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EXTRACTS OF *ROSMARINUS OFFICINALIS, RHEUM RHAPONTICUM, AND ORIGANUM MAJORANA* EXHIBIT SIGNIFICANT ANTI-STAPHYLOCOCCAL ACTIVITY

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ABSTRACT:

Background: Bacterial resistance to antimicrobial agents is on the rise and this is causing serious complications resulting in increased morbidity and mortality of bacterial infections. There is a need for new antimicrobial molecules in order to fight against Multi-Drug-Resistant Organisms. The Mediterranean area is rich in a variety of medicinal plants and this may represent a potential for new compounds and molecules with enhanced antibacterial activity.

Methods: The antimicrobial effects of three traditionally used Lebanese plants were investigated against 24 clinical isolates of *Staphylococcus aureus* with different phenotypes of resistance. *Rosmarinus officinalis, Rheum rhaponticum, and Origanum majorana* where extracted with ethanol, then further subfractionated with petroleum ether, dichloromethane and ethyl acetate. The remaining aqueous fraction was also collected, thus a total of five extracts were studied for each plant. The MIC and MBC of these extracts were determined using the micro-dilution technique.

Results: *Rosmarinus officinalis* was the most effective against most of the strains studied including MRSA, QS, QR, MS and MR. The ethyl acetate fraction of *Rosmarinus officinalis, Rheum rhaponticum, and Origanum majorana* showed significant antibacterial activity against *S. aureus* with MIC ranging between 2.5 and 5µg/µl. The crude extract of *Rheum rhaponticum* was also highly effective at a low concentration of 4.25µg/µl.

Conclusions: Most extracts showed antimicrobial activity at low concentrations. Antimicrobial activity of the plant extracts varied with the profile of resistance of the bacterial isolate. Further studies need to investigate the active compounds in these extracts and their mode of action to make use of them as antibiotics and food preservatives.

INTRODUCTION: The Middle East area has witnessed a surge in methicillin-resistant *Staphylococcus aureus* over the past two decades [1]. Two major studies performed in the region concerning MRSA [2] and involving 62 hospitals from Algeria, Cyprus, Egypt, Jordan, Lebanon, Malta, Morocco, Tunisia and Turkey showed that the median MRSA prevalence was 39%. It is well known that infections with antibiotic-resistant organisms lead to higher morbidity and mortality rates than similar infections with antibiotic-susceptible strains [3, 4]. With the emergence of MRSA and its uprising resistance to presently

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available antibiotics, the drug of treatment shifted from penicillin to vancomycin. MRSA strains are resistant to several antibiotics including erythromycin, tetracycline, gentamicin and fluoro-quinolones [5-7].

The Mediterranean area is rich in a variety of potential medicinal plants. Several of these have been used traditionally in Lebanon and are passed down from one generation to another [8]. In addition, plants have several uses beyond the medical application; they serve as raw material for several products in the market place including fuels, cosmetics, shampoos, soaps, paper and clothing. Plant extracts are currently being investigated for their antimicrobial effects to be used in preserving different foods like vegetables, meat and seafood instead of using artificial preservatives and disinfectants [9, 10].

Rosmarinus officinalis, common name rosemary, belongs to the family Lamiacceae and is widely found in the Mediterranean region. It is a very ancient plant of several applications. Some of its traditional uses include antispasmodic, analgesic, antirheumatic, diuretic, and antiepileptic. Some of the identified secondary metabolites of *Rosmarinus officinalis* include: carnosic acid, carnosol, rosmarol, rosmaridiphenol, rosmanol, isorosmanol, epirosmanol, rosmariquinone, ursolic acid and terpenoids [11-15]. On the other hand, *Rosmarinus officinalis* has several applications especially in the food processing and preserving industry because of its natural antioxidant and antimicrobial effect [16].

Rosmarinus officinalis extracts exhibit antimicrobial activity against *Vibrio parahaemolyticus* [17], *Listeria monocytogenes, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, and other bacterial species* [18].

