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Isolation and identification of endophytic bacteria and associated compound from *Gloriosa superba* and their antibacterial activities

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ABSTRACT

Gloriosa superba L., which belongs to the genus *Gloriosa* and family Colchicaceae, is a climbing annual herb and tuberous poisonous tropical medicinal plant. This study was aimed to isolate possible endophytic bacteria from leaves, stems and tubers of *Gloriosa superba*. Thirty pure endophytic bacteria were isolated and subjected to biochemical characterization. Bacterial identification was conducted by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The structure of the isolated compound was characterized. The antibacterial activity was also evaluated. Majority (21, 70 %) of the isolates were Gram-positive. Certain of them are spore formers. Based on MALDI-TOF MS, 26 of the isolates were identified as *Bacillus* spp. (65.4 %), *Escherichia* spp. (30.8 %) and *Providencia* spp. (3.9 %). A 1-undecene was isolated from culture filtrate of *E. coli* (GST-5). The ethyl acetate extracts (1000 μg/mL) of GSL-5 and GST-2 culture filtrates recorded maximum inhibition zone against *E. coli* (9.4 ± 0.6 mm) and *S. aurous* ATCC 25923^T (8.4 ± 0.8 mm), respectively. The *Pseudomonas aeruginosa* ATCC 27853^T was prone to all ethyl acetate extracts. A 1-undecene showed a moderate activity against *E. coli* ATCC 25922^{T}and *P. aeruginosa* ATCC 27853^T at 50 μg/mL. The present finding would be a breakthrough to studies of similar works in Ethiopia since it may be for the first time.

1. Introduction

Medicinal plants have been known as reservoirs of certain numbers of endophytic microbial cells [1]. Endophytes are the plant-associated microorganisms that are symbiotically residing within the living tissues of their hosts without any visible harm [2]. The strong endophyte-host plant association enables endophytes to take advantage of producing a diversified number of secondary metabolites. It is expected that endophyte-originated bioactive compounds may have less toxicity effect on the normal cell, as they do not harm the eukaryotic host system. Therefore, it is noteworthy to explore endophytes and associated bioactive compounds from their

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Table 1
Biochemical tests and MALDI-TOF MS results for endophytic bacterial isolates.

S.N.	Isolates	GS	S	CC	CLT	CT	MT	IT	LD	MRT	ET	OR	PH	SH	ST	UT	VPT	PII
1	GSL-1	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
2	GSL-2	+	R	WG	++	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
3	GSL-3	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		–	**B. megaterium*
4	GSL-4	+	R	WG	+++	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
5	GSL-5	+	R	WP	+	+	+	+	–	+	+	+	+	+	+		–	*B. subtilis*
6	GSL-6	–	R	WP	+	–	+	+	–	+	–	+	–	NP	–	–	+	*E. coli*
7	GSL-7	+	C	WGE	+	+	+		–	+	–	+	+	+	NP		+	*B. subtilis*
8	GSL-8	–	R	O	+	–	+	+	–	+	–	+	+	–	–	–	+	*E. coli*
9	GSL-9	–	R	WB	+	–	+	+	–	+	–	+	+	–	–	–	+	*E. coli*
10	GSL-10	–	R	LO	+	–	+	+	–	+	–	+	+	–	–	–	+	*E. coli*
11	GSS-1	–	R	WG	+	–	+	+	–	+	–	+	+	+	+	–	NP	*Providenciarettgeri*
12	GSS-2	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
13	GSS-3	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	**B. subtilis*
14	GSS-4	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
15	GSS-5	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
16	GSS-6	+	R	WG	+++	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
17	GSS-7	+	R	WG	+	+	+	+	–	+	+	+	+	+++	+		+	*B. amyloliquefaciens*
18	GSS–8	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
19	GSS-9	+	R	WG	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
20	GSS-10	+	R	B	+	+	+	+	–	+	–	+	NP	NP	NP		NP	**Corynebacterium*sp.
21	GSS-11	–	C	W	+	–	+	+	–	+	–	+	–	–	–	–	NP	*E. coli*
22	GSS-12	+	R	B	+	+	+	+	+	+	+	+	+	+	+		+	*B. atrophaeus*
23	GST-1	+	R	WG	+	+	+	+	+	+	+	+	+	+	+		+	*B. subtilis*
24	GST-2	–	R	LO	+	–	+	+	–	+	–	+	+		–	–		*E. coli*
25	GST-3	+	R	WG	+	+	+	+	NP	NP	+	NP	+	+	+		+	*B. amyloliquefaciens*subsp. *plantarum*
26	GST-4	–	R	LB	+	–	+	+	–	+	–	+	+	–	–	–	NP	*E. coli*
27	GST-5	–	R	LB	+	–	+	+	–	+	–	+	+	–	–	–	NP	*E. coli*
28	GST-6	+	R	LB	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtilis*
29	GST-7	+	R	LB	+	+	+	+	–	+	+	+	+	+	+		+	*B. subtils*
30	GST-8	+	R	B	+	+	+	+	–	+	+	+	+	+	+		+	**B.subtilis*

