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RESEARCH PAPER

Antibacterial Activity and Chemical Composition of Crude Extract and Oil of *Zygophyllum (Fagonia) luntii* (Baker) 1894 (Family Zygophyllaceae)

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النشاط المضاد للبكتيريا والتركيب الكيميائي للمستخلص الخام وزيت زيغوفيللوم (فاجونيا) لونتي (بيكر) ١٨٩٤ (عائلة زيغوفيللاسي)

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Abstract. Wild plants such as *Zygophyllum luntii*, from the Zygophyllaceae family, have traditionally been used for medicinal purposes in Oman. The present study investigated (i) the antibacterial activity of the crude extracts (leaves, stem and roots) and the oil (leaves); and (ii) the hydrocarbon contents and fatty acid methyl ester (FAME) components from *Z. luntii*. These extracts were tested against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using well diffusion assays utilizing Muller Hinton Agar (MHA). Antibacterial activity was observed with the *Z. luntii* leaf extract and significant differences (F=14.06, df=2, P=0.002) were found among *E. coli*, *P. aeruginosa* and *S. aureus*. The highest inhibition was observed against *P. aeruginosa*, with an inhibition zone of 15.5 ± 2.6 mm, followed by *E. coli* and *S. aureus* with inhibition zones of 11.3 ± 1.5 mm and 3.5 ± 4.7 mm, respectively. The *Z. luntii* extracts showed effectiveness within 50-60% against *E. coli* and *P. aeruginosa* as compared to Ciprofloxacin. The hydrocarbon contents and the FAME components of the extracts were determined with Gas Chromatography-Mass Spectrometry (GC-MS). A total of 20, 19, and 8 compounds were detected from leaf, stem, and root extracts, respectively. Heneicosane, docosane, and tricosane were found in the highest concentration in the leaves, HOP-22(29)-EN-3.BETA.-OL and β-sitosterol were found in the stems, and docosane and tricosane were found in the roots of *Z. luntii*. Nine types of fatty acids methyl esters were detected in the oil extracted from leaves with methyl esters of palmitic acid, linolenic acid, and oleic acid constituting 90% of the oil. This is the first report on antibacterial activity and chemical composition of *Z. luntii*.

Keywords: *Zygophyllum luntii*, Antibacterial activity, Gas Chromatography- Mass Spectrometry (GC-MS), Fatty Acid Methyl Ester (FAME)

المستخلص: يتم استخدام النباتات البرية مثل زيغوفيللوم لونتي *Zygophyllum luntii* المسماة السقطرية ، من عائلة زيغوفيللاسي Zygophyllaceae بشكل تقليدي في عمان للأغراض الطبية. بحثت هذه الدراسة عن النشاط المضاد للبكتيريا للمستخلصات الخام من الأوراق والساق والجذوروالزيت المستخلص من الأوراق. تم الكشف على مركبات من الهيدروكربونات والأحماض الدهنية في مستخلص الأوراق لنبتة *Z. luntii*. تم اختبار هذه المستخلصات ضد بعض الأنواع من البكتيريا مثل *Escherichia coli* و *Staphylococcus aureus* و *Pseudomonas aeruginosa* بطريقة فحوصات الانتشار الجيد بإستخدام أجار مولر هينتون المناسب لنمو هذه الأنواع من البكتيريا. لوحظ نشاط مضاد للجراثيم من مستخلصات نبات *Z. luntii* ووجدت فروق ذات دلالة إحصائية بين *E. coli* و *P. aeruginosa* و *S. aureus* . فقد لوحظ أعلى تثبيط ضد *P. aeruginosa* مع منطقة تثبيط ١٥,٥ ± ٢,٦ مم ، تليها *E. coli* و *S. aureus* مع مناطق تثبيط ١١,٣ ± ١,٥ مم و ٣,٥ ± ٤,٧ مم على التوالي. أظهرت مستخلصات *Z. luntii* فعالية في حدود ٥٠–٦٠ ٪ ضد *E. coli* و *P. aeruginosa* مقارنة مع النموذج التجاري سيبروفلوكساسين. تم تعريف مركبات الهيدروكربونات والأحماض الدهنية في المستخلصات بإستخدام جهاز الفصل الكروماتوجرافي الغازي المرتبط بمستشعر الطيف الكتلي (GC-MS) . تم الكشف عن مامجموعه ٢٠ و ١٩ و ٨ من المركبات من مستخلصات الأوراق والساق والجذور على التوالي. تم العثور على الهيدركربونات هينيكوسان و دوكوسان وتريكوسان كأعلى تراكيز للمركبات في الأوراق ، وتم العثور على مركب ٢٢-HOP (٢٩) –EN–٣. BETA.–OL ومركب بيتا سيستيرول في السيقان ، كما تم العثور على مركبات دوكوسان وتريكوسان في الجذور الموجودة في *Z. luntii* . تم الكشف عن تسعة أنواع من الأحماض الدهنية في الزيت المستخرج من الأوراق مع نسب عليا لمجموع تراكيز حمض البالمتيك وحمض اللينولينيك وحمض الأوليك شكلت مانسبته ٩٠٪ من الزيت. يعتبر هذا هو التقرير الأول على مستوى الدراسات البحثية عن النشاط المضاد للبكتيريا والتركيب الكيميائي لنبات *Z. luntii*.