Origanum majorana, known as sweet majorana, belongs to the family Lamiaceae. It is a perennial herbaceous plant [19]. *Origanum* genus consists of 38 species among them 75% are restricted to the Eastern Mediterranean area. They are characterized by a wide range of volatile secondary metabolites. It is commercially used as a spice and condiment and also used in perfumes [19]. It is traditionally used to treat asthma, indigestion, headache, rheumatism, dizziness, gastrointestinal disorder and migraine [20].

Rheum rhaponticum, common name rhubarb, belongs to the Polygonaceae family. It is spread in Europe and Asia. There are around 50 different species of *Rheum rhaponticum (Rhubarb)* is used for therapy, cooking and decoration [21]. It is highly beneficial for the liver, spleen and gallbladder problems. It is a laxative and diuretic, used to treat kidney stones, gout and liver diseases. *Rheum rhaponticum* has estrogenic, anti-mitotic, anti-microbial and hypotensive properties [21]. It is widely used in traditional Chinese medicine as a good remedy for ulcer, cancer, upper intestinal bleeding and toothache [22]. *Rheum rhaponticum* exhibits antimicrobial effect against Gram positive cocci at concentrations ranging from 125 to >500µg/µl. This inhibitory activity is attributed to anthraquinone derivatives, including aloe-emodin, emodin and rhein.

The aim of this study was to assess and quantify the antimicrobial activity of plant extracts from three traditional Lebanese plants including: *Rosmarinus officinalis*, *Origanum majorana*, and *Rheum rhaponticum* against *Staphylococcus aureus* strains of different phenotypes of resistance isolated from Lebanese patients with different clinical presentations.

Methods:

1. **Bacterial Strains:** Twenty four strains of *Staphylococcus aureus* isolated from clinical samples of Lebanese patients were used in this study. These were grouped in two main groups of patterns: Group 1 including strains that are susceptible to Methicillin (MSSA- Methicillin Susceptible *Staphylococcus aureus*) and Group 2 including the Methicillin resistant *Staphylococcus aureus* (MRSA). The MRSA were subdivided into 5 main subgroups: QS-MRSA: Quinolones susceptible MRSA, QR-MRSA: Quinolones resistant MRSA, MS-MRSA: Macrolides susceptible MRSA, MR.-MRSA: Macrolides resistant MRSA and MDR-MRSA: Multi-drug resistant MRSA, these strains are simultaneously resistant to Methicillin, Quinolones and Macrolides.

2. **Selection of Plants:** The herbal sample consisted of three different Lebanese plants: leaves of *Rosmarinus officinalis* (Rosemary), leaves of *Origanum majorana* (Marjoram) and

roots of *Rheum rhaponticum* (rhubarb). They were collected directly from nature, identified, and characterized by a taxonomist. The name of the plant, time, place and date of collection were recorded.

3. **Preparation of Crude Extract:** Fresh plants were dried in the shade at room temperature and ground in a coffee bean grinder. The dried plant material was weighed and then soaked in 80% ethanol for 7 days with continuous shaking in a shaker at room temperature. At day seven the plant material was filtered and the filtrate collected. This was repeated and the filtrates were combined and concentrated in a rotary evaporator to obtain the crude extract (fraction 1).

4. **Fractionation Method:** The crude extract of each plant was further partitioned by extraction with different solvents in a 1:1 (v/v) ratio in order to sub-fractionate the plant components according to their polarity: petroleum ether (fraction 2), dichloromethane (fraction 3), and ethyl acetate (fraction 4). Extractions were repeated three times and fractions were combined. The remaining aqueous layer was collected as fraction number 5.

   Fractions 1 and 5 were dried using a freeze dryer, but fractions 2, 3 and 4 were dried under the hood to dryness due to the inconvenience of introducing vapor solvent into the freeze dryer. Controls were prepared for each fraction by drying the same amount of solvent and following the same subfractionation method without plant extract (solvent control). In view of solubility related issues, the fraction 2 (petroleum ether) was discarded from all experiments.

