Isolation and characterization of a new oxygenated homoditerpenoid from leaves of *Centaurothamnus maximus* with antimicrobial potential

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**Abstract:** A new bioactive oxygenated homoditerpenic compound along with one known compound from the antimicrobial active ethanol extract of leaves of an endemic plant *Centaurothamnus maximus* was isolated. The n-hexane, dichloromethane, ethyl acetate and ethanol fractions of *C. maximus* leaves were evaluated for their antimicrobial potential by using standard agar well diffusion method against various microorganisms viz. *B. subtilis, S. aureus, E. coli, P. aeruginosa, C. albicans* and *M. smegmatis*. The results revealed that only ethanol extract was active against all microbes except the fungus *C. albicans*. A new compound 2\(\alpha\), 3\(\alpha\)-dihydroxy-8\(\alpha\)-methoxy-15-hydroxy-methylene-

\(~\)pimar-5,9(11)-diene (CM-1) was isolated along with a known compound 6\(\alpha\)-D-xylose (CM-2) from ethanol extract by reverse phase (RP-18) column chromatography and 1D and 2D NMR (DEPT, COSY, HMBC and HSQC) aided by EIMS mass and IR spectra were used to establish the structure. CM-1 was found to be active against *B. subtilis, S. aureus* and *M. smegmatis* (P>0.005) at MIC 20 \(\mu\)g/ml. Findings of this study may provide a lead for synthesis of more potent antimicrobial agents to serve the humanity against multidrug-resistant bacterial infections.

**Keywords:** *Centaurothamnus maximus*, Asteraceae, isolation, homoditerpenoid, antimicrobial.

**INTRODUCTION**

*Centaurothamnus maximus* (Asteraceae) is a paleoendemic species grown in Saudi Arabia. It is found only in two localities in Saudi Arabia and in a few high altitude cliffs of Yemen. Compared to the populations in Yemen, the density and distribution of this species in Saudi Arabia are highly restricted, represented by not more than 200 plants (Basahi et al., 2010). *C. maximus* is about 1.5 m tall leafy shrub (Collenette, 1985) and its aerial parts are reported to contain three cytotoxic sesquiterpene lactones namely guainolides including chlorojariner, cynapricrin and jainerin (Muhammad et al., 2003). Diterpenoids were reported to be found in Asteraceae (Guo et al., 2006). Methanolic extract of *C. maximus* was found to possess a noteworthy growth inhibitory effect at IC50 (<50 \(\mu\)g/mL) against the human lung cancer, urinary bladder cancer and breast cancers cell lines and also exhibited excellent antimicrobial property against Gram-positive multiresistant bacteria at MIC values < 500\(\mu\)g/mL\(^1\). However *C. maximus* exhibited good antioxidant property at high concentrations (Mothana et al., 2009). Some naturally occurring diterpenoids, including taxodione, salvinolone, 14-deoxycoleon U, were isolated from *Taxodium distichum* cones and they possess significant antimicrobial activity when evaluated *in vitro* against *Mycobacterium phlei* (IFO 3158) (Kusumoto et al., 2014). A Clerodane diterpenoid and a spinasterol isolated from *M. angolensis* exhibited significant antibacterial activities against *Enterococcus faecalis* (Tamokou et al., 2009). There are a large number of oxygenated diterpenoids reported from Genus *Isodon* (Family: Lamiaceae) with antibacterial activity (Sun et al., 2006).

**MATERIALS AND METHODS**

**General**

ATI Mattson genesis series Fourier transform (FT–IR) spectrophotometer was used to record IR spectra. Hewlett Packard 8452A diode array spectrophotometer was used to obtain UV spectra. JASCO DIP- 370 digital polarimeter was used to record the optical rotation at ambient temperature. Bruker Avance DRX 500 spectrometer was used to obtain 1D and 2D NMR spectra for 1H and 13C. Bruker Bioapex FT–MS was used to obtain the EI mass spectra. Silica gel (70-230 mesh) and LiChroprep RP-18 [40-63 \(\mu\)m; octadecyl silica (ODS) gel] (Merck) were employed for column chromatography. The AR grade chemicals n-Hexane, ethyl acetate, chloroform, methanol, ethanol, water, sulphuric acid and vanillin were procured from Faisal Zouman Al-Anazi Trading Est., Riyadh, Saudi Arabia. TLC was carried out on glass backed silica gel F\(_{254}\) plate (E. Merck) and derivatization of plates were carried out by using \(p\)-anisaldehyde solution as a spray reagent.