لكلمات المفتاحية: زيغوفيللوم لونتي ، النشاط المضاد للبكتيريا ، جهاز الفصل الكروماتوجرافي الغازي المرتبط باالطيف الكتلي (GC-MS) ، الأحماض الدهنية الميثيل إستر (FAME)

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Introduction

Around 80% of the world population use traditional medicine which is often based on medicinal plants (Martins, 2013; Oyebode et al., 2016). Around 75% of commercial drugs launched in the world

global market yearly are extracted or isolated from natural resources and about 25% of the prescribed pharmaceutical drugs are based on plant chemicals (Orhan, 2012). Plants are the major source of secondary metabolites, which are used to treat various diseases (Hossain et al., 2013; Akhtar et al., 2017; Raqiya and Hossain, 2017; Asma et al., 2017; Hossain, 2018; Said et al., 2018). Medicinal plants are found in many places in the world; however, they are found more in tropical regions (Al-Salt, 2012).

Family Zygophylllaceae includes many medicinally important plants spices including several species of *Zygophyllum*. *Zygophyllum* have antitumor, antioxidant and analgesic properties, and have been used for the treatments of cancer, fever, asthma, urinary discharges, toothache, stomach problems and kidney diseases (Ahsan et al., 2007; Satpute et al., 2009). *Zygophyllum* species were found to be potent antifungal and antibacterial agents (Zhang et al., 2008; Gupta et al., 2009) and contained many biologically active chemical constituents, such as alkaloids, saponins, terpenoids, sterols, flavonoids, coumarins and trace elements (Beier, 2005).

Zygophyllum luntii distribution is restricted to the Horn of Africa region, including Djibouti, Oman, Somalia and Yemen (Beier, 2005). It grows on sand as well as gravel, from sea level up to 1950 m altitude. In Oman, *Z. luntii* is found in the foot of Dhofar mountains along with several other species of *Zygophyllum (Z. bruguieri, schweinfurtii, indica, mahrana* and *ovalifolia)* (Mosti, et al., 2012).

The current study explored two objectives. Firstly, antibacterial activity of the *Z. luntii* extract (leaves, stems and roots) and leaf oil against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were investigated. Secondly, hydrocarbon contents of the plant extracts and lipids fatty acid methyl ester were determined (FAME) components of the oil extracted from leaves by gas chromatography- mass spectrometer (GC-MS).

Materials and Methods

Collection and Preparation of Plant Materials

Roots, leaves, and stems of *Z. luntii* were gathered from the Botanical Garden at Sultan Qaboos University, Oman. These parts were cleaned with tap water followed by distilled water to remove any dust and soil. The plant parts were then further divided into two portions; one was dried in the oven at 70°C for 8 h and then ground into a fine powder and other portion was kept fresh in a refrigerator at 4°C.

Plant Extracts Preparation for Anti-bacterial Test

The dried powder of the leaves, stem and roots was dissolved in 70% methanol (1:3, w/v) and then kept in a shaker for extraction at room temperature for 24 h. Then, methanol was dissipated from the sample using oven to get the crude extract which was re-suspended in dimethylsulfoxide (DMSO) for application in the antibacterial test.