5. **Study of Antimicrobial activity of the Plant Extracts***:* The dried plant material was weighed and dissolved in sterile distilled water. The solutions were filtered through 0.22 µm sterile filter membranes and stored at 4 ˚C for further use. The concentration of the original solution of the plant extract/fraction corresponds to the concentration obtained after re-suspension of the dried plant extracts. This was used as the stock solution for the preparation of the Minimum Inhibitory Concentration MIC series.

6. **Determination of the inhibitory and bactericidal concentrations:** The Micro-dilution Broth Method was used for the determination of the MIC of plant extracts as recommended by the Clinical and Laboratory Standards Institute [23]. Broth (100µl) was dispensed in each well of a sterile microdilution tray. An appropriate volume of plant extract suspension was added to the first tube in each series (after removing the same volume of broth) in order to achieve the desired concentration after the addition of the bacterial inoculum.

   A standardized bacterial inoculum was prepared and adjusted to 0.5 McFarland and then diluted to 10^6 CFU/ml. Within 15 minutes, the wells were inoculated with 100µl of this inoculum resulting in a 1:2 concentration of the content of the well in plant extract and of the bacterial suspension ($5x10^5$ CFU/ml). A routine bacterial count was performed in duplicates to verify the bacterial concentration. Positive and negative control wells were used. The negative control well consisted of 200 µl of Mueller Hinton Broth (MHB), the positive control consisted of 200 µl MHB with a bacterial suspension but without plant extract. The microdilution tray was incubated at 35°C for 18-24 hours after which the MIC was recorded as the highest dilution of each plant extract that still retained an inhibitory effect resulting in no visible growth or in other terms absence of turbidity observed with the naked eye. The Minimum Bactericidal Concentration (MBC) was determined by sub-culturing samples from the tubes with concentrations above the MIC on new plates of Mueller Hinton Agar (MHA).

   The MBC corresponded to the lowest concentration of the extract associated with no bacterial culture. All experiments were performed three independent times in duplicate form. The MIC_{90} is defined as the Minimum Inhibitory Concentration necessary to inhibit the growth of 90% of organisms; it was calculated as the percentile below which 90% of the individual MICs values fall. In view of the relatively small population of tested bacteria, it was not advantageous to calculate MIC_{50}.

RESULTS:

Antimicrobial activity of *Rosmarinus officinalis*: The crude extract, the dichloromethane, the ethyl acetate, and the aqueous fractions of *Rosmarinus officinalis* exerted both inhibitory and bactericidal activity on *Staphylococcus aureus* isolates. *Rosmarinus officinalis* MICs were recorded and the MIC_{90} was determined. The extracts had the MIC mean values ranging between 2.3 and 11.15µg/µl. The lowest MIC was recorded for the ethyl acetate fraction at 0.156µg/µl (**Table 1**). The concentration at which the bactericidal activity was observed for the majority of the strains was 2.5µg/µl. The MBC values were below 80µg/µl (Table 1). The crude extract and the dichloromethane fraction exhibited bactericidal activity on 96% of the strains. While the aqueous extract and ethyl acetate fractions were bactericidal against 92% and 100% of the strains, respectively.

