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Terpenes from the Fresh Stems of *Commiphora gileadensis* with Antimicrobial Activity

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Abstract: Phytochemical study directed by antimicrobial activity of the fresh *Commiphora gileadensis* stems $CHCl_3$ extract resulted in the isolation of compounds **1**-**9**. The structures of the isolated compounds were elucidated utilizing various spectroscopic methods including 1D, 2D NMR and HRESIMS. Compounds **1**, **3**-**5** were identified as the *ent*-verticillane-type diterpenes *(1S,3E,7E,11R)*-(+)-verticilla-3,7,12(18)-triene (**1**), *(13S,14S)-Ent*-13,14-epoxyverticillol (**3**), *(9S,10S)-Ent*-9,10-epoxyverticillol (**4**) and *Ent*-Verticillol (**5**) for the first time from *Commiphora* species. Compound **9** was identified as a diterpene with new skeleton and was given the name "gileadenol". In addition, four know triterpenes were also identified as friedelin (**2**), oleanonic aldehyde (**6**), canyophyllal (**7**) and urs-12-en-3-one-28-al (**8**). The three tested *ent*-verticillane-type diterpenes expressed promising antimicrobial activity especially against *K. pneumonia*. The new diterpene as well as canyophyllal were less active.

Keywords: *Commiphora gileadensis*; antimicrobial; *ent*-verticillane-type diterpenes; gileadenol; triterpenes.

1. Introduction

Genus *Commiphora* is represented by about 7 species in Saudi Arabia particularly in the southwest region [1]. *Commiphora gileadensis* (L) C.Chr. is synonymous with *Commiphora opobalsamum* [2]. The plant bark is the source of the balm of Gilead. Different parts of the plant are used in the traditional medicine. The dried layer under the bark was used to treat infected wounds [3]. The resins of

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Commiphora species are used for the treatment of microbial infections, wounds, inflammation, gastrointestinal diseases, tumors, obesity, pain, arthritis and fractures [4].

The methanol extract of *C. gileadensis* bark as well as the callus extracts of the plant showed a broad-spectrum antimicrobial effect against Gram-positive, Gram-negative bacteria and fungi [5,6]. *C. gileadensis* can interfere with bacterial lectin-dependent adhesion important for the function and survival of *P. aeruginosa*. These properties support the old claims of using the balsam as anti-infective agent [7]. The methanol extract of *C. gileadensis* leaves demonstrated antiviral activity via target the enveloped viruses (HSV-2 and RSV B) [8]. *C. gileadensis* leaf and twig aqueous extracts controlled alloxan-induced diabetes in hypercholesterolemic male rats [2]. The plant also expressed potential to protect against acute gastric ulcers induced by different necrotizing agents in rats [9] and protective effect against carbon tetrachloride induced hepatotoxicity as well [10].

Previous phytochemical study of the genus revealed the presence of pimarane, abietane diterpenoids and dammarane triterpenoids [11,12]. Triterpenoids are the major constituents isolated from *Commiphora* species. More than twenty-one dammarane triterpenoids were identified in the resins of four *Commiphora* species namely; *C. kua*, *C. dalzieli* [13], *C. confuse* and *C. myrrha* [14]. The flavonoids of this genus occurs mainly in the flowers, stems and barks, but not in the resinous exudates. Phytochemical study of the stem bark of *C. opobalsamum* resulted in the identification of six prenylated flavonoids comophorin A-E with cytotoxic activity against MCF-7 and HepG-2 cell lines [15].

The current study designed to identify the antimicrobial constituents from the stems of *C. gileadensis* by conducting detailed biologically directed phytochemical study.

2. Materials and Methods

2.1. General

Melting points were measured using open capillary tubes Thermosystem FP800 Mettler FP80 central processor supplied with FP81 MBC cell apparatus and were uncorrected. Infrared (IR) spectra were recorded on FT-IR spectrophotometer (Perkin Elmer) as film. ^{1}H, ^{13}C-NMR, and 2D-NMR data were collected on a Bruker UltraShield Plus 500 MHz spectrometer at the NMR Unite, College of Pharmacy, Prince Sattam Bin Abdulaziz operated at 500 MHz for protons and 125 MHz for carbons, respectively. Chemical shift values were reported in δ (ppm) relative to the residual solvents peaks. Coupling constants (*J*) were reported in Hertz (Hz). HRESIMS were determined by direct injection using Thermo Scientific UPLC RS Ultimate 3000 - Q Exactive hybrid quadrupole-Orbitrap mass spectrometer combined with high-performance quadrupole precursor selection with high resolution, accurate mass (HR/AM) Orbitrap™ detection. MPLC was performed using a Buchi medium pressure system composed of Buchi pump module C-605 controlled by Buchi control unit C-620 equipped with Buchi fraction collector C-660. The column eluate was detected by Buchi UV photometer C-640. Column 15/460-044032 was used and the system was operated by Sepacore control chromatography software. Sephadex LH20 (Amersham Biosciences, Uppsala, Sweden), Silica gel 60/230-400 mesh (EM Science), (Loba Chemie PVT. LTD. 100- 200 Mesh) and RP18 silica gel 40-63/230-400 mesh (Fluka) were used for column chromatography. The thin-layer chromatography (TLC) analysis was performed on Kiesel gel 60 F254 and RP18 F254 (Merck) plates. A UV lamp (entela Model UVGL-25) operated at 254 nm was used for spots detection on the TLC plates.

2.2. Plant Materials

The plants of *Commiphora gileadensis* (L.) C.Chr (Syn. *Commiphora opobalsamum* (L.) Engl.) were collected from “Badr” area near Al-Madinah city during November, 2019. The plants were identified by Prof Saniya Kamal, Prof of Taxonomy, College of Science, Alexandria University. Voucher specimen (ALX 111119) was deposited at the Department of Botany, College of Science, Alexandria University.

2.3. Extraction and Isolation

Fresh Stems (5.8 kg) were extracted with $CHCl_3$ at room temperature till exhaustion to yield 117.5 g of the $CHCl_3$ extract after evaporation of the solvent under reduces pressure. The stems were then extracted with MeOH at room temperature till exhaustion. Evaporation of the solvent under reduced pressure yielded 226.7 g of the MeOH soluble extract.

Column chromatography for 20 g of the $CHCl_3$ extract was preformed using silica gel (400 g, 5 cm i.d.) eluting with 5% EtOAc in hexane with increasing the percentage of EtOAc in a gradient system. Fractions of 250 mL each were collected, screened with TLC and similar fractions were pooled to yield ten fractions A- J.

Fraction A (4.69 g) eluted with 5% EtOAc was subjected to fractionation over Sephadex LH20 column (150 g, 3 cm i.d.) starting with petroleum ether followed by petroleum ether EtOAc mixtures with 25% increase in EtOAc contents each time. Fractions of 15 mL each were collected, screened with TLC and similar fractions were pooled. Fraction A-7 (136 mg) was subjected to Preparative TLC (PTLC) on silica gel plates using 9:1 petroleum ether / EtOAc mixture as developing system to yield 10 mg of compound **1**. Fraction A-13 (64 mg) was subjected to PTLC on silica gel plates using 9:1 petroleum ether / EtOAc mixture developing system to yield 52 mg of compound **2**.

Fraction B (4.99 g) eluted with 10% EtOAc was subjected to column chromatography on silica gel column (150 g, 3 cm i.d.) eluting with 5% EtOAc. Fractions of 100 mL each were collected, screened with TLC and similar fractions were pooled. Fraction B-a (1.2 g) was fractionated over MPLC silica gel column (92 cm, 1.5 cm i.d.) eluting with petroleum ether followed by petroleum ether EtOAc mixtures in a gradient system. Fractions of 15 mL each were collected, screened with TLC and similar fractions were pooled. Fraction B-a-12 (625 mg) was further purified on MPLC RP18 column eluted with 40% MeOH in water with gradual increase of MeOH in a gradient system. Fraction Fractions of 10 mL each were collected, screened with TLC and similar fractions were pooled. Fraction 64 eluted with 80% MeOH afforded 8 mg of **3**. Fraction 67 eluted with 85% MeOH afforded 6 mg of **4**. Fractions 71-75 eluted with 85% MeOH afforded 213 mg of **5**. Fractions 80-83 eluted with 90% MeOH afforded 86 mg of **6**. Fractions 87-92 eluted with 95% MeOH afforded 16 mg of **7**. Fraction B-a-13 (161 mg) was repurified over silica gel column (20 g, 1.5 cm) eluting with petroleum ether followed by petroleum ether EtOAc mixtures in a gradient system. Fractions 19- 20 afforded compounds **6** (8 mg), **8** (16 mg) and 20 mg of **9** after PTLC using silica gel plates and 9:1 petroleum ether / EtOAc mixture as developing system. Plates were developed for four times. Fraction B-a-14 (132 mg) was subjected to silica gel column (20 g, 1.5 cm) eluting with petroleum ether followed by petroleum ether EtOAc mixtures in a gradient system to afford 26 mg of **5**.

*Gileadenol (**9**)*: $C_{20}H_{32}O$. Oily**;** $[\alpha]^{20}_D$ =+112° (*c* 0.63, $CHCl_3$). IR (film) υ_{max}: 3611 (OH), 1651 (C=C), 1212 cm^{-1} (C-O). ^1H and ^{13}C NMR: Table 1. HRESIMS: *m/z* 287.2363 (*Calcd.* 287.2375) $[M^+-1]$, 271.2414 (Calcd. 271.2426) $[M^+-OH]$.

2.3. Antimicrobial Activity

2.3.1. Bacterial Strains

The antimicrobial assays were performed using reference strains of the American Type Culture Collection (ATCC) available in the Microbiology Laboratory of College of Pharmacy/Prince Sattam University (Al-Kharj- Arabia Saudi). The standard microbial strains used were as follows: gram positive bacteria (*Bacillus subtilis* ATCC10400, *Staphylococcus aureus* ATCC35501), gram-negative bacteria (*Escherichia coli* ATCC25992, *Klebsiella pneumonia* ATCC138222) and *Candida albicans* ATCC14053.

2.3.2. Antimicrobial Assay

The obtained bacteria were sub-cultured onto Mueller-Hinton agar for 24 h and the fungal strain *C. albicans* was cultured on Sabouraud-dextrose agar for 5 days at 37 ℃. Colonies from sub-cultured

plates were then grown in M_H broth and S-D broth to match the 0.5 McFarland turbidity standard equivalent to 1.5 _ 10 ^{8} CFU/mL. M_H agar and S-D agar were used for antibacterial and antifungal assays, respectively. The agar-well diffusion method was used for inhibition zone determination [16] using ampicillin as positive control. MIC were measured following the reported broth dilution method and the Clinical and Laboratory Standards Institute guidelines [17]. Serial dilutions were prepared and tested in from 7.812µg/mL to 50µg/mL. Inoculum of 10 µL from each tested microbes were added to each tested dilution. Mueller-Hinton broth (MHB, Scharlau) alone was tested as sterility control, and untreated microbes inoculated on MHB alone and with different concentration of the solvent DMSO as negative control. All tubes were incubated at 37^{0}C for 24 h and the lowest concentration of antimicrobial agent that inhibited visible growth MIC was determined.

2.3.3 Data Analysis

Values of three replicates are presented as mean ± standard deviation. The statistical parameters applied were the Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

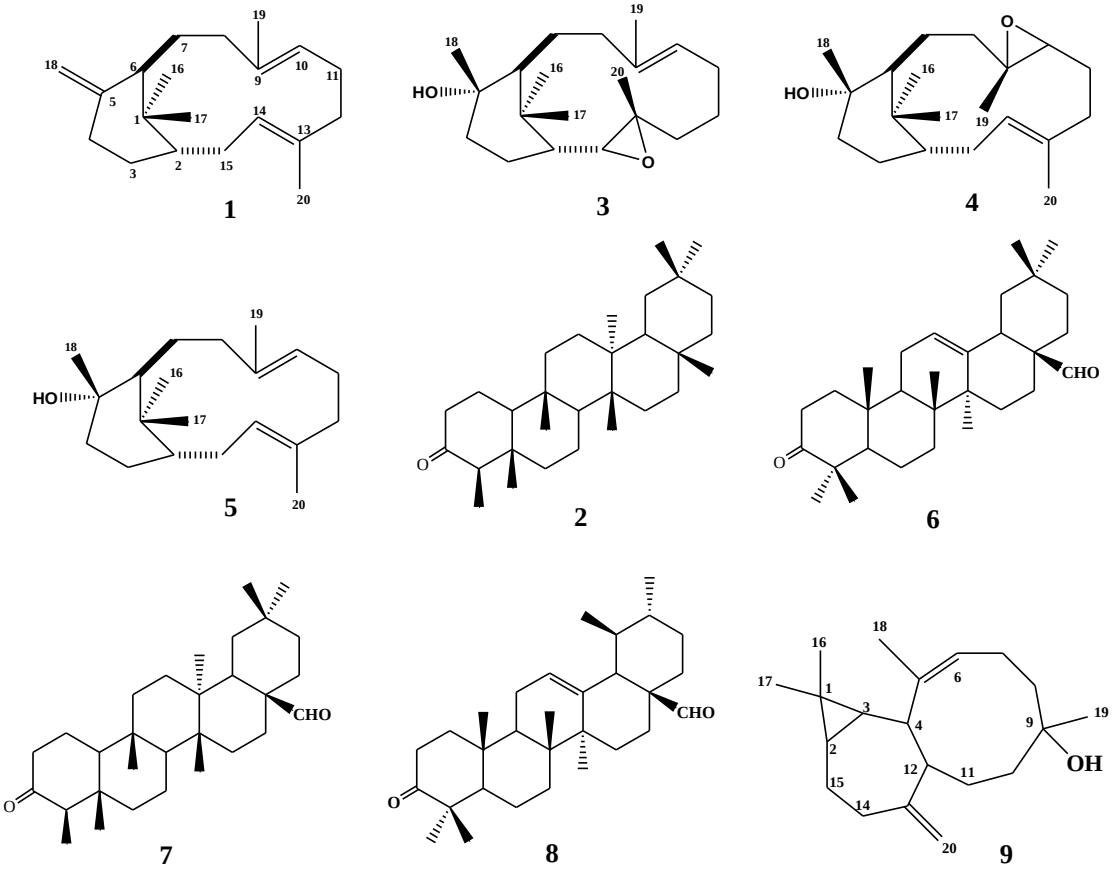


Figure 1. Chemical structures of compounds **1- 9**

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** showed 20 carbon resonances in the ^{13}C NMR spectrum (Table S2) sorted by DEPT135 experiment into 4 X CH_3, 8 X CH_2, 4 X CH and 4 quaternary carbons. The chemical shifts of the carbon atoms pointed out to a hydrocarbon skeleton free from oxygenation. The molecular formula supported by HRESIMS $C_{20}H_{32}$ indicated 5 degree of unsaturation. The data of **1** (Tables S1, S2) were very similar to those reported for (1*S*,3*E*,7*E*,11*R*)-(+)-verticilla-3,7,12(18)-triene [18, 19].

^{13}C NMR of **5** (Table S1, Figure S12) showed twenty carbon resonances sorted by DEPT135 experiment (Figure S13) into 5 X CH_3, 7 X CH_2, 4 X CH and 4 quaternary carbons. Similar to **1** the ^{1}H NMR of **5** (Table S2, Figure S11) showed two doublets for olefinic protons at δ_H 5.55, 4.80 correlated in an HSQC experiment (Figure S15) to carbons at δ_C 127.67 and 129.88 ppm. The main differences between **5** and **1** were the disappearance of the signals assigned for the exocyclic methylene moiety; instead one extra methyl singlet at δ_H 1.16; δ_C 24.04 ppm and an oxygenated quaternary carbon signal at δ_C 75.79 ppm were observed. HRESIMS data (Figure S16) showed ion peak at 273.2594 *m/z* representing M^+-OH. Comparison of the obtained data of **5** with those reported for verticillol and its different isomers revealed a close similarity with *ent*-Verticillol [20-22].

Compounds **3** and **4** ^{13}C NMR (Table S2, Figures S2, S7) showed two more oxygenated carbon signals at δ_C 63.60, 64.32 and 62.24, 66.37 ppm, respectively when compared with **5**. The two oxygenated carbons in DEPT135 experiment (Figures S3, S8) were sorted into one CH and one quaternary carbon in **3** and **4**. The HRESIMS showed an M^+-OH at *m/z* 289.2538 (Figure S5) and 289.2541 (Figure S10) for **3** and **4**, respectively, supporting an oxirane ring in each compound. The ^{1}H and ^{13}C NMR spectrum (Figures S1, S2) of **3** revealed the disappearance of the olefinic signals at δ_H 5.55, δ_C 127.67 and the quaternary carbon at δ_C 132.90 ppm in **5**, instead a proton signal at δ_H 3.45 (d, *J*= 9.5 Hz) correlated in an HSQC experiment (Figure S4) to the oxygenated carbon signal at δ_C 64.32 ppm and quaternary oxygenated carbon signal at δ_C 63.60 ppm assigned for C13-C14 oxirane ring structure. The data of **3** were identical with those reported for (13*S*,14*S*)-*Ent*-13,14-epoxyverticillol [22]. The ^{1}H and ^{13}C NMR spectrum (Figures S6, S7) of **4** revealed the disappearance of the olefinic signals at δ_H 4.64, δ_C 128.37 and the quaternary carbon at δ_C 133.90 ppm in **5** and they were replaced by a proton signal at δ_H 2.95 (d, *J*= 9.9 Hz) correlated in an HSQC experiment (Figure S9) to the oxygenated carbon signal at δ_C 66.37 and quaternary oxygenated carbon signal at δ_C 62.24 ppm assigned for C9-C10 oxirane ring structure. The data of **4** were closely related with those reported for (9*S*,10*S*)-*Ent*-9,10-epoxyverticillol [22]. This is the first report of the *ent*-verticillane-type diterpenes from the genus *Commiphora*.

Table 1. ^{1}H and ^{13}C NMR data for compounds **9** ($CDCl_3$, δ in ppm, *J* in Hz).

Pos.	^{1}H	^{13}C	Pos.	^{1}H	^{13}C
1	-	24.31	**11**	1.49 m [a], 1.81 m [a]	26.34
2	0.63 m [a]	26.73	**12**	2.12 bq [d] (6.2)	53.01
3	0.40 dd [b] (9.8, 10.8)	29.22	**13**	-	153.43
4	1.26 m [a]	53.68	**14**	1.95 m [a] 2.33 dd [b] (6.0, 8.2)	38.87
5	-	131.08	**15**	0.92 m [a], 1.87 m [a]	24.78
6	5.01 bt [c] (7.0)	124.86	**16**	0.95 s [e]	13.66
7	1.95 m [a]	25.26	**17**	1.59 s [e]	25.72
8	1.05 m [a], 1.26 m [a]	43.29	**18**	1.52 s [e]	17.52
9	-	81.07	**19**	1.21 s [e]	25.90
10	1.48 m [a], 1.68 m [a]	41.58	**20**	4.58 bs [f], 4.61 bs [f]	106.30

[a] multiplet, [b] doublet of doublet, [c] broad triplet, [d] broad quartet, [e] singlet, [f] broad singlet.

^{13}C NMR of **9** (Table 1, Figure S18, S19) showed 20 carbon resonances sorted by DEPT135 experiment (Figure S20) into 4 X CH_3, 7 X CH_2, 5 X CH and 4 quaternary carbons. The HRESIMS data (Figure S33) showed M^+-1 at *m/z* 287.2363 and M^+-OH at *m/z* 271.2414 for the molecular formula $C_{20}H_{32}O$ with 5 degrees of unsaturation. One of the degree of unsaturation was assigned from the NMR data (Figures S17-S20) of **9** as exo-methylene moiety at δ_H 4.59, 4.61 (each as broad singlet) correlated in HSQC experiment (Figure S23) with the carbon signal at δ_C 106.30 and the quaternary carbon at δ_C 153.43 ppm. Another degree of unsaturation represented by the olefinic signals at δ_H 5.01 (bt) correlated in an HSQC experiment with the CH at δ_C 124.86 and the quaternary carbon at δ_C 131.08 ppm. With the absence of other Sp^2 carbon signals the remaining unsaturation were accounted for three ring structures.

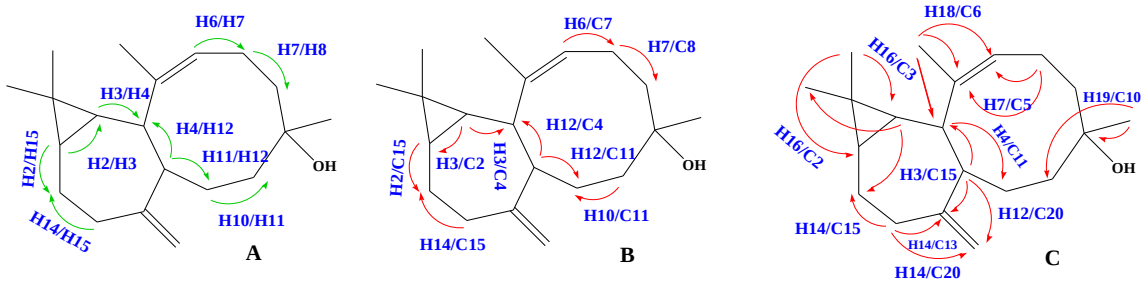
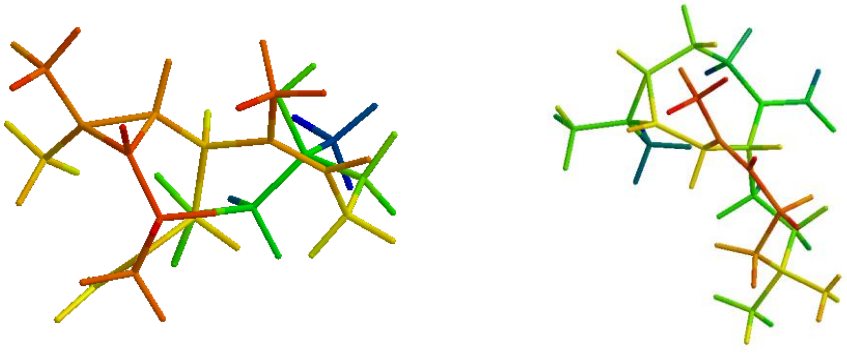


Figure 2. ^{1}H-^{1}H COSY (**A**), H2BC (**B**) and selected HMBC (**C**) correlations of compound **9.**