**Plant material**

The collection of *C. maximus* leaves was done in March 2006 from Aqabaat Al-Makhwah, after tunnel # 13, Saudi
Arab. The Identification of plant was done by Field Taxonomist, Pharmacognosy Department, College of Pharmacy, KSU, Riyadh. Voucher Specimen (Voucher # 15024) is deposited in herbarium of Pharmacognosy Department.

**Extraction and isolation**
The collected leaves (1.0 kg) were air dried, powdered and exhaustively extracted in soxhlet apparatus with n-hexane. This process was repeated, until the complete exhaustion of the plant material. Rotary evaporator was then used to concentrate the extract under reduced pressure. Remaining marc was dried and extracted with the same apparatus till exhaustion of the drug material using dichloromethane and concentrated with rotary evaporator. The same procedure was followed for extraction with ethyl acetate and ethanol (90%). The obtained extracts were concentrated to dryness, weighed and investigated for antibacterial activity. Bioactive ethanol extract was subjected for isolation by reverse phase column chromatography using LiChroprep RP-18 as stationary phase and water, methanol, chloroform, ethyl acetate and n-hexane used in different combinations as eluent. CM-1 (1.3g) was obtained using gradients of chloroform: ethyl acetate (80:20) as solid off white crystalline powder, soluble in methanol and CM-2 (267 mg) with methanol: chloroform (90:10) as white crystalline solid.

**Antimicrobial activity**
Sample Preparation
For the antimicrobial screening a concentration of 512 μg/mL for different extracts of *C. maximus* and 40μg/mL of CM-1 was used. The concentrations for extracts were achieved by dissolving 2.56mg of extracts in 5 ml solvent and for CM-1 it was achieved by dissolving 1mg of CM-1 in 25mL Chloroform (NCCLS, 2003).

**Sources and maintenance of organisms**
Gram-positive organisms *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 29213), *M. smegmatis* (ATCC 35797), Gram-Negative organisms *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 15442) and a fungal yeast *C. albicans* (ATCC 90028) were maintained at Microbiology lab of Central Research Laboratory, College of Pharmacy, KSU, Riyadh, K.S.A. All the Organisms were maintained on Mueller- Hinton Agar medium (Oxoid, UK). Each of Gram-positive organisms *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 6633), *S. chromogenes* (ATCC 6099), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 15442) and a fungal yeast *C. albicans* (ATCC 35797), *C. neoformans* (ATCC 90028) were maintained at Microbiology lab of Central Research Laboratory, College of Pharmacy, KSU, Riyadh, K.S.A. All the Organisms were maintained on Mueller- Hinton Agar culture medium (Oxoid, UK). Each of Gram-positive organisms *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 6633), *S. chromogenes* (ATCC 6099), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 15442) and a fungal yeast *C. albicans* (ATCC 35797), *C. neoformans* (ATCC 90028) were maintained at Microbiology lab of Central Research Laboratory, College of Pharmacy, KSU, Riyadh, K.S.A. All the Organisms were maintained on Mueller- Hinton Agar culture medium (Oxoid, UK). Each of Gram-positive organisms *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 6633), *S. chromogenes* (ATCC 6099), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 15442) and a fungal yeast *C. albicans* (ATCC 35797), *C. neoformans* (ATCC 90028) were maintained at Microbiology lab of Central Research Laboratory, College of Pharmacy, KSU, Riyadh, K.S.A. All the Organisms were maintained on Mueller- Hinton Agar culture medium (Oxoid, UK).