TABLE 1: ANTIBACTERIAL ACTIVITIES OF THE DIFFERENT FRACTIONS OF *ROSMARINUS OFFICINALIS*

Bacteria	*Crude*		*Dichloro-methane*		*Ethyl acetate*		*Aqueous*	
	MIC (µg/µl)	MBC	MIC (µg/µl)	MBC	MIC (µg/µl)	MBC	MIC (µg/µl)	MBC
Sa002SGH	5	40	1.27	40	2.5	20	10	80
Sa006SGH	2.5	5	2.5	20	0.625	2.5	10	20
Sa006SGH	0.625	1.27	1.27	2.5	2.5	5	2.5	5
Sa007SGH	2.5	5	10	10	0.625	0.625	5	5
Sa007SGH	5	5	1.27	5	2.5	2.5	2.5	2.5
Sa009SGH	2.5	2.5	0.625	1.27	0.625	0.625	2.5	2.5
Sa011SGH	2.5	2.5	0.625	0.625	0.625	0.625	2.5	2.5
Sa013SGH	5	40	2.5	20	0.625	2.5	5	10
Sa014SGH	5	10	1.27	2.5	0.625	5	5	10
Sa018SGH	10	20	10	20	1.27	2.5	20	40
Sa020SGH	2.5	5	1.27	1.27	0.625	0.625	40	80
Sa023SGH	20	20	2.5	>80	5	10	2.5	2.5
Sa026SGH	0.625	1.27	0.312	0.312	2.5	5	1.27	1.27
Sa027SGH	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sa027SGH	40	>80	20	20	2.5	10	>80	>80
Sa032SGH	0.625	0.625	0.312	0.312	0.156	0.156	0.312	0.625
Sa035SGH	2.5	2.5	1.27	2.5	1.27	5	5	10
Sa037SGH	5	20	1.27	20	0.625	1.27	5	10
Sa038SGH	5	5	1.27	2.5	1.27	1.27	1.27	1.27
Sa039SGH	1.27	2.5	0.625	1.27	2.5	5	2.5	2.5
Sa042SGH	1.27	2.5	0.625	2.5	1.27	5	2.5	10
Sa058SGH	5	10	1.27	1.27	1.27	2.5	1.27	1.27
Sa077SGH	1.27	1.27	2.5	4	1.27	2.5	2.5	5
Sa105SGH	80	>80	40	>80	20	>80	80	>80
Mean	8.67		4.46		2.30		11.15	
MIC_{90}	17		10		2.5		18	
SD	17.34		8.77		3.92		17.63	

MIC_{90} is the concentration at which 90% of the strains MIC lie. The mean was also calculated for each extract against all the tested strains of *Staphylococcus aureus*.

Table 2 shows the variation of MIC with respect to the difference in bacterial resistance pattern. The ethyl acetate fraction had the lowest MIC_{90} (2.5µg/µl) with MSSA; while the crude, dichloro-methane and aqueous fractions exhibited higher recordings of MIC_{90}. Quinolone-susceptible MRSA showed more susceptibility than Quinolone-resistant MRSA vis-à-vis the crude, dichloro-methane and ethyl acetate extracts. Macrolide resistant strains showed a lower MIC_{90} than macrolide susceptible strains with the crude, ethyl acetate and aqueous extracts (**Table 2**).

Antimicrobial activity of *Origanum majorana*: Both inhibitory and bactericidal effects of *Origanum majorana* crude extract, dichloro-methane, ethyl acetate and aqueous fractions were observed against different strains of *S. aureus*. *Origanum majorana officinalis* MICs were recorded and the MIC_{90} was determined. The mean was also calculated for each extract against all the tested strains of *Staphylococcus aureus*. The concentrations at which most of the bacterial suspensions were cleared were 2.5µg/µl for the crude extract and the aqueous fraction and

0.625µg/µl for the dichloromethane and ethyl acetate fractions (**Table 3**). The ethyl acetate fraction had the lowest mean (1.77µg/µl) and lowest MIC_{90} (5µg/µl). The highest mean and MIC_{90} values were recorded by the crude extract (Table 3). The lowest individual MIC (0.625µg/µl) was recorded for the dichloromethane, ethyl acetate and aqueous fractions.

TABLE 2: MIC_{90} OF THE DIFFERENT FRACTIONS OF *ROSMARINUS OFFICINALIS* ACCORDING TO BACTERIAL RESISTANCE PATTERNS

	MIC $_{90}$ (µg/µl)						
	MSSA	**MRSA**					
		All	**QS**	**QR**	**MS**	**MR**	**MDR**
Crude	8	9.5	2.5	15.5	13	4.25	4.62
Dichloromethane	10	2.5	1.14	2.5	2	2.25	2.37
Ethyl acetate	2.5	3.25	1.14	4.25	4	1.27	1.27
Aqueous	17	3.25	4.4	2.5	4	2.25	2.37

MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin resistant *Staphylococcus aureus*, All: All MRSA, QS: quinolones susceptible MRSA, QR: quinolones resistant MRSA, MS: Macrolides susceptible MRSA, MR: Macrolide resistant MRSA and MDR: Multi-drug resistant MRSA.