Key notes: **B**-Brown, **C**-*Cocci*, **CC**-Colony color, **CLT**-Catalase test, **CT-**Citrate test, **GS-**Gram staining, **ET**-Endospore test, **IT-**Indole test, **LB**-Lightbrown,**LD-** Lysine decarboxylase test, **LO-**Light orange, **MRT-**Methyl red test, **MT**-Motility test, **NP-** not performed**, O-**Orange**, Or**-Ornithine test, **OT**-Oxidase test, **PH-** Phenylalanine test, **PII-**Presumptive identified isolate, **R**-Rod, **S**-Shape, **SH-**Starch hydrolysis, **ST-**Salt tolerance test, **WB-**White brown, **WGE-**White green, **WG-**White grey, **WP-**White purple, **UT**-Urease test, **VP**-Vogues Proskauer test, + = slight response, ++ = moderate response, +++ = strong response, - = negative response, *not identified by MALDI-TOF MS.

host plants [3]. *G. superba* L. (also synonymic to *G. simplex*, *G. abyssinica* and *G. speciosa*) belongs to the genus G*loriosa* and family Colchicaceae. It is an erect or climbing annual herb and tuberous poisonous tropical medicinal plant growing up to 3 m height [4,5]. It is commonly known as glory lily in English. It exhibits a hollow stem about 6 m, emerging from its tuberous underground stem every year in rainy season. Leaves are etiolated, alternate, sessile, lanceolate and spear shaped with curved end, which helps them to climb and creep. The flowers of *G. superba* have characteristics of brilliant wavy edged yellow and red colors that have been observed every year from November to March [6]. Fruits of this poisonous plant are oblong, ellipsoid capsule and its seeds are numerous and rounded [5]. *G. superba* grows widespread in *Acacia-Commiphora*, *Combretum-Termianlia* and wood-land, about 400–2500 m altitudes [4].

In Africa, it is widely distributed in Senegal, Ethiopia, Somalia and South Africa. *G. superba* has been known for its wide ethnomedicinal uses to treat various ailments. Various parts of the plant are used to treat spleen complaints, sores, tumors, syphilis, CNS depressant and sexual dysfunction [5,7]. *G. superba* is also used as an embolic in labor, and purgative, and anthelminthic. This medicinal plant has been employed to cure certain diseases and insects such as chronic ulcers, leprosy, colic, head lice hemorrhoids, skin parasites [8]. The tuberous root of the plant is used to reduce a joint pain affected with arthritis [9]; and also applied to treat bruises, impotence, intestinal worms, infertility, inflammation, leprosy, skin diseases, snake bites and ulcers. In the Eastern Ethiopia, the leaves of *G. superba* are used for the treatment of epilepsy, skin cyst, toothache, tumor or "*keledo*", gallstone and gangrene [8].

Some endophytic fungi were isolated and identified from various parts of *G. superba*. For instance, a total of 233 fungal endophyte isolates were identified and reported from a total of 450 segments of roots, stems and leaves parts [10]. *Bipolaris Cynodontis*, *B. specifera*, *Fusarium oxysporum* and *F. solani* were found as dominant colonizers of the roots part together with *Talaromyces pinophilus*, *Oidiodendron* spp. and *Colletotrichum Gloeosporioides. Phomopsis* species was another endophytic fungus reported from the tuber part of *G. superba*, which is used to produce the known anticancer agent, colchicine [11,12]. Two endophytic fungi, namely, *Alternaria solani* and *Penicillium funiculosum*, were also identified from the plant and used in the biosynthesis of antimicrobial Silver nanoparticles [13]. However, to the best of our knowledge no endophytic bacteria were isolated and reported from this plant. In the present study, preliminary work was done to isolate endophytic bacteria and associated compound from stems, leaves and tubers of *G. superba* to shed light on the study of endophytes in the context of Ethiopia. The isolated endophytic bacteria were identified using the MALDI-TOF MS instrument to complement the classical methods of bacterial identification. This MALDI-TOF MS tool has been established for the identification of mainly various types of bacterial isolates, and rarely for yeast and mold. It works based on the generation of mass spectral data of protein profile of corresponding bacterial isolates and comparison with those from the database. MALDI-TOF MS has many advantages over the conventional and molecular techniques including its simplicity, rapidness and cost-effectiveness, and it does not need special expertise [14,15].