**Isolation**
The preparation of culture media was carried out as per the instruction given by the manufacturer. Culture media was then autoclaved and 20 ml of culture media was dispensed in each plate of 12x12cm Petri dishes. To ensure the sterility of set plates they were incubated overnight before use.

**Antimicrobial assay**
The microbial suspensions were formed in normal sterile saline and adjusted to 0.5 Macfarland standards (10 Cfu/ml). A serial dilutions of 256, 128, 64, 32, 16, 8, 4 µg/mL were made from the 512µg/mL of stock solutions of different extracts and 30, 20, 10, 5, 2.5, 1µg/mL of serial dilutions were made from the 40μg/mL of stock solution of CM-1 (NCCLS, 2003). A sterile cotton swab was used to evenly inoculate the test microorganisms on the surface of every labeled medium plate by streaking to get a lawn growth. A sterile cork borer of 5mm diameter was used to make wells on the medium. In each well 0.1ml of the different extracts and CM-1 concentrations were filled and labeled appropriately (Shahidi Bonjar, 2004). For the proper diffusion of extracts in to the agar medium the inoculated plates were kept in the refrigerator for 1 hour (Atta et al., 2003). The Agar plates were then incubated at 37°C for 24 hours. The diameter of zone of inhibition and minimum inhibitory concentration (MIC) were used to determine antimicrobial potential of extracts as well as CM-1 (Prescott et al., 1999). 0.05% of Ciprofloxacin was used as positive control and blank plate containing only medium was used as negative control (NCCLS, 2003).

**STATISTICAL ANALYSIS**
The values of antimicrobial activity of the leaves extract of *C. maximus* were expressed as mean ± standard deviation (n=3) for each sample. Data were analyzed by one-way ANOVA and p values were considered significant at P<0.005.

**RESULTS**

**Isolation**
Compound CM-1 was obtained as solid off white crystalline powder from gradients of chloroform and ethyl acetate (80:20) eluents.

M.P.: 233.2°C; [α]D = +0.0356° (CHCl3); IR νmax (KBr): 3412, 3325, 2921, 2850, 1625, 1465, 1350, 1261 and 1048 cm⁻¹; ¹H NMR (MeOD): δ 5.36 (1H, d, J= 5.2 Hz, H-6), 5.22 (1H, m, H-11), 3.78 (1H, d, J= 5.1 Hz, H-3β), 3.72 (1H, ddd, 5.1, 3.1, 5.2 Hz, H-2β), 3.35 (3H, brs, OMe), 3.22 (2H, d, J= 8.5 Hz, H-7α), 2.63 (1H, d, J=5.2 Hz, H-7α), 2.59 (1H, J=6.8 Hz, H2-7β), 2.37 (1H, d, J=5.6 Hz, H-12α), 2.32 (1H, d, J=7.7 Hz, H-12β), 1.95 (1H, s, H-13α), 1.83 (1H, s, H-13β), 1.70 (1H, m, H-10α), 1.66 (1H, m, H-1β), 1.49 (1H, m, H-15), 1.20 (3H, brs, Me-18), 1.08 (3H, brs, Me-21), 0.95 (3H, brs, Me-19), 0.82 (3H, brs, Me-20), 0.79 (3H, d, J=6.5 Hz, Me-16); ¹³C NMR (MeOD): δ 89.06 (C-1), 78.01 (C-2), 70.12 (C-3), 46.24 (C-4), 137.72 (C-5), 124.15 (C-6), 37.65 (C-7), 84.33 (C-8), 155.91 (C-9), 30.79 (C-10), 117.72 (C-11), 31.36 (C-12), 47.86 (C-13), 21.65 (C-14), 30.52 (C-15), 18.11 (C-16), 68.35 (C-17), 20.75 (C-18), 22.78 (C-
The diameter of zone of inhibition for susceptible bacteria subtilis only ethanol fraction has the prominent activity against zone of inhibition and MIC values (table 1) revealed that inactive against all the mentioned bacterial and fungal aeruginosa. It was assessed by determination of zone of inhibition and Antimicrobial assay (40.1).

Compound CM-2: Elution of column with methanol-chloroform (9:1) gave colourless prismatic crystals of CM-2, recrystallized from methanol.