TABLE 3: ANTIBACTERIAL ACTIVITIES OF THE DIFFERENT FRACTIONS OF *ORIGANUM MAJORANA*

Bacteria	**Crude**		**Dichloro-methane**		**Ethyl acetate**		**Aqueous**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
	(µg/µl)		**(µg/µl)**		**(µg/µl)**		**(µg/µl)**	
Sa002SGH	20	40	10	10	5	5	10	40
Sa006SGH	2.5	5	0.625	0.625	1.27	2.5	1.27	10
Sa006SGH	5	5	0.625	1.27	0.625	0.625	2.5	5
Sa007SGH	2.5	2.5	0.625	0.625	0.625	2.5	0.625	0.625
Sa007SGH	10	20	0.625	1.27	0.625	0.625	2.5	5
Sa009SGH	2.5	2.5	2.5	5	2.5	2.5	5	5
Sa011SGH	2.5	5	0.625	0.625	0.625	0.625	1.27	2.5
Sa013SGH	5	5	1.27	5	0.625	1.27	1.27	20
Sa014SGH	2.5	10	10	20	1.27	1.27	5	5
Sa018SGH	1.27	10	1.27	1.27	0.625	1.27	1.27	2.5
Sa020SGH	5	5	20	80	5	40	2.5	2.5
Sa023SGH	2.5	2.5	2.5	40	5	10	10	80
Sa026SGH	5	5	0.625	0.625	0.625	1.27	2.5	2.5
Sa027SGH	20	20	0.625	0.625	0.625	1.27	2.5	5
Sa027SGH	2.5	2.5	2.5	2.5	1.27	1.27	0.625	0.625
Sa032SGH	10	10	0.625	0.625	1.27	2.5	2.5	5
Sa035SGH	2.5	5	2.5	2.5	1.27	1.27	2.5	2.5
Sa037SGH	2.5	5	2.5	2.5	1.27	10	2.5	5
Sa038SGH	10	10	0.625	0.625	0.625	0.625	2.5	2.5
Sa039SGH	10	40	1.27	2.5	2.5	2.5	2.5	10
Sa042SGH	10	20	1.27	2.5	2.5	5	2.5	5
Sa058SGH	5	10	5	5	5	10	2.5	2.5
Sa077SGH	5	10	1.27	2.5	0.625	1.27	2.5	5
Sa105SGH	20	>80	80	80	1.27	1.27	10	10
Mean	6.82		6.22		1.77		3.28	
MIC_{90}	17		10		5		8.5	
SD	5.86		16.33		1.59		2.79	

MIC_{90} is the concentration at which 90% of the strains MIC lie. The mean was also calculated for each extract against all the tested strains of *Staphylococcus aureus*.

The MIC and MBC were detected mostly within one dilution. Both the ethyl acetate and the aqueous fractions had the lowest MIC_{90} (4µg/µl) against MSSA (**Table 4**). The dichloromethane fraction had a MIC_{90} of 10µg/µl against MSSA, whereas the crude extract had MIC_{90} of 16µg/µl. Quinolone-susceptible MRSA had lower MIC_{90} than that of the Quinolone-resistant MRSA with the dichloromethane, ethyl acetate and aqueous extracts. Macrolide-susceptible MRSA had a lower MIC_{90} in comparison to macrolides-resistant MRSA with the crude, dichloromethane and ethyl acetate extracts.

TABLE 4: MIC_{90} OF THE DIFFERENT FRACTIONS OF *ORIGANUM MAJORANA* ACCORDING TO BACTERIAL RESISTANT PATTERNS

	MIC $_{90}$ (µg/µl)						
	MSSA	MRSA					
		All	QS	QR	MS	MR	MDR
Crude	16	10	8.5	5	8	9	5
Dichloromethane	10	3.25	2.5	4.25	2.5	4.25	4.62
Ethyl acetate	4	5	2.25	5	4	4.25	4.56
Aqueous	4	6.5	4.5	7.75	8	2.5	2.5

MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin resistant *Staphylococcus aureus*, All: All MRSA, QS: quinolones susceptible MRSA, QR: quinolones resistant MRSA, MS: Macrolides susceptible MRSA, MR: Macrolide resistant MRSA and MDR: Multi-drug resistant MRSA.

