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Anti-bacterial activity of secondary metabolites from *Chrysanthemum cinerariaefolium*

Caroline J. Kosgei[1*], Festus Tolo[3], Josphat C. Matasyoh[2], Meshack Obonyo[1], Peter Mwitari[3], Lucia Keter[3], Richard Korir[3] and Beatrice Irungu[3]

[1]Department of Biochemistry, Faculty of Science, Egerton University, P. O. Box 536-20115 Egerton, Kenya.
[2]Department of Chemistry, Faculty of Science, Egerton University, P. O. Box 536-20115 Egerton, Kenya.
[3]Kenya Medical Research Institute, P. O. Box 54840-00200, Nairobi, Kenya.

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This study evaluated antibacterial activity of *Chrysanthemum cinerariaefolium* (pyrethrum) flower dichloromethane crude extract, fractions and isolated compounds; pyrethrin II, jasmolin I and cinerolone against *methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella sonnei*. The isolated compounds were obtained by carrying out column chromatography on dichloromethane extract and purifying the fractions using preparative High Perfomance Liquid Chromatography (HPLC). The structures of the isolated compounds were elucidated using 1D and 2D NMR. The bioactivity of crude extract, fractions and isolated compounds were determined using disc diffusion assay at a concentration of 100 mg/mL. The MIC and MBC were determined using microdilution method. The bioassay results showed that individually isolated compounds were not active on all the micro-organisms except Jasmolin I which showed slight activity on *P. aeruginosa* with 7.7±0.6 mm. There was significant difference in the activity of the isolated compounds as a mixture and the activity of individual compounds on MRSA, *S. aureus*, *P. aeruginosa*, with P= 0.01, P= 0.0002, P= 0.0007 respectively (α =0.05, Tukey's test). Isolated compounds and isolated compounds as a mixture in a ratio of (1:1:1) were not active on *S. sonnie*. Those fractions and isolated compounds which caused inhibition zones of above 10 mm were subjected to MIC and MBC. The lowest MIC and MBC observed was for fraction 3 against MRSA which were 6.5 and 12.5 mg/mL respectively. The compound mixture had MIC and MBC of 25 and 50 mg/ml respectively against *P. aeruginosa*.

Key words: MIC (minimum inhibitory concentration), MBC (minimum bacteriostatic concentration), bioassay, *Chrysanthemum cinerariaefolium*, Jasmolin, Pyrethrin II, Cinerolone.

INTRODUCTION

Microbial infections continue to be a growing concern in the world due to resistance to current antimicrobial agents. According to estimates by Centre for Disease Control and Prevention (CDC), at least more than 2.8

*Corresponding author. E-mail: rocachep@gmail.com. Tel: +254-707-309-338.

million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result (CDC, 2019). In sub-Saharan Africa, 2.6 million babies required treatment for severe bacterial infection in the first month of life in 2012 (Anna et al., 2014). Bacteria are the causative agents of food borne illness globally in approximately 60% of cases requiring hospitalization (Sapkota et al*.,* 2012).

Antibacterial agents currently used to treat bacterial infections work by inhibiting the growth of micro-organisms or kill them by interfering with cell wall synthesis and DNA replication (Senka et al*.,* 2008). Regrettably, overuse and misuse of these antibacterial agents have led to the development of resistance by bacteria thus rendering these agents ineffective in treating infections associated with these micro-organisms (Davis and Davis, 2010). The concerns about the development of resistance to antimicrobial agents by bacteria drive efforts in bioprospecting for new novel compounds and formulations that can be used to target these resistant microorganisms. Plants remain the potential source of antimicrobial agents since time immemorial with about 60 to 90% of populations in the developing countries being reported to use plant-derived medicine as a traditional form of medicine against various bacterial infections (Alviano and Alviano, 2009). The potency of plant extracts against microbial agents is due to the presence of a variety phytochemicals in plants which include tannins, terpenoids, alkaloids, and flavonoids active against the microbial organisms (Talib and Mahasneh, 2010).

The pyrethrum plant, *Chrysanthemum cinerariaefolium* has a long history of use as an insecticide (Duchon et al*.,* 2009). This is because the plant majorly produces pyrethrins that possess insecticidal properties besides other secondary metabolites. During the1980s and 1990s, Kenya was a global leader in pyrethrum production, contributing over 70% to the global market. The sub sector was a major foreign exchange contributor with earnings rising up to Ksh 2.1 billion in 1996 (Kariuki, 2013). A decline has been seen in the production of pyrethrum globally in the recent years with countries such as Kenya, Papua New Guinea (Corbett, 2015), Tanzania (UNIDO, 2001), Australia (Moslemi, 2017) and Rwanda (Jongschaap, 2018) being affected. This is attributed to introduction of low cost synthetic pesticides known as pyrethroids (Grdiša et al*.,* 2009).

Besides insecticidal properties, the genus *Chrysanthemum* has been reported to possess other medicinal importance (Jung, 2009). For example, flowers of *Chrysanthemum morifolium* Ramat and its herbal infusions are used in the treatment of bacterial and viral infections, sinusitis, blood pressure, digestive, skin problems, influenza virus PR3, leptospira, HIV-1, human colon cancer, headache, dizziness, sore throat, hypertension, flu, cough etc. (Yeasmin et al*.,* 2016). Previous study on *C. cinerariaefolium* has shown that pyrethrin esters isolated from the plant inhibited multiple drug resistant (MDR) *Mycobacterium tuberculosis* at concentrations of 33 and 100 µg/ml (Rugutt et al*.,* 1999).