Fig. 1: Chemical structure of 2α, 3α-dihydroxy-8α-methoxy-15-hydroxy- methylene-pimar-5, 9(11)-diene (CM-1).

Fig. 2: Chemical structure of α-D-xylose (CM-2)

M.P.: 144-145ºC; [α]D = +22.5º (CHCl3); IR vmax (KBr): 3480, 3398, 2925, 1652, 1401, 1103 and 1015 cm⁻¹; ¹H NMR (MeOD): δ 4.89 (1H, d, J=1.6 Hz, H 2-14, and OMe with C-8; and Me-18, Me-16, H-15 and 3.35, 1.20, 1.08, 0.95 and 0.82 attributed correspondingly to methoxy and tertiary C-18, C-21, C-19 and C-20 methyl protons and a three-proton doublet at δ 3.22 (J=8.5 Hz) accounted to β-oriented carbinol H-2β and H-2β and hydroxymethylene H 2-17 protons, respectively, five three-proton broad singlets at δ 3.35, 1.20, 1.08, 0.95 and 0.82 attributed correspondingly to methoxy and tertiary C-18, C-21, C-19 and C-20 methyl protons and a three-proton doublet at δ 0.79 (J=6.5 Hz) accounted to secondary C-16 methyl protons, all located on the saturated carbons. The ¹³C NMR spectrum of CM-1 exhibited the presence of 22 carbon atoms assigned to vinylic H-6 and H-11 protons, respectively, three one-proton signals as a doublet at δ 5.36 (J= 5.2 Hz) and as a multiplet at δ 5.22 assigned to vinylic H-6 and H-11 protons, respectively, three one-proton signals as a doublet at δ 3.78 (J=5.1 Hz), as a triplet doublet at δ 3.72 (J= 5.1, 3.1, 5.2 Hz) and as a doublet doublet at δ 3.22 (J=8.5 Hz) ascribed to β-oriented carbinol H-2β and H-2β and hydroxymethylene H 2-17 protons, respectively, five three-proton broad singlets at δ 3.35, 1.20, 1.08, 0.95 and 0.82 attributed correspondingly to methoxy and tertiary C-18, C-21, C-19 and C-20 methyl protons and a three-proton doublet at δ 0.79 (J=6.5 Hz) accounted to secondary C-16 methyl protons, all located on the saturated carbons. The ¹³C NMR spectrum of CM-1 exhibited the presence of 22 carbon atoms assigned to vinylic carbons between δ 155.91-117.72, carbinol carbons at δ 78.01 (C-2) and 70.12 (C-3), oxygenated quaternary carbon at δ 84.33 (C-8), methoxy carbon at δ 50.46, and methyl carbons from δ 29.22 to 18.11. The DEPT spectrum of CM-1 showed the presence of five methyl, one methoxy, five methylene, five methane and six quaternary carbons. The ¹H-¹H COSY spectrum of CM-1 exhibited correlations of H-2 with H 2-1 and H-3; H-6 with H 2-7; H 2-7, H-11 with H 2-12; H 2-12 with H 2-14; H 2-14 with H 2-12 and Me-18; H-15 with H 2-17 and Me-16.

The HMBC spectrum of CM-1 showed interactions of H-2, H-3 and Me-16 with C-4; Me-21 and H-2-14 with C-5; H-6 and H 2-7; H-11 with H 2-12; H 2-12 with H 2-14 and Me-18; H-15 with H 2-17 and Me-16. The HSQC spectrum of CM-1 exhibited key-correlations between the proton at δ 5.36 (H-6) and carbon signal at δ 124.15 (C-2), between the proton at δ 5.22 (H-11) and carbon signal at δ 117.72 (C-11), between H-2 at δ 3.72 with C-2 at δ 78.01, between H-2-12 at δ 3.22 with C-17 at δ 68.35 and methyl protons with their respective carbons. On the basis of these evidences the structure of CM-1 has been formulated as 2α, 3α-dihydroxy-8α-methoxy-15-hydroxy- methylene-pimar-5, 9(11)-diene. This is a new homoditerpenoid.