Antimicrobial Activity of *Rheum rhaponticum*: *Rheum rhaponticum* manifested inhibitory as well as bactericidal effects on *Staphylococcus aureus* the best inhibitory activity which is represented by the lowest MIC_{90} was observed with the crude fraction at 4.25µg/µl (**Table 5**). The lowest MIC was recorded for the dichloromethane fraction at 0.312µg/µl (Table 5). The concentrations at which most of the strains were inhibited were 2.5µg/µl for the crude extract, 5µg/µl for the dichloromethane fraction, 0.625µg/µl for the ethyl acetate fraction and 5µg/µl for the aqueous fraction. The concentrations at which bactericidal activity was observed for the majority of the strains were 10µg/µl for the aqueous fraction and 5µg/µl for the crude extract, the dichloromethane fraction, and the ethyl acetate fraction (Table 5).

TABLE 5: MICS OF DIFFERENT FRACTIONS OF *RHEUM RHAPONTICUM*

Bacteria	Crude		Dichloro-methane		Ethyl acetate		Aqueous	
	MIC (µg/µl)	MBC (µg/µl)	MIC (µg/µl)	MBC (µg/µl)	MIC (µg/µl)	MBC (µg/µl)	MIC (µg/µl)	MBC (µg/µl)
Sa002SGH	2.5	5	10	>80	20	40	2.5	80
Sa006SGH	2.5	40	5	5	0.625	20	2.5	80
Sa006SGH	1.27	2.5	0.312	0.321	0.625	1.27	10	10
Sa007SGH	2.5	20	2.5	2.5	0.625	5	1.27	5
Sa007SGH	1.27	1.27	0.625	2.5	0.625	2.5	10	10
Sa009SGH	2.5	2.5	1.27	1.27	5	5	10	20
Sa011SGH	2.5	2.5	5	5	5	10	5	5
Sa013SGH	2.5	10	5	>80	1.27	10	1.27	5
Sa014SGH	2.5	10	5	10	2.5	10	2.5	2.5
Sa018SGH	2.5	5	5	5	0.625	1.27	1.27	2.5
Sa020SGH	2.5	5	20	40	5	>80	2.5	2.5
Sa023SGH	2.5	2.5	10	10	2.5	2.5	5	10
Sa026SGH	1.27	2.5	0.625	5	0.625	2.5	5	5
Sa027SGH	1.27	2.5	0.625	1.27	0.625	0.625	5	10
Sa027SGH	5	10	5	5	2.5	10	5	10
Sa032SGH	1.27	2.5	1.27	2.5	0.625	5	10	20
Sa035SGH	2.5	5	5	40	2.5	40	2.5	40
Sa037SGH	2.5	5	5	5	20	80	5	10
Sa038SGH	5	5	1.27	5	1.27	1.27	10	20
Sa039SGH	1.27	5	1.27	2.5	0.625	5	5	10
Sa042SGH	2.5	5	1.27	10	1.27	5	10	10
Sa058SGH	2.5	5	0.625	0.625	2.5	10	5	5
Sa077SGH	1.27	5	0.625	2.5	0.625	5	10	10
Sa105SGH	20	40	5	20	2.5	80	20	80
Mean	3.07	5	3.98	>80	3.33	40	6.09	80
MIC_{90}	4.25	40	10	20	5	20	10	80
SD	3.74	2.5	4.82	0.321	5.33	1.27	4.39	10

MIC_{90} is the concentration at which 90% of the strains MIC lie. The mean was also calculated for each extract against all the tested strains of *Staphylococcus aureus*.

Table 6 shows that the the crude extract of *Rheum rhaponticum* had the most prominent fraction with antimicrobial activity against MSSA with an MIC_{90} of 3.75 µg/µl. The dichloromethane and the aqueous fractions had similar MIC_{90} of 10µg/µl against MSSA. While the ethyl acetate fraction exhibited a high MIC_{90} of 14 µg/µl, the ethyl acetate and the aqueous fractions of *Rheum rhaponticum* were more effective against quinolones resistant MRSA than the quinolones susceptible MRSA. The crude extract was equally effective against the QS and QR strains, with an MIC_{90} of 2.5µg/µl for both patterns of resistance (Table 6).

TABLE 6: MIC_{90} OF THE DIFFERENT FRACTIONS OF *RHEUM RHAPONTICUM* ACCORDING TO BACTERIAL RESISTANT PATTERNS

	MIC $_{90}$ (µg/µl)						
	MSSA	MRSA					
		All	QS	QR	MS	MR	MDR
Crude	3.75	2.5	2.5	2.5	2.5	2.25	2.37
Dichloromethane	10	6.5	4.25	7.18	8	1.41	0.625
Ethyl acetate	14	3.25	4.5	2.5	4	2.12	2.31
Aqueous	10	10	10	8.5	10	10	9.5

MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin resistant *Staphylococcus aureus*, All: All MRSA, QS: quinolones susceptible MRSA, QR: quinolones resistant MRSA, MS: Macrolides susceptible MRSA, MR: Macrolide resistant MRSA and MDR: Multi-drug resistant MRSA.

DISCUSSION: Bacteria have been able to develop resistance and rapidly disseminate it to other bacteria [24]. *Staphylococcus aureus* is an excellent example of species that have been able to acquire resistance very quickly over time. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent causes of hospital and community-associated infections. A great number of people die each year from nosocomial infections. The increasing occurrence of *S. aureus* resistance not only to methicillin but to a wide range of antimicrobial agents, including all β- lactams, has made this pathogenic bacterium more virulent and therapy more difficult [25, 26].

Plants are an excellent source of biological active components; they contain a variety of secondary metabolites that can be easily solubilized by soaking in ethanol or methanol [27, 28]. 70% Ethanol was used in this study for extraction as described in different studies [29-32].

The sub-fractionation of the ethanol extract aimed at separating different compounds. Each solvent selected in the study extracted a number of compounds based on the polarity of the molecules. The petroleum ether isolated the least polar compounds and the ethyl acetate extracted the most polar compounds.

Petroleum ether extracts mostly terpenoids, essential oils, fatty acids, vitamins, flavonoids and coumarins. Dichloromethane extracts phenols, terpenoids, steroids, flavonoids and aldehydes. Ethyl acetate extracts phenols, steroids, terpenoids, flavonoids and coumarins [33].

This study reveals the potential of developing new antibiotics from *Rheum rhaponticum*, *Origanum majorana* and *Rosmarinus officinalis* to extract new antibiotics. The *Rosmarinus officinalis* ethyl acetate extract was the most effective among all the extracts tested recording the lowest MIC_{90} of 2.5 µg/µl, followed by the crude extract of *Rheum rhaponticum* (MIC_{90} of 4.25µg/µl) and the ethyl acetate extracts of *Origanum majorana* (MIC_{90} 5 µg/µl). The lowest MBC was recorded for the dichloromethane, ethyl acetate and aqueous extract of *Origanum majorana* which were all 0.625µg/µl. The MIC_{90} recorded by all the extracts were between 2.5µg/µl and 18µg/µl. The MIC_{90} of the crude ethanol extracts of the studied plants was between 4.25-17µg/µl.

Nitta *et al* [34] also studied the effect of plant extracts against the growth of MRSA. Their study involved 181 species of tropical and subtropical plants. A total of 505 extracts were tested, among which 53 (10.5%) inhibited the growth of MRSA.

The active compounds were stilbene derived from barks of *Shorea hemsleyana* and roots of *Cyphostemma bainessi*. The most active stilbene was hemsleyanol isolated from *Shorea hemsleyana* acetone extract with an MIC of 2µg/µl and genetin that was derived from methanol extract of *C. bainessi* with an MIC of 4µg/µl. These values are comparable to the MIC values that were recorded in this study, which were between 0.312 – 80 µg/µl.