2. Materials and methods

2.1. Plant material collection

Plant parts of *Gloriosa superba* (leaves, stems and tubers) (photo shown in Fig S_1) were collected from Dengego mountains, Dire Dawa, Ethiopia. These samples were aseptically collected in sterile plastic boxes and brought to microbiology laboratory, School of Medical Laboratory Sciences, Haramaya University, and were preserved in refrigerator (4 °C) for 24 h. The botanical name of the plant was identified and a specimen was preserved with voucher number of AHU110 at herbarium of Haramaya University.

2.2. Endophytic bacteria isolation

The plant parts were thoroughly washed with running water to remove attached debris and excess epiphytes. Subsequently, plant tissues were surface sterilized by sequential immersion in 95 % (v/v) ethanol (10s), 5 % (v/v) aqueous solution of sodium hypochlorite (2–5 min) and 70 % (v/v) ethanol (2 min). Finally, the surface sterilization was ended by rinsing the plant tissues three times with autoclaved distilled water (to remove excess sterilants) and dried on aluminum foil. All steps in the sterilization procedure were conducted under aseptic conditions. Surface sterilized plant explants were cut into small segments (2 cm) using sterilized knife and 15 segments of each part were seeded on Tryptic soy agar (TSA) (g/L) (pancreatic digest of casein, 15.0; peptic digest of soybean meal, 5.0; sodium chloride, 5.0; agar, 15.0; pH 7.3 ± 0.2 at 25 °C) medium augmented with antifungal drug (carbendazim). At the same time, aliquot of last tissue washing was inoculated on TSA for sterility checking. All plates were incubated at 37 °C for 48 h in duplicates using incubator (Binder B28, Germany). After incubation, emergence of endophytic bacteria was observed and the number of segments colonized by bacterial endophytes was counted. No colony growth was observed in the last washing of the plant tissues, indicative of safe surface sterilization. Of the emerging bacterial colonies, thirty of colonies with distinct features (10 from leaves, 12 from stems and 8 from tubers) were sub-cultured several times in fresh TSA until getting pure colonies. Finally, pure colonies were kept at 4 °C for biochemical and MALDI-TOF MS characterizations. Other duplicates of these pure colonies were preserved at −20 °C using 50 % glycerol (v/v) for sub-culture.

2.3. Morphological and microscopic characterization

The colony morphological color, on the Petri dish, of all the thirty endophytic bacterial isolates were noted and presented in Table 1. Besides, each isolates were subjected to Gram-staining analysis using standard staining procedure; and their response toward Gram-staining reagent and shape were identified using Olympus microscope. Those spore bearing isolates were further subjected to spore staining test using Malachite green reagent to confirm their spore forming ability.

2.4. Biochemical tests

In order to complement the morphological and microscopic characterization, both the Gram-positive and Gram-negative endophytic bacterial isolates were subjected to various biochemical tests such as catalase, starch hydrolysis (amylase), motility, citrate, oxidase, lactose and glucose fermentation, Vogues-Proskauer (VP), methyl red (MR), indole, ornithine, lysine, decarboxylase (LDC), phenylalanine and urease tests based on the flow chart of Bergey's manual of determinative bacteriology [16] for systematic identification.=

2.5. Effect of salt concentration

The endophytic isolates were also checked for their growth tolerance to salt concentration by inoculating few colonies of each isolate onto Tryptic soy agar (g/L) (pancreatic digest of casein, 15.0; peptic digest of soybean meal, 5.0; sodium chloride, 5.0; agar, 15.0; pH 7.3 ± 0.2 at 25 °C) medium supplemented with 6.5 % NaCl concentration. After 24 h incubation, growth of each isolate was checked.

2.6. Identification of endophytic bacterial isolates based on protein profile using MALDI-TOF MS

Pure isolates of the endophytic bacteria were identified following the methods of [15]. Matrix-assisted laser desorption ionization time of flight mass-spectroscopy (MALDI-TOF MS) identification was carried out at NAHDIC, Ethiopia. Isolate classifications were carried out using the direct transfer method (MALDI Biotyper 3.1. User Manual, Bruker Daltonics Inc.). The representative single colonies of each isolated bacteria were smeared as a thin film directly into a spot on MALDI target plate using a tooth applicator. The MALDI-TOF MS target plate was overlaid with 1 μL of 70 % (v/v) of formic acid and allowed to dry at a room temperature. Immediately the spot was overlaid with 1 μL of matrix solution α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile (CHCA) solution and allowed to dry at room temperature. The resulting spectra were compared with reference spectra by using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.0 for a given species were considered High confidence identification to the species level, 1.70–1.99 were considered Low confidence identification, and 0.00–1.69 were characterized as No organism identification possible. *E. coli* ATCC 25922^T was used as a standard for calibration and quality control.