Compound CM-2 (fig. 2) gave positive tests of sugar. Its IR spectrum showed absorption bands for hydroxyl groups (3480, 3398 cm⁻¹). The mass spectrum of CM-2 displayed a molecular ion peak at m/z 150 corresponding

Antimicrobial assay

It was assessed by determination of zone of inhibition and MIC values (table 1) for Hexane, ethyl acetate, dichloromethane and ethanol fractions of leaves of C. maximus against B. subtilis, S. aureus, E. coli, P. aeruginosa, M. smegmatis and C. albicans. The obtained zone of inhibition and MIC values (table 1) revealed that only ethanol fraction has the prominent activity against B. subtilis, S. aureus, M. smegmatis, E. coli and P. aeruginosa but the remaining extracts were found to be inactive against all the mentioned bacterial and fungal strains. The isolated compound CM-1 from bioactive ethanol extract was found to be highly active against B. subtilis, S. aureus and M. smegmatis at MIC 20μg/ml. The diameter of zone of inhibition for susceptible bacteria on treatment with CM-1 were observed in the following order B. subtilis (26±1.28 mm) > S. aureus (18±1.32 mm) > M. smegmatis (9±0.57 mm). P. aeruginosa, E. coli and C. albicans were found to be highly resistant to CM-1.

DISCUSSION

Compound CM-1 (fig. 1) had IR absorption bands for hydroxyl groups (3412, 3325 cm⁻¹) and unsaturation (1625 cm⁻¹). Its molecular ion peak was determined at m/z 364 on the basis of mass and ¹³C NMR spectra which was consistent to the molecular formula of a tetra-oxygenated homoditerpenoid C22H36O4. The ¹H NMR spectrum of CM-1 showed two one-proton deshielded signals as a doublet at δ 5.36 (J= 5.2 Hz) and as a multiplet at δ 5.22 assigned to vinylic H-6 and H-11 protons, respectively, three one-proton signals as a doublet at δ 3.78 (J=5.1 Hz), as a triplet doublet at δ 3.72 (J= 5.1, 3.1, 5.2 Hz) and as a doublet doublet at δ 3.22 (J=8.5 Hz) ascribed to β-oriented carbinol H-2β and H-2β and hydroxymethylene H 2-17 protons, respectively, five three-proton broad singlets at δ 3.35, 1.20, 1.08, 0.95 and 0.82 attributed correspondingly to methoxy and tertiary C-18, C-21, C-19 and C-20 methyl protons and a three-proton doublet at δ 0.79 (J=6.5 Hz) accounted to secondary C-16 methyl protons, all located on the saturated carbons. The ¹³C NMR spectrum of CM-1 exhibited the presence of 22 carbon atoms assigned to vinylic carbons between δ 155.91-117.72, carbinol carbons at δ 78.01 (C-2) and 70.12 (C-3), oxygenated quaternary carbon at δ 84.33 (C-8), methoxy carbon at δ 50.46, and methyl carbons from δ 29.22 to 18.11. The DEPT spectrum of CM-1 showed the presence of five methyl, one methoxy, five methylene, five methane and six quaternary carbons. The ¹H-¹H COSY spectrum of CM-1 exhibited correlations of H-2 with H 2-1 and H-3; H-6 with H 2-7; H-11 with H 2-12; H 2-12 with H 2-14 and Me-18; H-15 with H 2-17 and Me-16.

The HMBC spectrum of CM-1 showed interactions of H-2, H-3 and Me-16 with C-4; Me-21 and H-2-14 with C-5; H-6 and H 2-7; H-11 with H 2-12; H 2-12 with H 2-14 and Me-18; H-15 with H 2-17 and Me-16. The HSQC spectrum of CM-1 exhibited key-correlations between the proton at δ 5.36 (H-6) and carbon signal at δ 124.15 (C-2), between the proton at δ 5.22 (H-11) and carbon signal at δ 117.72 (C-11), between H-2 at δ 3.72 with C-2 at δ 78.01, between H-2-12 at δ 3.22 with C-17 at δ 68.35 and methyl protons with their respective carbons. On the basis of these evidences the structure of CM-1 has been formulated as 2α, 3α-dihydroxy-8α-methoxy-15-hydroxy- methylene-pimar-5, 9(11)-diene. This is a new homoditerpenoid.
to a molecular formula of a pentose sugar C_{5}H_{10}O_{5}. The ion peak generated at \( m/z \) 133 [M-OH] indicated the removal of the hydroxyl group at anomeric carbon. The 1H NMR spectrum of CM-2 exhibited a one-proton doublet at \( \delta \) 4.89 \( (J=4.5 \text{ Hz}) \) assigned to \( \alpha \)-oriented anomeric H-1 proton. Three one-proton multiplets at \( \delta \) 3.71, 3.69 and 3.59 were accounted to hydroxymethine protons H-2, H-3 and H-4, respectively. A two-proton doublet at \( \delta \) 3.33 \( (J=1.6, 1.6 \text{ Hz}) \) was attributed to oxygenated methylene protons (H-2,5). The 13C NMR spectrum of CM-2 showed carbon signals for anomeric H-1 proton. Three one-proton multiplets at \( \delta \) 95.81 with H-1, 61.96 (C-5). The 1H-1H COSY spectrum of CM-2 showed correlations of H-1 with H-2, H-3 and H-4, respectively. Its HMBC spectrum exhibited interactions of H-2, H-3 and H-2,5 with C-1. The HSQC spectrum of CM-2 indicated the interactions of C-1 at \( \delta \) 95.81 with H-1 at \( \delta \) 4.89, carbinal carbons with their respective protons and oxygenated methylene carbon at \( \delta \) 61.96 with the methylene protons H-2,5 at \( \delta \) 3.33. On the basis of these evidences and chemical reactions the structure of CM-2 has been determined as \( \alpha \)-D-xylose.

The gram-positive bacteria belonging to genus Staphylococcus plays an important role as pathogen, and generally exhibits multiple resistance to antimicrobial drugs. The new homoditerpenoid (CM-1) isolated from C. maximus exhibited very potent (P<0.005) antibacterial activity against gram positive bacteria like B. subtilis, S. aureus and M. smegmatis out of which B. subtilis was found to be most susceptible among all three while the compound CM-1 was not showing antimicrobial effect (P<0.005) against other tested gram negative bacteria as well as C. albicans. The antimicrobial activity of CM-1 also supports the previous researches on diterpenoidal compounds being antibacterial (Kusumoto et al., 2014). This type of studies is much needed in developing countries. It encourages the scientific research in the country as well as promotes the use of natural resources for prophylactic and remedial purposes.

### Table 1: Antimicrobial activities of different C. maximus leaves extract and compound CM-1 against some microorganisms

<table>
<thead>
<tr>
<th>Organisms</th>
<th>ZOI (mm)</th>
<th>MIC (μg/mL)</th>
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<tr>
<td></td>
<td>HE</td>
<td>DCME</td>
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<tr>
<td>Bacillus subtilis</td>
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<tr>
<td>Staphylococcus aureus</td>
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<td>Mycobacterium smegmatis</td>
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<td>Escherichia coli</td>
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<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Candida albicans</td>
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</table>

Abbreviations: All values are mean ± SD, n=3 (P<0.005), ZOI=Zone of inhibition in mm ± S.D. HE: n-hexane extract; DCME: Dichloromethane extract; EAE: Ethyl acetate extract; EA: Ethanol extract; CM-1: New homoditerpenoid compound; CIP: Ciprofloxacin; mm: Millimeter; MIC-Minimum inhibitory concentration (μg/ml)

### CONCLUSION

Infection is the root cause of many diseases and sometimes it becomes uncontrollable. The herbal anti-inflammatory agents are the first choice for majority of the population because these preparations produce lower level of toxicity and side effects in comparison to the synthetic medicines. This study may provide a good option to the people of Saudi Arabia to combat the infective diseases and encourage herbal remedies against multdrug-resistant bacterial infections.

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### REFERENCES