To the best of the authors' knowledge antibacterial activity of *C. cinerariaefolium* against *(MRSA), Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella sonnei* has not been ascertained. Therefore, finding new biomedical uses of crude extracts, fractions and isolated compounds from *C. cinerariaefolium* would help in curbing bacterial infections besides increasing the demand of pyrethrum; hence help in reviving and revamping the pyrethrum industry in Kenya and other countries that had suffered from upsurge of pyrethroids.

MATERIALS AND METHODS

Collection of plant materials

Pyrethrum flowers were collected from local farmers in Elgeyo-marakwet County which is located at latitude 00 10' to 00 52" N, Longitude 350 25" to 350 45" E and altitude of 8389 m above sea level. The flowers were identified by a botanist Mr Patrick Mutiso and a voucher specimen (Tolo/Mwitari/Keter/002) was deposited at the Center for Traditional Medicine and Drugs Research (CTMDR) at Kenya Medical Research Institute (KEMRI), Nairobi.

Extraction of pyrethrum crude extracts

Air dried and ground plant material was extracted by repeated soaking (2 × 48 h) in a mixture of methanol and dichloromethane (1:1) at room temperature and evaporated to dryness under reduced pressure. Vacuum liquid chromatography (VLC) using solvents of increasing polarity and silica gel (thin layer chromatography grade) was carried out to yield six VLC extracts as follows; VLC 1 (n-hexane), VLC 2 (1:1 n-hexane/dichloromethane), VLC 3 (dichloromethane), VLC 4 (1:1 dichloromethane/ethyl acetate), VLC 5 (ethyl acetate) and VLC 6 (methanol). The VLC extracts were then subjected to antibacterial activity against *P. aeruginosa*, *MRSA*, *S. sonnie,* and *S. aureus*. The most active extract was VLC 3 (dichloromethane extract) and was therefore subjected to column chromatography after a solvent system had been determined using thin layer chromatography (TLC).

Thin layer chromatography

The dichloromethane dry extract was first dissolved in dichloromethane solvent followed by spotting on 2×5 aluminum backed TLC plates using a capillary tube. The solvent mixture that gave optimum separation for the dichloromethane extract was acetone: petroleum ether (A: P) 5: 5.

Column chromatography

The dry dichloromethane extract was placed on top of silica in the column and the solvent system obtained from TLC analysis was then added to the reservoir attached to the column. The column was eluted gradually and the flow rates maintained at approximately 15 ml/5 min. A total of 20 fractions of equal volume were collected and TLC analysis of each fraction performed. Fractions with similar TLC patterns were pooled together resulting in 4 fractions as shown in Figure 1. After carrying out preliminary bioassay, fraction 4 was the most active fraction with a yield of 2.5 g.

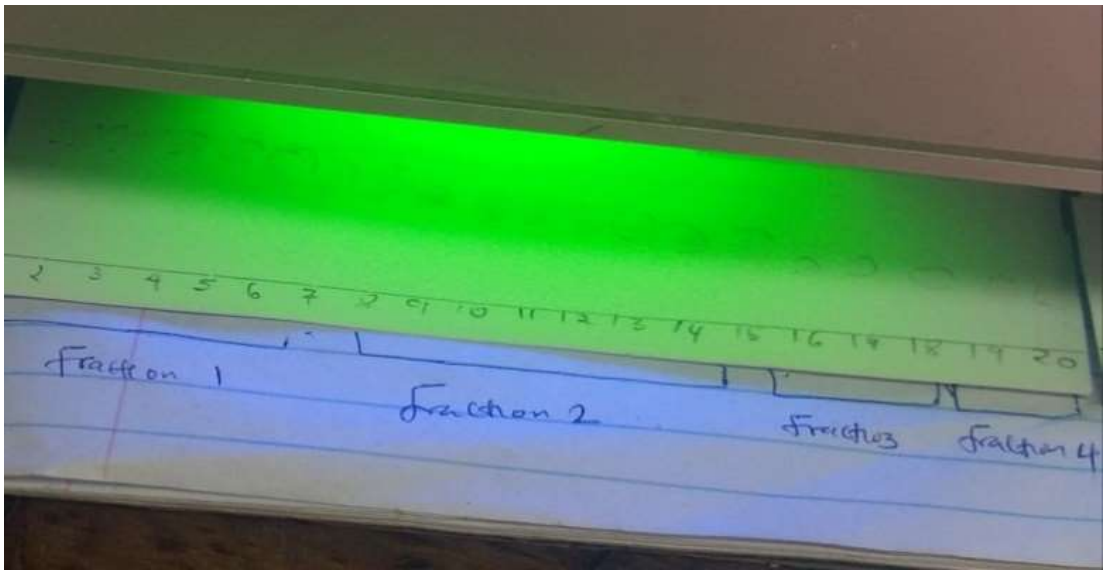

Figure 1. TLC chromatogram showing four fractions obtained from column chromatography of the dichloromethane extract.

The fraction was therefore subjected to preparative high performance liquid chromatography (HPLC) to obtain the pure compounds.

Preparative high performance liquid chromatography (HPLC)

Fraction 4 of dichloromethane extract was purified using preparative HPLC equipped with UV-vis detector with a stationery phase Grom-Sil 120 ODS-5 (250 × 20 mm; 10 µm; Grace Davison, Deerfield, IL, USA) column. The gradient applied was t_0-t_{20} = 5-100% B, t_{21}-t_{23} = 100% B and re-equilibration at 15% (B) till t_{25} with a flow rate of 15 mL/min. Solvents used were water and acetonitrile spiked with 0.1% HCOOH. The compounds were eluded at different times as follows, Compound 1 (0.028 g, t_R 22.5 min), Compound 2 (0.6 g, t_R 19 min) and Compound 3 (0.3 g, t_R 12.5 min). The compounds were then divided into two portions; one portion of each compound was used for 1D and 2D high field NMR spectroscopy analysis while the other portions were subjected to assays against selected bacteria.

Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H, ^{13}C, DEPT, HSQC, COSY and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrometer at the Technical University of Berlin, Germany. The readings were done in DMSO and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The structures were then simulated using ACD NMR manager program to obtain the chemical shifts of proton. The off- diagonal elements were used to identify the spin - spin coupling interactions in the ^{1}H-^{1}H COSY (Correlation spectroscopy). The proton-carbon connectivity, up to three bonds away, was identified using ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) spectrum. The ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectrums were used to determine the connectivity of hydrogen to their respective carbon atoms.

Antibacterial bioassay

Test micro-organisms

Four bacterial strains *methicillin-resistant Staphylococcus aureus* (MRSA) (Clinical isolate), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923) and *S. sonnei* (ATCC 25931), were used for assay of antibacterial activities. The four micro-organisms were obtained from Kenya Medical Research Institute (KEMRI).

Disc diffusion assay

The disc diffusion method for antibacterial susceptibility testing was carried out according to NCCLS (2000). Mueller-hinton agar was prepared according to the manufacturer's instruction and dispensed at 20 ml per plate in a Petri dish. Suspension of selected micro-organism was made in a sterile normal saline and adjusted to 0.5 Mc Farland standard (10^8 cfu/ml). Each labeled medium plate was then inoculated with *P. aeruginosa, S. sonnie, S. aureus,* and *MRSA* using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. Dichloromethane crude extract, fractions, and isolated compounds equivalent to 100 mg/mL were applied to sterile paper discs (6 mm diameter) and the discs deposited on the surface of the inoculated agar plates and incubated for 24 h at 37°C. Mixture of the isolated compounds in a ratio of (1:1:1) at the same concentration of 100 mg/ml were also used in the bioassay. Zones of inhibition were measured in millimeter after 24 h of growth. The inhibition zones less than 10 mm in diameter were not considered for the antibacterial MIC and MBC analysis. For each extract, 3 replicates were assayed. The negative control used in this experiment was 1% dimethyl sulfoxide (DMSO) whereas 30 µg/disc chloramphenicol discs were used as the positive control. All tests were performed in triplicates.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined using the microdilution method as

described by Clinical and Laboratory Standards Institute (2009). Serial two-fold dilutions of all the extracts were prepared with sterile saline in a 96-well microtiter plate, obtaining a concentration range from 100 to 6.5 mg/mL. It was followed by addition of 5 µL of *P. aeruginosa, S. sonnei*, *MRSA* and *S. aureus* suspension which were added to the wells containing the dilutions. Each dose was assayed in triplicate. Uninoculated wells containing sterile saline and extract were used as controls. After incubation for 24 h at 37°C, the samples were observed. MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity. To estimate the MBC, an aliquot of each well that did not show microbial growth in the prior tests was swabbed on the entire surface of Muller Hinton Agar plates and then incubated under the growth conditions described before. The lowest concentration that prevented the bacterial growth was registered as MBC.

Data analysis

Significant difference between means of zone of inhibition of the isolates by the extracts were determined using Tukey's test (α =0.05)

RESULTS AND DISCUSSION

Structure elucidation of the isolated compounds

The three isolated compounds, Compounds 1, 2, and 3 were subjected to 1D and 2D NMR spectroscopic studies such as ^{1}H NMR, HMBC, COSY and DEPT NMR, as described in the methodology. This resulted in the elucidation of the compounds using spectral data summarized in Table 1.

The three isolated compounds in this study have been previously isolated and characterized (Bramwel et al*.,* 1969; Crombie et al*.,* 1969; Rugutt et al*.,* 1999). The ^{1}H NMR spectrum of compound **1** displayed signals at δ_H 4.98 (1H, dq, *J* = 8.1, 1.4 Hz), 1.96 (1H, dd, *J* = 8.1, 5.3 Hz) and δ_H 1.55 (1H, dd, *J* = 5.3Hz) assignable to the olefinic proton H-7, and the cyclopropane ring protons H-3 and H-1, respectively, deduced to be part of a *cis*-chrysanthemate moiety (Patenden et al*.,*1973). The protons for the two methyl groups attached to the cyclopropane ring resonated at δ_H 1.22 (3H, s) and 1.12 (3H, s). Additionally, the ^{1}H NMR spectrum showed another signal at δ_H 5.65 (1H, dd, *J* = 18.6, 1.7 Hz), 2.82 (1H, dd, *J* = 18.6, 6.3 Hz) and δ_H 2.13 (1H, dd, *J* = 6.3, 1.7 Hz) corresponding to the cyclopentenone ring protons H-1′, H-5a′ and H-5b′, respectively. Further, a doublet due to the exocyclic methylene protons was observed at δ_H 2.92 (2H, d, *J* = 7.4, H-7′), two sets of doublets of triplets due to the olefinic protons (designated H-8′ and H-9′) in a *Z* configuration based on the coupling constant (10.5 Hz), a methylene proton multiplet at δ_H 2.15 (2H, m, H-10′), and a saturated methyl triplet at δ_H 0.96 (3H, t, *J* = 7.5, H-11′) for the protons in its side-chain (Patenden et al*.,*1973).

The ^{13}C NMR, DEPT and HSQC analysis confirmed the presence of 21 carbons attributable to a carbonyl carbon at δ_C 203.1 (C-4′), an ester carbonyl carbon at δ_C 171.4 (C-4), four quaternary carbons at δ_C 164.8 (C-2′), δ_C 141.5 (C-3′), δ_C 134.5 (C-8) and δ_C 28.2 (C-2), six methine carbons at 132.4 (C-9′), δ_C 124.3 (C-8′), δ_C 120.9 (C-7), δ_C 72.9 (C-1′), δ_C 33.6 (C-1) and δ_C 32.2 (C-3), six methylene carbons at δ_C 41.5 (C-5′), δ_C 21.6 (C-10′) and δ_C 20.7 (C-7′), and six methyl carbons at δ_C 25.3 (C-10), δ_C 20.1 (C-6), δ_C 20.0 (C-5), δ_C 18.1 (C-9), δ_C 13.9 (C-11′) and δ_C 13.7 (C-6′). The HMBC cross-peaks from H-5 and H-6 methyl protons to C-1 and C-2 suggested the location of these methyl groups at C-2 while the attachment of the 2-methylprop-1-en-1-yl group to C-3 was suggested by the HMBC cross-peaks from H-7 to C-1. The pent-2-en-1-yl group was determined to be at C-2′ based on the HMBC cross-peaks from H-7′ to C-2′, C-3′, C-4′ and those from H-8′to C-3′. From the above evidence and previous studies, the structure of compound **1** was elucidated as (Z)-2-methyl-4-oxo-3-(pent-2-en-1-yl)cyclopent-2-en-1-yl2,2-dimethyl-3-(2-methylprop-1-en-1-yl)cyclopropane-1-carboxylate, commonly known as jasmolin I (Bramwel et al*.,* 1969). The structure, HMBC and ^{1}H-^{1}H COSY correlations for Compound **1** are shown in Figures 2 and 3 respectively. The ^{1}H NMR and ^{13}C NMR spectra of Compound **2** closely resembled those of compound **1** except for a few noted differences. First, there was a disappearance of the C-9 methyl carbon signal accompanied by the appearance of methyl ester carbon signals at δ_C 167.6 (C-9) and δ_C 52.1 (9-OCH_3) attached to C-8 in compound **2** (Rugutt et al*.,* 1999). The attachment of this group at C-8 was suggested by the HMBC cross-peaks from the olefinic proton H-7 to the ester carbonyl carbon at C-9. Second, there was the replacement of the pent-2-en-1-yl substituent in compound 1 with the penta-2,4-dien-1-yl substituent in 2 as evident from the doublet at δ_H 3.10 (2H, d, *J* = 7.7, H-7′), a doublet of triplet at δ_H 6.85 (1H, dt, *J* = 16.8, 10.8, H-8′), a triplet at δ_H 6.01 (1H, t, J = 10.8, H-9′), a multiplet at δ_H 5.36 (1H, m, H-10′) and a set of two doublet of doublets for the two terminal olefinic protons at δ_H 5.27 (IH, dd, *J* = 16.8, 2.2, H-11a′) and δ_H 5.20 (IH, dd, *J* = 10.2, 2.2, H-11b′). The direct bonding of proton to carbons was derived from the HSQC spectra and the structure of compound **2** was elucidated to be 2-methyl-4-oxo-3-((Z)-penta-2,4-dien-1-yl)cyclopent-2-en-1-yl-3-((E)-3-methoxy-2-methyl-3-oxoprop-1-en-1-yl)-2,2-dimethylcyclopropane-1-carboxylate, commonly known as pyrethrin II (Rugutt et al*.,* 1999). The structure, HMBC and ^{1}H-^{1}H COSY correlations for Compound **2** are shown in Figures 4 and 5 respectively.

The ^{1}H NMR spectrum of Compound **3** displayed signals at δ_H 4.52 (1H, d, *J* = 6.3 Hz), 2.63 (1H, dd, *J* = 18.1, 6.3 Hz) and δ_H 2.06 (1H, m) for the cyclopentenone ring protons H-4, H-5a and H-5b, respectively. Additionally, the ^{1}H NMR spectrum also displayed signals for a doublet due to the exocyclic methylene protons at δ_H 2.90 (2H, d, *J* = 6.2, H-1′), two sets of multiplets for the

Table 1. NMR spectroscopic data for isolated compounds (1, 2, 3).