2.7. Culture cultivation and ethyl acetate extraction

Six endophytic bacteria isolates, namely, GST-8, GST-2, GST-5, GST-4, GSL-5 and GSS-7, were selected as representatives and subjected to the fermentation process to cultivate and scale up the respective cultures for extraction of bioactive compounds. A single colony of each isolate was picked up and inoculated in separate Erlenmeyer flask (500 mL) containing tryptone soya broth (TSB, 250 mL) aseptically and placed on orbital shaking incubator (Hy-5A, Movel Scientific Instrument CO. Ltd., China) (121 rpm at 28 °C) for 8 days. Fermented cultures were filtered using muslin cloth and cell free culture filtrates were liquid-liquid partitioned with equal amounts of ethyl acetate. The ethyl acetate phases were separated; dried over Na_2SO_4 anhydrous and concentrated using rotavapor leading to 0.2, 2.6, 3.5, 0.044, 0.064 and 0.16 g crude yield for GST-8, GST-2, GST-5, GST-4, GSL-5 and GSS-7 isolates, respectively. The chemical profile of each ethyl acetate extract of each isolate was monitored with TLC analysis using DCM/EtOAc/Acetic acid (3:1:0.1) as developing solvent and UV-lamp followed by vanillin/MeOH/H_2SO_4 (0.3g/95/5) as visualizing techniques. All the tested endophytic bacteria isolates exhibited similar TLC profiles. Accordingly, the obtained ethyl acetate extract of *Escherichia coli* (GST-5) isolate was fractionated on silica gel column chromatography as follows.

2.8. Fractionation of Escherichia coli (GST-5) ethyl acetate extract

Yellowish power (3.0 g) of ethyl acetate extract was reconstituted in EtOAc (30 mL), adsorbed on 10 g of silica gel and applied onto CC packed with DCM saturated silica gel (150 g). Elution was started with DCM followed by gradient of DCM/EtOAc and ended with EtOAc/MeOH (95/5) to collect one hundred thirteen fractions. Collected fractions were analyzed for their TLC profiles using the solvent systems of DCM/EtOAc/AcOH (9:1:0.1 and 3:1:0.1) under the visualizing techniques of UV-lamp (254 nm) and iodine vapor. Even though spots were not clearly visible in the TLC slides, fractions expected to have similar chemical profiles were combined and their TLC analysis was performed. Unfortunately, all the combined fractions (fr1-12, fr12-40, fr41-65, fr65-80 and fr81-113) exhibited similar TLC profile both under iodine and vanillin/MeOH/H_2SO_4 (0.3g/95/5) reagents with DCM/EtOAc/AcOH (3:1:0.1) developing solvent system. Hence, all the fractions (1–113) were recombined, concentrated (200 mg) and subjected to silica gel CC sub-fractionation as follows.

Fr1-113 (200 mg) was sub-fractionated on small CC packed with 30 mg of silica gel solvated with DCM after dissolved in ethyl acetate and adsorbed on silica gel. Fifty sub-fractions were eluted using DCM, DCM/EtOAc (80/20 and 60/40) and EtOAc solvent systems. After TLC examination under the solvent systems of DCM and DCM/EtOAc/AcOH (9:1:0.1) and detection method of vanillin, sub-fraction1-5, sub-fraction 9–12, sub-fraction 13–16, sub-fraction 17–19 and sub-fraction 21–50 were mixed and re-subjected to TLC analysis. Among the sub-fractions, sub-fraction1-5 was showed better purity and yielded 1-undecene (20 mg). It was obtained as colorless oily semi volatile (20 mg) with R_f value of 0.87 (DCM/*n*-hexane, 3:1). See Table 2 for the NMR spectral data. 1D-NMR spectroscopy was employed. The ^{1}H (400 MHz), ^{13}C and DEPT-135 (100 MHz) NMR experiments were conducted on a BRUKER

ACQ 400 AVANCE spectrometer equipped with a 5 mm proton probe and running topspin 2.1 Software operating at 298 K. All chemical shifts (δ_{ppm}) of the spectra were recorded relative to an internal TMS reference. Acquired spectra were further processed using MestReNova software (Mestrelab Research S.L. