tested by disc diffusion method as described by Mukhtar and Ghori (2012)[21] with some modifications. One mL of a bacterial suspension (adjusted to a bacterial density of 10^8 C.F.U/ml is seeded in the Petri dishes containing Mueller-Hinton agar. A sterile paper disc (6mm diameter) was aseptically placed on the inoculated plates on which 10µL of previously prepared essential oil were added. Then, plates were kept for 15 min at room temperature. After 18 hr of incubation at 37°C, the inhibition zones were measured in mm. Disc impregnated with 40% v/v of DMSO was used as negative control. Both assays of standard and clinical bacteria were done in triplicate. The sensitivity of bacterial strain and isolate to the essential oil was classified as not sensitive for a diameter smaller than 8mm, moderately sensitive for a diameter range from 8 to 14mm, sensitive for a 14-20mm diameter, and very sensitive for a diameter larger than 20mm (Mukhtar and Ghori, 2012)[21]. The same assay was used to test the activity of Antibiotics discs as positive references for bacteria and compare their activity with that of the tested essential oil.

Determination of Minimum inhibitory concentration (MIC)

Andrews, (2006)[22] agar dilution method was adopted in this study to determine the minimum inhibitory concentration of the essential oil which can inhibit the growth of the seeded clinical isolates of extended spectrum B-lactamse producer Methicillin resistant *Staphylococcus aureus* bacteria on the Mueller-Hinton agar media. Serial dilutions were prepared for the plant essential oil in decreasing concentrations in the following order: 200, 100, 50, 25, 12.5, 6.25, and 3.13mg/ml. In sterile covered glass bottles, 5ml Melted double strength Mueller-Hinton agar cooled to 45°C were mixed with 5ml of each dilution of the tested plant essential oil to get a final serial dilution of 100, 50, 25, 12.5, 6.25, 3.13 and 1.65mg/ml of the essential oil. The mixture was poured to sterile small petri dishes, left to solidify, a loop full of standard loop (0.01ml) from each tested bacterial fresh suspension adjusted with McFarland 0.5 solution was spotted in duplicate spots onto the surface of each agar plate.

The inoculum allowed to be absorbed into the agar before incubation and then the plates incubated at 37°C for 18 hours. After the incubation period the least concentration mg/ml of the plant extract that inhibits the growth of organism was considered as (MIC).

RESULTS AND DISCUSSION

As showed in Figure No.1, the essential oil revealed a variable antibacterial activity against both tested standard and clinical bacteria with inhibition zones of 31.5mm ± 0.5 and 29.5mm ± 0.1 respectively.

This result is in agreement with Bertella *et al*, (2018)[23] who also proved that the essential oil of *Artemisisa herba alba* has active growth inhibition performance against both standard and clinical *Staphylococcus aureus* with growth inhibition zones of 16.3 ± 0.5 and 28 ± 1, respectively. Also Lakehal *et al*, 2016[24] reported closed inhibition zone of 33 ± 0.43 with *Artemisia herba* essential oil against clinical *Staphylococcus aureus*. This activity performance of the plant essential oil could be attributed to the bioactive constituents of the oil, which it has been documented that the essential oil of *Artemisia herba alba* contains camphor and α/β thujone as major compounds they has antimicrobial activities (Lakehal *et al*, 2016[24] and Chouhan *et al*, 2017)[25]. However this study results disagreed with Bertella *et al*, (2018)[23] in that they found that the antibacterial performance of *Artmesia herba* oil was higher on clinical isolate than standard strain of *Staphylococcua aureus* bacteria, on the contrary of this research in which the clinical isolate showed lower susceptibility than tested standard strain. These differences may contribute to different time and different geographical area the plant was collected in and from. The environmental factors (climate, seasons and soils), the geographic conditions, the genetic diversity of the species, the harvesting period and the extraction technique are parameters could be lead to the variation in the biological activities of plant extracts (Salvagnini *et al*, 2008[26], Ben Ghnaya *et al*, 2013)[27]. These results showed a probability of that the essential oil of *Artemisia herba alba* inhibit the growth of *Staphylococcus aureus* either via cell wall disruption as the peptidoglycan Vancomycin do or may have the ability to bind with the 30S subunit of the bacterial ribosome by which inhibit the protein synthesis as the aminoglycoside gentamicin do or it may have good penetration power and good affinity to the protein binding proteins (PBP 2a) which produced by MRSA in order to block cell wall permeability to the Betalactams; Augmentin, Ceftazidime and Ceftriaxone. Another probability to which this study referred the activity of the agent is that the essential oil of *Artemisia herba* inhibits the growth by interfering of bacteria biofilm formation. The multidrug-resistant clinical isolates of *S. aureus* have a greater likelihood of developing biofilms (Gwang, 2008)[28].

The agar dilution assay used in this study showed that the growth of tested clinical multidrug resistant ESBP-MRSA was inhibited from *Artemisia herba* essential oil with MIC of 3.125mg/ml, the concentration which is lower than the MIC 7.5mg/ml reporteded with Bertella *et al*, 2018[23], and higher than the MIC 0.1mg/ml founded by Lakehal *et al*, 2016[24] from *Artmesia herba* oil against the clinical Methicilln resistant *Staphylococcus aureus*.

The antibacterial activity of the essential oil of *Artemisia herba alba* against of the tested clinical ESBP-MRSA bacteria compared with the selected antibiotics (Table No.1 and Figure No.2) figured out that the antibacterial activity of the essential oil was higher than the activity showed from Vancomycin (30ug) and Gentamicin (10ug) which showed large diameters inhibition zones of 19.1 and 19.6mm respectively. Furthermore, if we take in consideration the side effects of both antibiotics and their commonly unfavourable parenteral route, this result could offer this essential oil as anti-*Staphylococcus aureus* instead of Gentamicin and Vancomycin. On the other hand, no growth inhibition activity had shown from the Betalactam; the penicillin derivative Augmentin 30µg (Amoxicillin/Clavulanic acid) and the 3rd generation Cephalosporines; Ceftazidime 30µg and Ceftriaxone 30µg which revealed zero (0) inhibition zones compared with the largest inhibition zone 29.5 mm revealed from the essential oil of aerial parts of *Artemisia herba alba*. LSD Post hoc statistical analysis showed high significant differences (P value ≤0.01) between the growth inhibition performance of essential oil of the aerial parts of *Artemisia herba alba* and the all antibiotics

used. No significant differences (P value≥ 0.05) shown between the performance of Vancomycin (30ug) and Gentamicin (10ug) on the clinical tested ESBP-MRSA while both antibiotics performances appeared high significant differ (P value ≤0.01) from other tested Beta-lactams. Also no significant differences (P value≥ 0.05) seen in the performance of Augmentin (Amoxicillin/Clavulanic acid), Ceftazidime and Ceftriaxon on tested bacteria (Table No.1). The results which suggested that this essential oil of *Artemisia* has promising anti extended spectrum Beta-lactamase producer Methicillin *Staphylococcus aureus* and then could be used as alternative to the resisted Beta-lactam antibiotic with the note that the oil should be used at therapeutic doses, where it has been reported that the oil was slightly toxic with a median lethal dose of 615 mg/kg (Bertella *et al*, 2018)[23]. Further study should be carried out to study the mechanism/s of action and to determine the effective therapeutic dose of essential oil of *Artemisia herba*.

Table No.1: Antibacterial activity of essential oil of aerial parts of *Artemisia herba* and used antibiotics references against clinical ESBP-MRSA

S.No	Antibacterial agent	Mean of Diameter of Inhibition zone (mm) ± Standard deviation
1	Essential oil of Artemisia herba alba 100mg/ml	$29.5^{a} \pm 0.50$
2	Vancomycin 30ug	$19.1^{b} \pm 0.15$
3	Augmentin 30ug	$0^{c} \pm 0.00$
4	Ceftazidim 30ug	$0^{c} \pm 0.00$
5	Ceftriaxon 30ug	$0^{c} \pm 0.00$
6	Gentamicin 10ug	$19.6^{b} \pm 0.10$
7	Sig.	**

ESBP-MRSA = extended spectrum B-lactamase producer Methicillin resistant *Staphylococcua aureus*

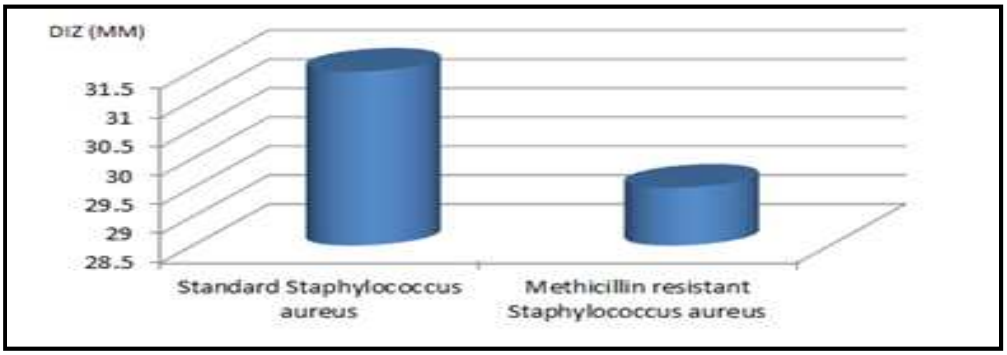

Figure No.1: Antibacterial activity of essential oil aerial parts *Artemisia herba* against standard and clinical Methicillin resistant *Staphylococcus aureus* bacterial

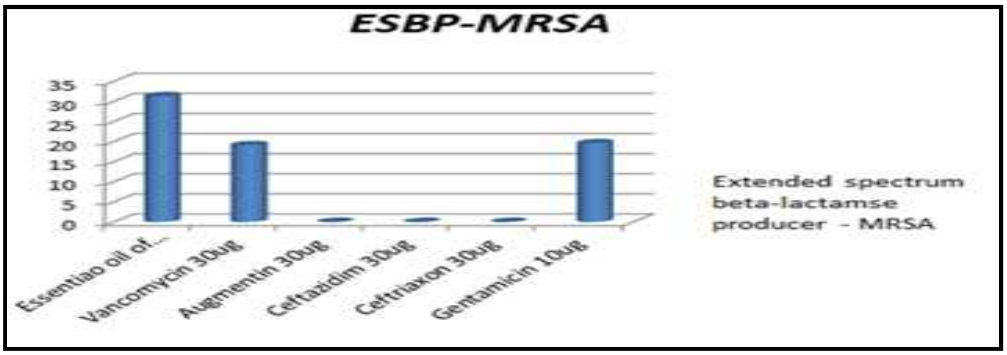

Figure No.2: Antibacterial activity of essential oil of aerial parts of *Artemisia herba* and used antibiotics references against clinical ESBP-MRSA

CONCLUSION

An important promising antibacterial activity of essential oil of *Artemisia herba-alba* plant was proved in this study against extended spectrum Beta-lactamase producer methicillin resistant *Staphylococcus aureus*. Also the study output suggests that the essential oil could be used as alternative to Gentamicin and to Vancomycin; the drug of choice to treat infections caused by methicillin resistant *Staphylococcus aureus*. In addition this study suggests that this oil could be used as alternative to Beta-lactam antibiotics to treat infectious diseases caused by this multi-drug resistant *Staphylococcus aureus*.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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