The group of signals representing two CH, one quaternary carbon and two CH_3 groups at δ_H 0.63, δ_C 26.73 (C2); δ_H 0.40, δ_C 29.22 (C3); δ_C 24.31 (C1); δ_H 0.95, δ_C 13.66 (C16); δ_H 1.59, δ_C 25.72 ppm (C17) were typical for 1,1-dimethyl cyclopropane moiety [23]. Combination of COSY and H2BC connectivity experiments (Figures 2, S21, S22, S25-S28) enable the identification of a CH-CH-CH spin system at δ_H 0.40, δ_C 29.22 (C3); δ_H 1.26, δ_C 53.68 (C4) and δ_H 2.12, δ_C 53.01ppm (C12). Another spin system was detected between CH at 0.63, δ_C 26.73 (C2), CH_2 at δ_H 0.92, 1.87, δ_C 24.78 (C15) and CH_2 at δ_H 1.95, 2.33, δ_C 38.87 ppm (C14). The terminal CH (C12) and CH_2 (C14) in these two spin systems showed two ways 3 bonds HMBC correlations (Figures 2, S29-S32) with the exo-methylene moiety at δ_H 4.59, 4.61, δ_C 106.30 (C20). H14 protons showed 2 bonds HMBC correlations with the quaternary carbon at δ_C 153.43 ppm (C13). This arrangement enables the assignment of a 7 membered ring fused with the 1,1-dimethyl cyclopropane ring structure. With the remaining two methyl groups signals at δ_H 1.52, δ_C 17.52 and δ_H 1.21, δ_C 25.90 ppm the rest of carbons must form a 9 membered ring coupled with the 7 membered ring via the two methines at δ_H 1.26, δ_C 53.68 (C-4) and δ_H 2.12, δ_C 53.01ppm (C12). The 9 membered ring contains the olefinic array and oxygenated quaternary carbon at δ_C 81.07 ppm. The positions of the two substituents was undoubtfully assigned based on COSY, H2BC and HMBC experiments results. The methyl signal at δ_H 1.52 (C18) showed HMBC correlations with carbon signals at δ_C 131.08, 124.86 and 43.29 ppm assigned for C-5, C-6 and C-8, respectively. Clear H2BC were observed between the H-8 protons at δ_H 1.05, 1.26 and the C7 carbon at δ_C 25.26 ppm (Figure S26). The methyl group protons signal at δ_H 1.21 showed 2 bonds HMBC correlations with the carbons at δ_C 81.07 and 3 bonds correlations with the carbon at δ_C 41.58 ppm assigned for C-9 and C-10, respectively (Figure S31). COSY and H2BC correlations were observed between C10 (δ_H 1.48, 1.68; δ_C 41.58 ppm), C11 (δ_H 1.48, 1.81; δ_C 26.34 ppm) and C12 (δ_H 2.12; δ_C 53.01ppm) (Figures 2, S21, S22, S25-S28) confirming assignments of their position. In the HMBC experiment long range correlations were observed through 5 and 6 bonds away. This correlation can be explained by the "W" coupling [24] that could happen with trans fused 7 and 9 membered rings with α-oriented H-4. However, from the available data the complete stereochemistry cannot be determined.



A **B**

Figure 3. Proposed stereo models of **9** to explain the long range HMBC correlations. **A**: Trans ring junction with *α*-oriented H-4 in favour of "W" coupling. **B**: Trans ring junction with *β*-oriented H-4 not in favour of "W" coupling.

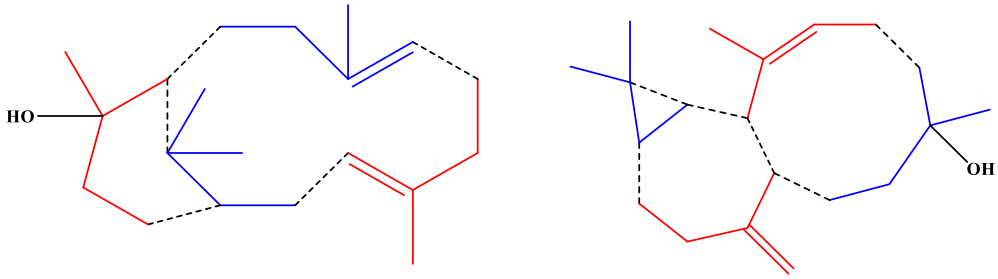


Figure 4. Proposed biosynthetic origin of compound **9**

The structure of **9** represents a rearranged verticillene skeleton identified for the first time from natural source. The name "gileadenol" was given to the new compound **9** with "gileadene" novel diterpene skeleton. The novel skeleton of **9** proposed to be biosynthesized from four isoprene unites in similar fashion as verticillene diterepenes. However, different cyclization pattern takes place to produce such newly discovered skeleton (Figure 4).

The four isolated triterpenes were identified as friedelin (**2**) [25, 26], oleanonic aldehyde (**6**) [27, 28], canyophyllal (**7**) [29] and urs-12-en-3-one-28-al (**8**) [30]. Compounds **6-8** are reported for the first time from *Commiphora* species.

Table 2. MIC (µg/mL) of different extracts of *C. gileadensis*

	Staph. aureus	***B. subtilis***	***E. coli***	***K. pneumonia***	***C. albicans***
Fresh stem $CHCl_3$ Ext.	750	750	750	250	125
Fresh stem MeOH Ext.	17000	35000	175	50000	16000
Fresh Leaves $CHCl_3$ Ext.	750	750	750	250	125
Fresh Leaves MeOH Ext.	17000	25000	175000	25	7500
3	NT [a]	NT [a]	NT [a]	46.8	NT [a]
4	NT [a]	NT [a]	NT [a]	25	NT [a]
5	NT [a]	10	NT [a]	93.8	NT [a]

[a] Not tested.

3.2. Antimicrobial Activity

MIC were determined for the different extracts obtained from the fresh, dried leaves and stems (Table 2) using Gram-positive bacteria (*Bacillus subtilis* ATCC10400, *Staphylococcus aureus* ATCC35501), Gram-negative bacteria (*Escherichia coli* ATCC25992, *Klebsiella pneumonia* ATCC138222) and *Candida albicans* ATCC14053. All the chromatographic purification steps were monitored by the antimicrobial testing using *E. coli* and *C. albicans* and activity were determined based on the inhibition zone measurement. The $CHCl_3$ extract of the fresh stem was the most active and further purified to afford the nine isolates from the active fractions. The antimicrobial activity of compounds **2** and **6** was previously reported [26, 27]. Compounds **3-5**, **7** and **9** were tested against the five organisms and the corresponding inhibition zones are presented in Table 3. Both **7** and **9** were less active against the tested microorganisms. MIC were determined for **3-5** against *K. pneumonia* and for **5** against *B. subtilis* as well (Table 2). Compound **4** was the most active with MIC= 0.025 mg/mL followed by **3** with MIC= 0.0468 mg/mL, while **5** was the least active at MIC= 0.0938 mg/mL against *K. pneumonia*. The MIC of **5** against *B. subtilis* was 0.01 mg/mL. The obtained results indicated that the compounds with verticillene skeleton were the most active. The presence of the epoxide moiety increased the activity against the Gram-negative bacteria *K. pneumonia* probably due to better penetration of the cell walls. Compound **5** lacking the epoxide function was more active against the Gram-positive bacteria *B. subtilis*. The antimicrobial activity of the isolated compounds supports the traditional uses of the plant to treat infected wounds [3, 4].

Table 3. Inhibition zones (mm) of compounds **3-5, 7** and **9** at 1 mg/mL against four bacterial strains

	Staph. aureus	***B. subtilis***	***E. coli***	***K. pneumonia***
Ampicillin[a]	17±0.2	20±0.1	16±0.3	18±0.1
3	11±0.3	16±0.2	13±0.3	18±0.2
4	12±0.2	16±0.2	13±0.3	20±0.2
5	13±0.2	18±0.3	12±0.2	15±0.1
7	11±0.3	15±0.2	13±0.3	15±0.2
9	12±0.4	13±0.4	13±0.2	16±0.4

[a] Ampicillin tested at 10 µg/mL

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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