Lipid Extraction from Leaves

Around 470g of the fresh leaves with 1 L of distilled water were grinded by a blender. The solution was mixed with the solvent (chloroform: methanol in 2:1 ratio) to separate the lipids and then evaporated in a rotary evaporator. The extract was filtered through charcoal. The obtained oil (1 g) was kept in storage at 4°C until utilization for further tests.

Anti-bacterial Assay

Muller Hinton Agar CM0337 from Oxiod (Part of Thermo Fisher Scientific) was used in well diffusion assay. MHA contains beef dehydrated infusion 300.0 g/L, casein hydrolysate 17.5 g/L, starch 1.5 g/L and agar 17.0 g/L. Amount of 38 g of MHA was suspended in 1 L of distilled water, boiled and sterilized by autoclaving at 121°C for 15 min.

Three strains of pathogenic bacteria *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923 were utilized as test microorganisms. These clinical isolates were obtained from Microbiology Laboratory at Sultan Qaboos University Hospital. These strains are for antibiotic testing and fall under the American type collection culture (ATCC). Furthermore, these strains were sub cultured in liquid broth for a period of 6 to 8 hours. The well diffusion assay was conducted utilizing Muller Hinton Agar (MHA). The assay of leaves, stem and roots extract activity was carried out in nine MHAs plates which were replicated three times. For every plate, four discs were used one each for leaves, stem, roots and an antibiotic standard (Ciprofloxacin) as positive control. Discs of 6 - 8 mm diameter were removed from agar with a sterile glass pasture pipette and filled with 30 µl of the sample extract or standard. At the same time, three MHAs plates were used for oil using a similar procedure where three discs were used for every plate; two discs for oil and one disc for antibiotic standard. Zone inhibition was investigated after incubating agar plates at 37°C.

Sample Preparation and Extraction for GC-MS Analysis

Fresh leaf, stem and root samples were weighed and grinded using a mechanical grinder. Then, 50 mL of 70% methanol was added to each grinded sample and placed in an ultrasonic water bath working at 50–60 kHz with power of 350 W for 30 min at room temperature. By using a rotary vacuum evaporator, the methanol was evaporated and the extracts were concentrated. The crude extracts were dissolved in hexane, filtered by microfiltration (0.45 µl syringe) and injected to GC-MS.

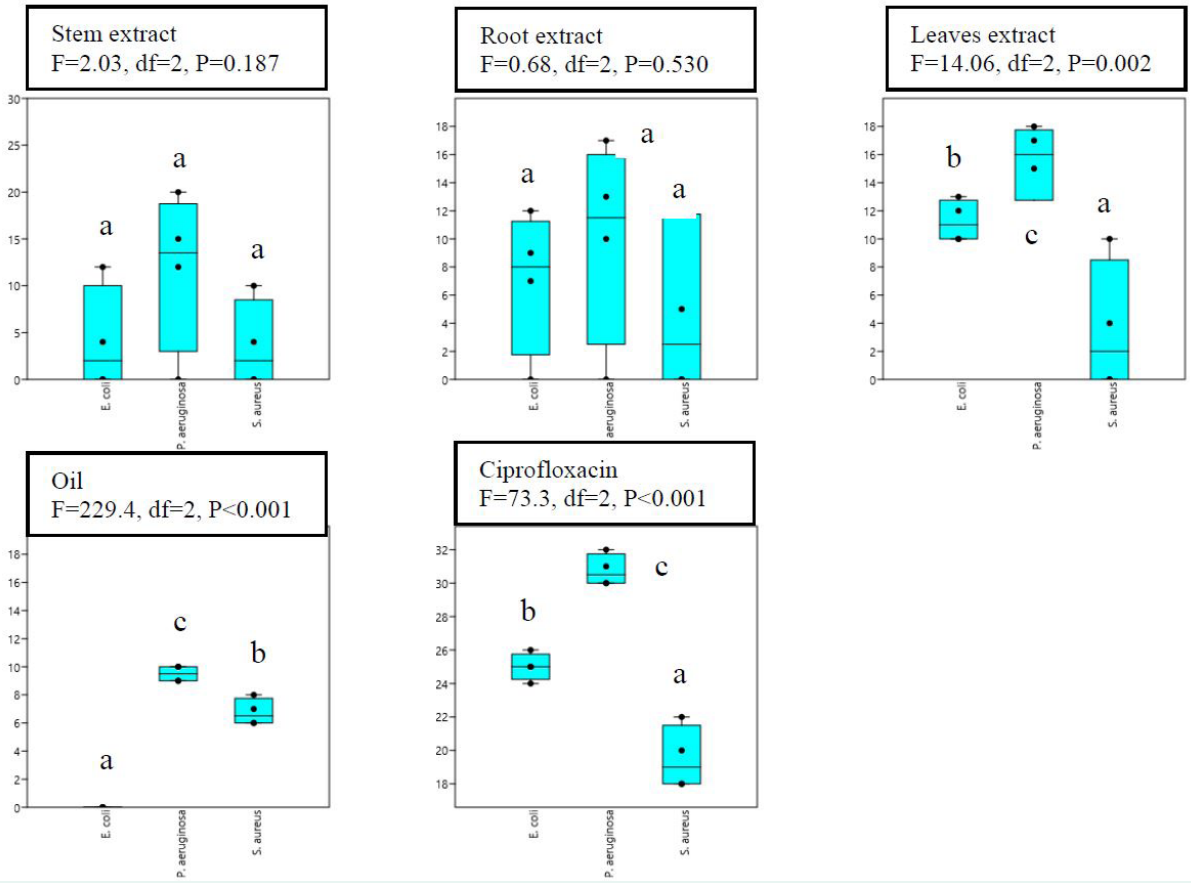

Figure 1. Comparison of anti-bacterial activity (inhibition zone in mm) among *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 in well diffusion assays exposed to *Zygophyllum luntii* extracts and Ciprofloxacin. Bars designated by the same letters are not statistically significant at $\alpha_{0.05}$

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

GC-MS conditions for samples extracted from leaves, stem and roots: GC-MS analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; maximum temperature, 350°C), coupled to a Perkin Elmer Clarus 600C MS. Ultra-high puri-

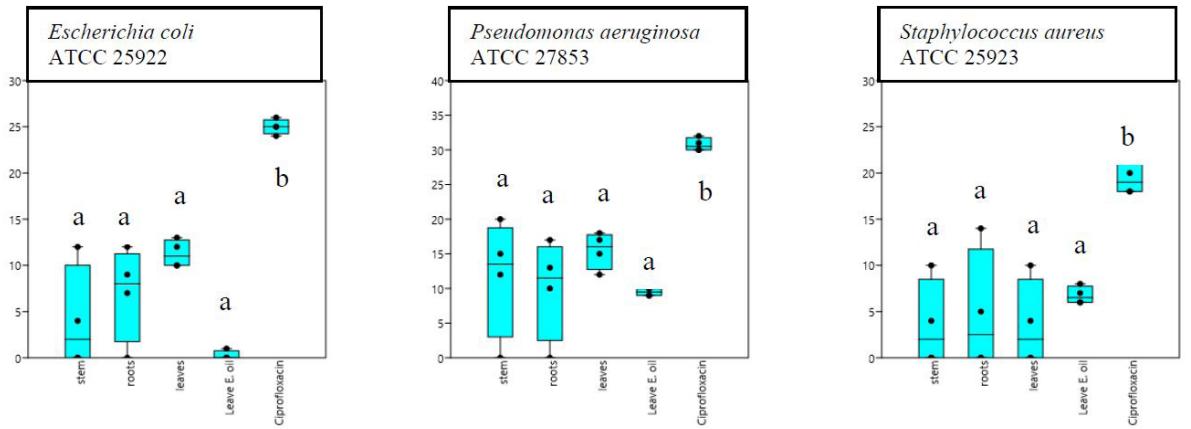

Figure 2. Comparison of anti-bacterial activity (inhibition zone in mm) of the *Zygophyllum luntii* extracts and Ciprofloxacin against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 well diffusion assays. Bars designated by the same letters are not statistically significant at $\alpha_{0.05}$

Table 1. Compounds identified in the leaf, stem and root extracts of *Zygophyllum luntii* by GC-MS

Name of compound	Retention Time (min)	Percent (%)
Leaves		
Pentadecane	13.64	0.33
Tetradecane, 2,6,10-trimethyl-	16.43	0.68
Oxalic acid, allyl octyl ester	16.81	0.32
Unidentified	17.02	0.61
Octadecane	17.79	0.34
Nonadecane	18.73	0.90
Eicosane	19.02	5.84
Heneicosane	21.57	12.30
Docosane	22.60	12.72
Tricosane	23.26	12.93
Tetracosane	24.24	9.94
Pentacosane	25.36	10.42
Hexacosane	26.21	7.63
Heptacosane	27.13	5.84
Octacosane	27.87	5.82
Nonacosane	28.70	5.03
Triacontane	29.62	4.10
Hentriacontane	30.71	1.84
Dotriacontane	31.99	2.42
Tetratriacontane	36.24	0.13
Stem		
Hexadecanal	18.33	2.60
Phytol	21.50	5.22
Unidentified	22.22	2.70
Unidentified	23.20	0.38
Unidentified	24.20	0.27
Unidentified	25.18	0.47
Heptacosane	26.90	0.35
Octacosane	27.80	0.22
Supraene	28.14	0.95
Unidentified	28.60	0.46
Triacontane	29.50	0.29
Hentriacontane	30.64	0.38
Vitamin E acetate	31.40	1.24
Dotriacontane	31.93	0.64
Tritriacontane	33.40	1.23
β-Sitosterol	34.36	11.02
Heptatriacontane	35.40	0.80
Hop-22(29)-En-3.Beta.-Ol	35.90	69.95
Octatriacontane	37.70	0.84
Roots		
Docosane	22.60	14.04
Tricosane	23.49	14.36
Tetracosane	24.43	12.54
Pentacosane	25.36	4.02
Pentacosane	25.54	5.33
Hexacosane	26.24	6.67
Octacosane	27.13	43.05
Tetratriacontane	36.24	1.15

ty helium (99.9999%) was used as carrier gas at a constant flow of 1.0 mL/min. The injector, transfer line and ion source temperatures were 280°C, 270°C and 270°C, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from auto tune. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The injected sample volume was 1 µl with a split ratio of 10:1. The oven temperature program was 60°C at a rate of 80°C per minute to 280°C hold for 25 minutes. The total run time was 53.5 minutes.

GC/MS conditions for the oil extracted from leaves: Fatty Acid Methyl Ester (FAME) compounds were detected by the GC-MS equipment as mentioned above. The helium gas flow rate was 0.7 mL/min. The injector, transfer line and ion source temperatures were 250°C, 250°C and 220°C, respectively. The initial oven temperature was set at 50°C (holds for 8 minutes) and increased to 250°C in a rate of 40°C per minute. All data were obtained by collecting the full-scan mass spectra within the scan range 35-500 amu. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition).

Statistical Analysis

Data on antibacterial assays from leaves, stems and roots, and the three bacterial strains were analysed separately using one-way analysis of variance (ANOVA) in Past (https://past.en.lo4d.com/windows). Means were separated by Dunn's Multiple Comparison Test and differences were considered significant at $p<0.05$.

Table 2. Compounds detected in the oil extracted from *Zygophyllum luntii* leaves by GC-MS

Name of compound	Retention Time (min)	Percent (%)
	Leaves	
Caprylic acid methyl ester	11.58	0.12
Capric acid methyl ester	18.27	0.19
Lauric acid, methyl ester	24.29	0.74
Myristic acid, methyl ester	29.83	0.81
Palmitic acid, methyl ester	34.93	34.55
Palmitoleic acid, methyl ester	35.90	1.70
Oleic acid, methyl ester	39.78	23.99
Linoleic acid, methyl ester	40.57	5.81
Linolenic acid, methyl ester	41.73	32.09

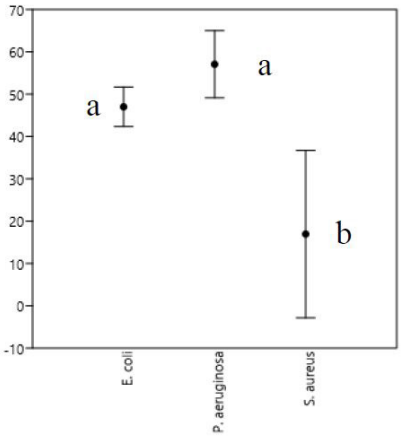

Figure 3. Percent effectiveness of the *Zygophyllum luntii* extracts compared to Ciprofloxacin against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853, and *S. aureus* well diffusion assays. Bars designated by the same letters are not statistically significant at $\alpha_{0.05}$

Result and Discussion

Antibacterial Assays

Significant antibacterial activity was observed in the *Z. luntii* leaves extract and significant differences (F=14.06, df=2, P=0.002) were found among *E. coli*, *P. aeruginosa* and *S. aureus* (Figure 1). The highest inhibition was observed against *P. aeruginosa* (15.5 ± 2.64 mm) then *E. coli* (11.25 ± 1.50mm) and *S. aureus* (3.50±4.72 mm). The inhibition zones of the stem (F=2.03, df=2, P=0.187) and root (F=0.68, df=2, P=0.530) extracts did not differ significantly among the three bacterial strains. Oil from leaves did not produce any inhibition in *E. coli* but had significantly higher inhibition (F=229.4, df=2, P<0.001) in *P. aeruginosa* (9.5±0.58mm) and *S. aureus* (6.75±0.96mm). Ciprofloxacin had significantly different inhibition zones (F=73.3, df=2, P<0.001) among the three bacterial strains. The highest inhibition zone by the commercial antibiotic was against *P. aeruginosa* (30.75± 0.96 mm) then *E. coli* of (25.0± 0.82 mm) and lowest against *S. aureus* (19.50±1.91 mm).

The inhibition in the stem, root and leaves extracts, and oil from leave was significantly lower than Ciprofloxacin against *E. coli* (F=29.8, df=4, P<0.001), *P. aeruginosa* (F=11.7, df=4, P<0.001) and *S. aureus* (F=9.9, df=4, P<0.001) (Figure 2). The stem extract was more active against *P. aeruginosa* while root extract was more active against *S. aureus*. Leaves extract showed more activity against *E. coli*. The oil showed some antibacterial activity against *P. aeruginosa* and *S. aureus* but not *E. coli*. Overall, the *Z. luntii* extracts were active against both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*), though they were more active against the latter. The ethanol extracts of intact leaf of *Z. arabica* showed an inhibition zone of only 6.08 mm against *E. coli* and did not inhibit the growth of *S. aureus* (Alam et al., 2010). The ethanol whole plant extract of *F. cretica* produced inhibition zones of 15 mm, 15 mm and 14 mm against *E. coli*, *P. aeruginosa* and *S. aureus*, respectively (Sajid et al., 2011) which are similar to our results. The crude extract of *Z. arabica* from Sinai showed broad antimicrobial spectrum against Gram-positive, Gram-negative, spore-forming and acid-fast bacteria (El-Hefnawi, 1999). It is obvious that leaves extracts from *Zygophyllum* spp including *Z. luntii* have antibacterial properties.

The percent effectiveness of the *Z. luntii* extracts were compared to Ciprofloxacin against *E. coli*, *P. aeruginosa* and *S. aureus* by subtracting the extract inhibition zone sizes from the Ciprofloxacin (Figure 3). The calculated percent effectiveness against *E. coli* was 47.0±4.8%; against *P. aeruginosa* was 57.1±8.1%; and against *S. aureus* was 19.4±24.2%. The percent effectiveness against *E. coli* and *P. aeruginosa* was significantly higher compared to *S. aureus* (F=10.54, df=2, P=0.005). Alam et al. (2010) produced callus of *Z. arabica* by tissue culture and found that the callus extract was more effective against *Serratia marcescens*, *E. coli* and *Acetobacter aceti* subsp. liquefaciens (inhibition zones = 32.67, 33.92 and 34.83 mm respectively) than the crude extract of the intact leaf (IZ=6.08 mm) suggesting higher antibacterial effects of callus extract against Gram – ve bacteria.

Several pathogens are increasingly developing resistance, particularly to broad-spectrum antibiotics (Kunin, 1993). Resistant *E. coli* isolates have been reported from humans using disk diffusion method against ciprofloxacin (22% with the highest of 52% reported from Iran), cefotaxime (31.2%–58%) and ceftazidime (10%–57.4%) (Pormohammad et al., 2019). Some of the gram-positive drug resistant bacteria include *S. aureus*, *Streptococcus*

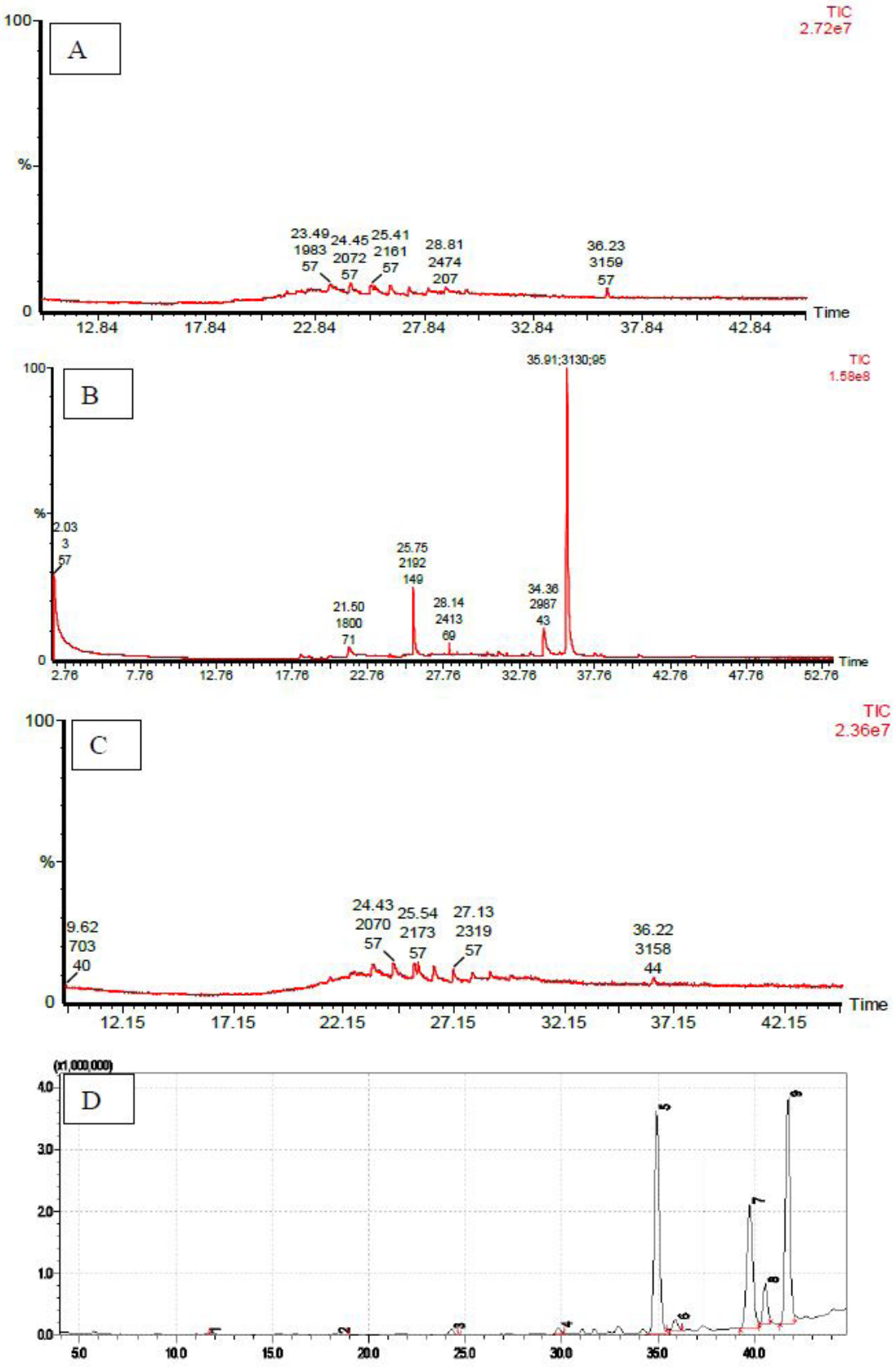

Figure 4. Chromatogram of compounds in leaves (A), stem (B) and roots (C), and fatty acids methyl esters (FAME) in oil (D) extracted from *Zygophyllum luntii* leaves detected by GC/MS.

pneumoniae, and *Enterococcus* spp., and the gram-negative drug resistant bacteria *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *E. coli,* and *P. aeruginosa* (Lister et al., 2009). Our results also indicated reduced sensitivity of *S. aureus* and *E. coli* to Ciprofloxacin. The *Z. luntii* callus extract may help in managing antibacterial resistant pathogens of different strains

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

Higher number of compounds was identified in the leaves (n=20) and stem (n=19) extract compared to roots (n=8) extracts of *Z. luntii* while 6 compounds could not be identified (Table 1). The retention time (RT) of all compounds varied between 13.64 to 37.7 minutes. The unidentified compounds were present in a relatively low amount. Heneicosane, Docosane and Tricosane were present in higher quantities in the leaves extract. Hop-22(29)-En-3.Beta.-Ol (69.95%) was present in higher quantities in the stem extract while the root extract had octacosane in high quantities. Palmitic acid, linolenic acid and oleic acid were the main components of oil extracted from leaves (Table 2).

Heptacosane, Heneicosane, Tetradecane have been reported with antimicrobial activity (Elshiekh and Abdelmageed, 2015). Beta-acids are an important component of hops (*Humulus lupulus* L. family Cannabaceae) soft resins and usually isolated as by-products during hop processing (McCallum et al., 2019). Hexahydro-β acids showed strong antibacterial activity and good stability (Liu et al., 2019). Noticeably, HOP-22(29)-EN-3. BETA.-OL had the highest percentage compared to other compounds.

Close retention time of 22-27 minutes in roots compounds showed their similar affinity to stationary phase. Both retention time and peak area (%) for Docosane (C22) and Tricosane (C23) (22.60 minutes and 23.26 minutes, and 12.72% and 12.93%, respectively) did not show wide variation. These two compounds were present in leaves and roots but not in stems. The n-alkane fractions (hydrocarbons C22-C35) was detected in the leaves extract of *Z. luntii* which have been detected in vegetable oils by GC/MS (Troya et al., 2015). These compounds are more common in plant extracts.

Species of *Zygophyllum* have been found to contain saponins (Abdel-Khalik et al., 2001), alkaloids (Sharawy and Alshammari, 2009), terpenoids (Perroni et al., 2007), sterols (Shoeb et al., 1994), flavonoids (Ibrahim et al., 2008), proteins and amino acids (Sharma et al., 2010), coumarins (Alam et al., 2010) and trace elements (Fatima et al., 1999). The presence of such chemical ingredients in *Zygophyllum spp*. Would contribute to the medical properties, including stimulating the immune system in humans, treating and preventing the development of chronic diseases (Beier, 2005), and the vitality to resist such types of pathogenic bacteria used in this study.

Oil Extract from Leaves

Nine different types of fatty acids methyl esters (FAME) were found in oil extracted from *Z. luntii* leaves. Palmitic acid, Linolenic acid, and Oleic acid were the main compounds and represented 90% of the oil (Fig.4). There was no study found on *Z. luntii* that explored the fatty acid content. However, seven fatty acids including the Oleic acid, Palmitic acid and Linoleic acid were found in other *Zygophyllum* species e.g. *F. arabica* L (Alam et al., 2010) and *F. cretica* (Soad, 1994).

Conclusion

Extracts from leaves, stem, and roots of *Z. luntii* had significant antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*. The extracted oils had activity against only *P. aeruginosa* and *S. aureus*. The commercial antibiotic Ciprofloxacin had reduced activity against *S. aureus* and *E. coli*. The *Z. luntii* extracts showed about 50-60% effectiveness against *E. coli* and *P. aeruginosa* compared to Ciprofloxacin. HOP-22(29)-EN-3. BETA.-OL, Hexahydro-β acids, and FAME compounds could have contributed to the antibacterial activity. With further research on callus production, improving extraction process and antimicrobial activity assays against more pathogenic bacterial (including antibiotic resistant) species, *Z. luntii* can be promoted as a source of traditional medicine in Oman

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