Other studies also investigated the use of plant extracts to inhibit the growth of *S. aureus* [35, 36]. Antimicrobial activity of 52 plant oils and extracts was investigated against *Acinetobacter baumanii, Aeromonas veronii, Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica subsp. enterica serotype typhimurium, Serratia marcescens* and *Staphylococcus aureus* using the agar dilution method and microdilution method. Among the lowest MIC recorded was that of vetiver oil against *S. aureus* (0.008%). 77% of the plant extracts (40 plant extracts) inhibited the growth of *S. aureus* and *Candida albicans* [37].

In this study, **t**he petroleum ether extracts of the three plants yielded a mucous substance that could not be used in the micro-dilution method for MIC determination and was therefore excluded from our study. Kökdil [27] studied the antibacterial activity of the petroleum ether, dichloromethane and methanol extracts of eleven *Nigella L.* species seeds against Gram positive strains (*Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis*) and Gram-negative strains (*Escherichia* coli, *Pseudomonas aeruginosa*) by the agar disc-diffusion method.

In our study, *Origanum majorana* was highly inhibitory to the growth of *Staphylococcus aureus,* recording the lowest MIC in this study with the dichloromethane, ethyl acetate and aqueous extracts which were all 0.625µg/µl (Table 3). Similarly, Abdel-Massih *et al* [38] showed that the ethyl acetate fraction of *Origanum majorana* had the best antimicrobial effect on *E. coli* and *K. pneumoniae* exhibiting the lowest MIC_{90} (1.28 µg/µl). Leeja *et al* [28] also studied the antimicrobial activity of *Origanum majorana* against *Staphylococcus aureus* using the disc diffusion method. The antimicrobial activity of methanol extract of *Origanum majorana* exhibited a zone of inhibition between 17 and 23mm.

On the other hand, *Rheum rhaponticum* showed antimicrobial activity against *Staphylococcus aureus* with the crude extract being the most effective recording an MIC_{90} of 4.25µg/µl. Tegos *et al* [39] also showed that *Rheum rhaponticum* had antimicrobial effect against *Staphylococcus aureus* and *Staphylococcus epidermidis* at concentrations ranging from 125 to >500 µg/µl. Rhein is believed to be the main compound responsible for the antibacterial activity of *Rheum rhaponticum* and is possibly among the components present in our extracts leading to the antimicrobial activity against *Staphylococcus aureus*.

The crude extract of *Rheum rhaponticum* was the most effective against the MSSA and MRSA strains with an MIC_{90} of 2.5µg/µl and 3.75µg/µl, respectively. The crude extract of *Rheum rhaponticum* was also the most effective against MS strains recording a low value of 2.5µg/µl. The *Rosmarinus officinalis* and *Rheum rhaponticum* had the best antimicrobial activity against the QS (MIC_{90} of 2.5µg/µl for both) and MS strains (MIC_{90} of 2.25µg/µl and 4.25µg/µl respectively). All of the crude extracts of the three plants were effective at low doses against Multi Drug Resistant strains. Overall, *Rosmarinus officinalis* was the most effective against most of the strains including MRSA, QS, QR, MS and MR. It was also more effective against MRSA than *Origanum majorana*.

CONCLUSIONS: Antibiotics could potentially be developed from *Rosmarinus officinalis*, *Origanum majorana* and *Rheum rhaponticum* extracts as shown in this study. Further studies should be implemented to investigate the source of antimicrobial effect in these extracts and to highlight the active compounds and their mode of action. Synergistic studies could also be investigated by combining available drugs in use against *S. aureus* and plant extracts from our study. The combinations of these extracts with known drugs have the advantage of lowering the effective dose needed of the drug, and thereby minimizing the side effects of the drugs. Studies to assess the toxicity of these plants should also be performed. Further studies include the study of the effect of these extracts on other bacteria such as *Acinetobacter baumanii* and *Pseudomonas aeruginosa* that are leading causes of hospital acquired infections and are developing high resistance towards commonly used antibiotics.

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