Compound 1			Compound 2			Compound 3		
Position	**δ_H, mult. (*J* in Hz)**	**δ_C, type**	**Position**	**δ_H, mult. (*J* in Hz)**	**δ_C, type**	**Position**	**δ_H, mult. (*J* in Hz)**	**δ_C, type**
1	1.55, d (5.3)	33.6, CH	1	2.08, d (5.2)	35.2, CH	1		204.8, C=O
2		28.2, C	2		30.4, C	2		138.2, C
3	1.96, dd (8.1, 5.3)	32.2, CH	3	2.15, dd (9.7, 5.2)	32.6, CH	3		170.9, C
4		171.4, C	4		171.0, C	4	4.52, d (6.3)	69.9, CH
5	1.12, s	20.0, CH_3	5	1.20, s	22.1, CH_3	5a	2.63, dd (18.1, 6.3)	43.9, CH_2
6	1.22, s	20.1, CH_3	6	1.26, s	20.7, CH_3	5b	2.06, m	
7	4.98, dq (8.1, 1.4)	120.9, CH	7	6.50, dt (9.7, 1.4)	139.8, CH	6	2.01, s	13.6, CH_3
8		134.5, C	8		129.1, C	1′	2.90, d (6.2)	21.4, CH_2
9	1.68, d (1.3)	18.1, CH_3	9		167.6uu, C	2′	5.27, m	126.8, CH
10	1.71, d (1.4)	25.3, CH_3	10	1.89, d (1.4)	13.1, CH_3	3′	5.27, m	131.9, CH
1′	5.65, dd (18.6, 1.7)	72.9, CH	1′	5.67, dd (18.5, 1.7)	73.7, CH	4′	1.03, s	22.8, CH_3
2′		164.8, C	2′		166.6, C	4-OH	8.15, s	
3′		141.5, C	3′		141.5, C			
4′		203.1, C=O	4′		203.5, C=O			
5a′	2.82, dd (18.6, 6.3)	41.5, CH_2	5a′	2.85, dd (18.5, 6.3)	41.9, CH_2			
5b′	2.13, dd (6.3, 1.7)		5b′	2.15, dd (6.3, 1.7)				
6′	2.03, s	13.7, CH_3	6′	2.04, s	14.2, CH_3			
7′	2.92, dd (7.3, 1.7)	20.7, CH_2	7′	3.10, d (7.7)	22.1, CH_2			
8′	5.22, dt (10.5, 7.4)	124.3, CH	8′	6.85, dt (16.8, 10.8)	132.4, CH			
9′	5.39, dt (10.5, 1.7)	132.4, CH	9′	6.01, t (10.8)	130.5, CH			
10′	2.15, m	21.6, CH_2	10′	5.36, m	127.8, CH			
11′	0.96, t (7.5)	13.9, CH_3	11a′	5.27, dd (16.8, 2.2)	118.8, CH_2			
			11b′	5.20, dd (10.2, 2.2)				
			9-OCH_3	3.68, s	52.1, CH_3			

olefinic protons at δ_H 5.27 (1H, m, H-2') and δ_H 5.27 (1H, m, H-3'), and a singlet at δ_H 1.03 (3H, s, H-4') for the but-2-en-1-yl substituent. A signal for methyl protons was also observed at δ_H 2.01 (3H, s, H-6).

The ^{13}C NMR and HSQC data of Compound **3** exhibited 10 carbon signals which were classified as carbonyl carbon (δ_C 203.1, C-1), two quaternary carbons at δ_C 170.9 (C-3) and δ_C 138.2 (C-2), three methine carbons at δ_C 131.9 (C-3′), δ_C 126.8 (C-2′) and δ_C 69.9 (C-4), two methylene carbons at δ_C 43.9 (C-5) and δ_C 21.4 (C-1′), and two methyl carbons at δ_C 22.8 (C-4′) and δ_C 13.6 (C-6) (Crombie et al*.,* 1969). The HMBC cross-peaks from H-5 to C-1/C-3/C-4 and from H-4 to C-2/C-3 revealed that the carbonyl group was at C-1 and the hydroxyl group was attached to C-4. The HMBC cross-peaks from H-1′ to C-1′/C-2′/C-3′ were used to locate the but-2-en-1-yl substituent at C-2, while the HMBC cross-peaks from the methyl protons H-6 to C-2/C-3 suggested the attachment of this methyl group at C-3. Therefore, the compound was characterized as (*Z*)-2-(but-2-en-1-yl)-4-hydroxy-3-methylcyclopent-2-en-1-one, commonly known as cinerolone (Crombie et al*.,* 1969). The structure, HMBC and ^{1}H-^{1}H COSY correlations for

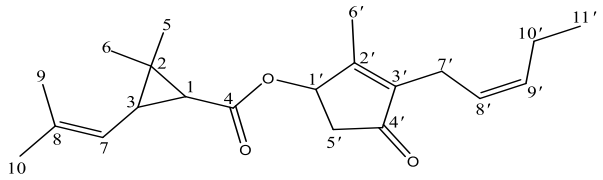

Figure 2. (Z)-2-methyl-4-oxo-3-(pent-2-en-1-yl)cyclopent-2-en-1-yl2,2-dimethyl-3-(2-methylprop-1-en-1-yl)cyclopropane-1-carboxylate (Jasmolin I).

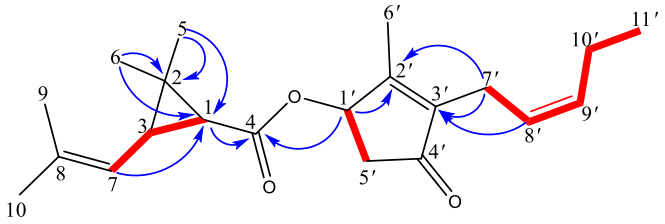

Figure 3. HMBC (blue) and ^{1}H-^{1}H COSY (red bold lines) correlations of Jasmolin I.

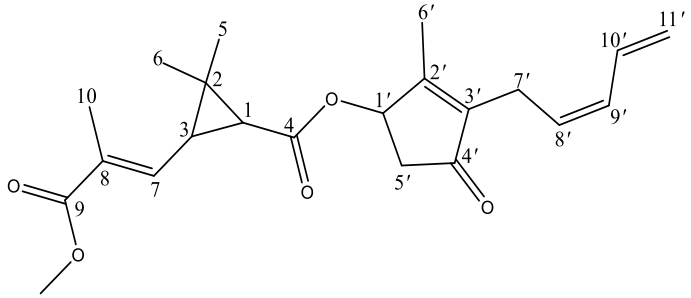

Figure 4. 2-methly-4-oxo-3((*Z*)-penta-2,4-diene-1-yl)cyclopent-2-en-1-yl 3-((*E*)-3-methoxy-2-methly-3-oxoprop-1-en-yl-2,2-dimethycyclopropane-1-carboxylate (Pyrethrin II).

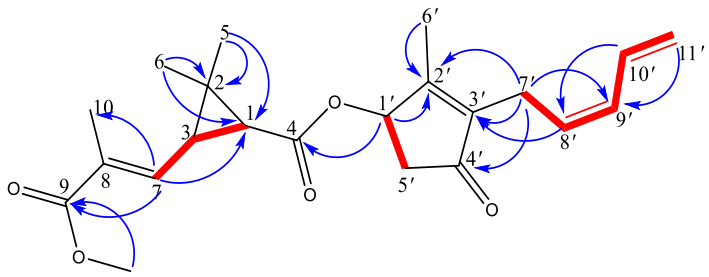

Figure 5. HMBC (blue) and 1H-1H COSY (red bold lines) correlations of Pyrethrin II.

Compound **3** are shown in Figures 6 and 7 respectively.

Antibacterial activity of pyrethrum extracts and isolated compounds

Results of the bioassay against the selected organism

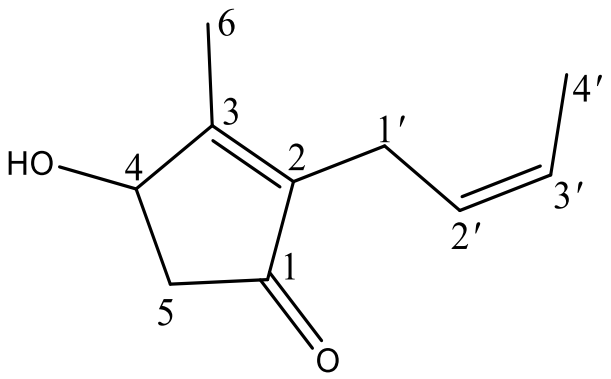

Figure 6. (Z)-2-(but-2-en-1-yl)-4-hydroxy-3-methylcyclopent-2-en-1-one (Cinerolone)

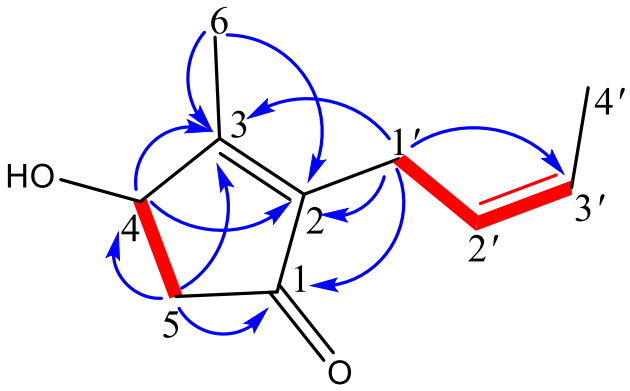

Figure 7. HMBC (blue) and ^{1}H-^{1}H COSY (red bold lines) correlations of cinerolone.

using disc diffusion assay are as shown in Table 2 and Figures 8 to 12. The values are the mean of three experiments ± S.D. Within a column, the inhibition zones of extracts sharing the same letter(s) were not significantly different while those with different letters are significantly different (*α =0.05, Tukey's test*). The extracts which caused inhibition zone of above 10 mm mean ± S.D were subjected to MIC and MBC. These extracts were fraction 1, 3 and 4 subjected to *MRSA* and fraction 1, 2, 3, 4 and isolated compounds as a mixture (1:1:1) subjected to *P. aeruginosa.* The MIC for fraction 1, 3 and 4 against *MRSA* was 12.5, 6.5 and 12.5 mg/ml respectively while the MBC for fraction 1, 3 and 4 against *MRSA* was 25, 12.5 and 25 mg/mL respectively. The MIC for fraction 1, 2, 3, 4 and compound mixture against *P. aeruginosa* was 12.5, 25, 25, 25 and 25 mg/ml respectively while the MBC for fraction 1, 2, 3, 4 and compound mixture against *P. aeruginosa* were 25, 50, 50, 50 and 50 mg/ml, respectively.

In the current study, dichloromethane extract of *C. cinerariaefolium* showed some degree of activity against

Table 2. Inhibition zones (mm) of different extracts on the test organisms.

Extracts	Test organisms			
	MRSA	***S. aureus***	***P. aeruginosa***	***S. sonnie***
DCM crude extract	10.7±1.2[a]	10.3±0.6[a]	7.2±0.3[a]	6.3±0.3[b]
Fraction 1	12±0.5[a]	7±0.5[b]	11.7±0.6[b]	6±0[b]
Fraction 2	7.6±0.6[b]	7.4±0.6[bf]	11.6±0.5[b]	6±0[b]
Fraction 3	11±1[a]	6.6±0.2[b]	17.3±1.2[c]	6±0[b]
Fraction 4	12.3±0.6[a]	9.8±1[af]	22.7±1.2[d]	6±0[b]
Compound 1	6±0[e]	6±0[b]	7.7±0.6[a]	6±0[b]
Compound 2	6±0[e]	6±0[b]	6±0[e]	6±0[b]
Compound 3	6±0[e]	6±0[b]	6±0[e]	6±0[b]
Compound mixture	7.3±0.6[b]	8.2±0.3[f]	14±0[g]	6±0[b]
Chloramphenical [P]	26.7±1.2	24.2±0.8	26±1	24.7±1.3
DMSO+distilled H_2O[Q]	6±0	6±0	6±0	6±0

***** Within a column similar letters shows no significant differences while different letters show significant difference**.** [P] Positive control, [Q] negative control.

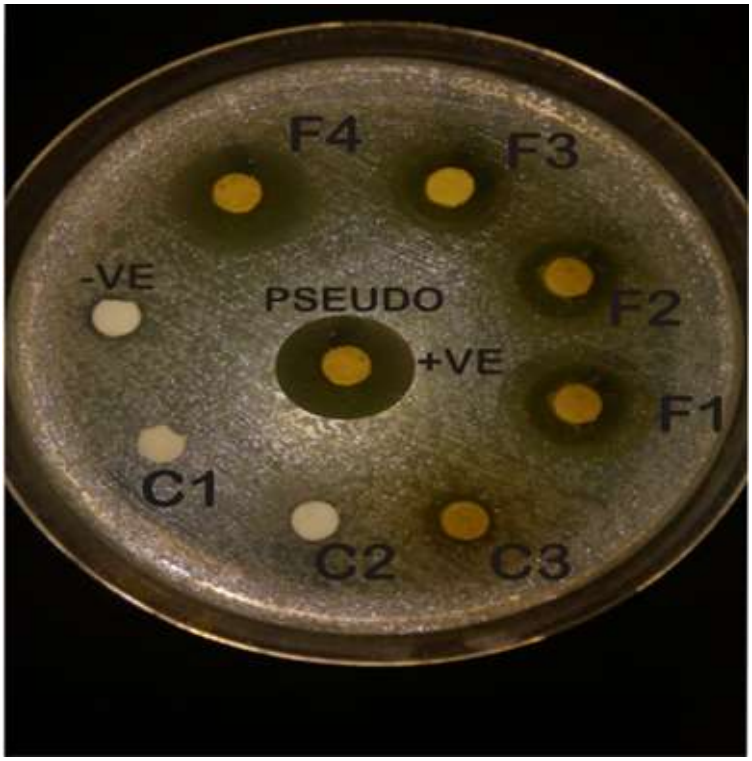

Figure 8. Screening of dichloromethane fractions (f1-f4) and isolated compounds (C1-C3) against *P. aeruginosa*.

the selected bacteria; an indication that the plant has some bioactivity against both gram negative and gram positive bacteria. This concurs with a previous study which showed that large numbers of *Chrysanthemum* extracts were active against both gram-positive and gram-negative bacteria (Sassi et al*.,* 2008). It is also coinciding with previous studies which have shown that flowers of members of the *Chrysanthemum* genus (*Asteraceae*) possess phytochemicals that are of medicinal importance (Jung, 2009). For example, flowers of *Chrysanthemum indicum* have been used in folk medicine for the treatment of several infectious disease

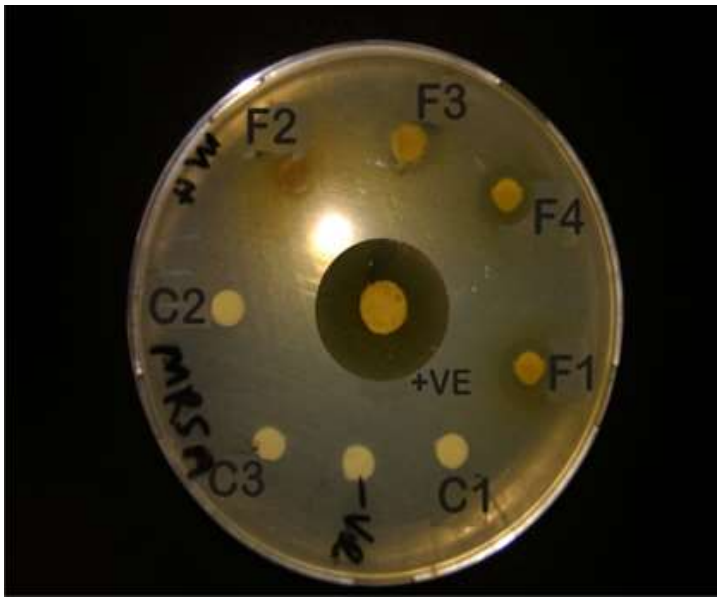

Figure 9. Screening of dichloromethane fractions (f1-f4) and isolated compounds (C1-C3) against *MRSA*.

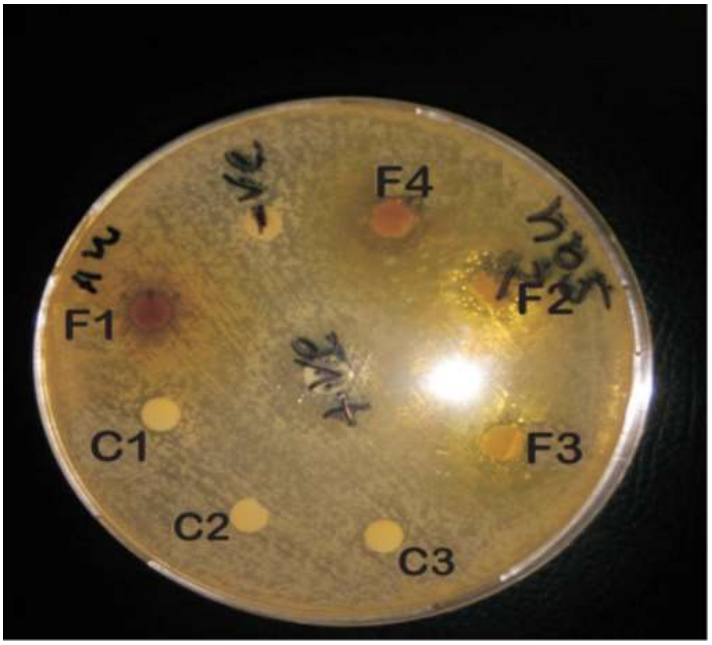

Figure 10. Screening of dichlorometahne fractions (f1-f4) and isolated compounds (C1-C3) against *S. aureus*

such as pneumonia, colitis, stomatitis, cancer, fever, sore and hypertensive symptom (Jung, 2009).

Generally, the dichloromethane crude extract was more active against the gram positive bacteria, that is, *MRSA* and *S. aureus* than the gram negative bacteria that is, *P. aeruginosa* and *S. sonnei*. This is because gram-negative bacteria have been reported to be less susceptible to crude extracts than the gram positive bacteria due to presence of cell membrane restricting the diffusion of compounds through its lipopolysaccharide layer (Perussi, 2007). The fractions and isolated compounds as a mixture in the ratio of (1:1:1) also showed some degree of bioactivity against all the selected micro-organsisms except *S. sonnei*. In contrast, to the bioactivity observed

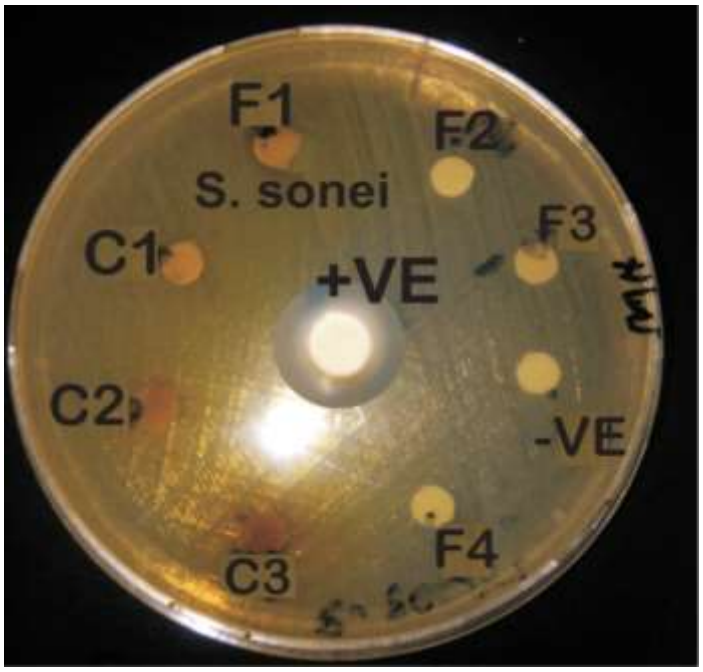

Figure 11. Screening of dichlorometahne fractions (f1-f4) and isolated compounds (C1-C3) against *S. sonnie*

Figure 12. Screening of isolated compounds as a mixture in triplicate at concentration of 100 mg/ml against *P. aeruginosa*.

in the crude extracts, the fractions were more active on gram negative bacteria *P. aeruginosa* than both gram positive bacteria. This observation may be attributed to the fact that the amount of the active components in the crude extract may have been diluted and fractionation may have increased their concentrations, hence reason for enhanced bioactivity in *P. aeruginosa*. There could also be possibility of antagonism among various

antibacterial compounds in crude extracts when lumped together thus fraction may have reduced leading to enhanced activity observed in *P. aeruginosa* (Kuete et al*.,* 2011).

Individually, the isolated compounds did not show any bioactivity against the selected microorganisms except Jasmolin I which showed some slight activity against *P. aeruginosa*. When the three compounds were mixed together at the same concentration and ratio, there was increased bioactivity on the selected micro-organisms suggesting synergy. The activity observed in the compounds as a mixture could be due to cyclopropyl fragment ring in jasmolin I and pyrethrin II. These molecules have been reported to improve the overall activity of majority of biologically important molecules that contain them by acting as potent alkylation agent (Peterson*,* 2001). The cyclopropyl fragment is a versatile player that frequently appears in preclinical/clinical drug molecules (Tanaji, 2016). These molecules include quinolone antibiotics such as ciprofloxacin, clinofloxacin gemifloxacin and moxifloxacin. Other molecules that have the cyclopropyl fragment include tyrosine kinase inhibitor (4/lucitanib), HCV NS3/4A protease inhibitors, HIV-1 reverse transcriptase inhibitor (Lumacaftor), calcitriol, calcipotriol, amitifadine and etomide pro-drugs among others (Tanaji, 2016). In molecular structure-activity relationship studies of quinolone antibiotics it is evident that a cyclopropyl at position 1 of these quinolones are the optimal substituents, regardless of the changes made at other sites (Peterson, 2001).

The cyclopropane fragment has been reported to possess spectrum of biological properties ranging from enzyme inhibitions to insecticidal, antifungal, herbicidal, antimicrobial, antibiotic, antibacterial, antitumor and antiviral activities. Previous studies have shown that cyclopropane associated with fatty acids have been proven to have antifungal activity (Pohl et al*.,* 2011). Another study showed cyclopropane associated with fatty acids from the marine bacterium *labrenzia* exhibited antimicrobial activity and it also activated orphan G-protein coupled receptor GPR84, which is vastly expressed on immune cells (Moghaddam et al*.,* 2018).

Fraction 4 was more active than the other fractions on *P. aeruginosa* since it contained all the isolatedcompounds besides other biomolecules. The isolated compounds showed significant bioactivity against *P. aeruginosa* as a mixture in the ratio of (1:1:1). The proportion of the isolated compounds in fraction 4 could have been high before the compounds were fractionated. This therefore suggests synergy among the isolated compounds in fractions 4 together with other compounds present in this fraction.

Conclusion

Results of this study show that pyrethrum crude extracts, fractions and isolated compounds possess antibacterial activity against both gram negative and gram positive bacteria. To the best of the authors' knowledge there is no previous work on antibacterial activity of *C. cinerariaefolium* on the selected bacteria; hence the findings will aid in exploitation of other uses of the plant besides aiding in the discovery of new antibacterial drugs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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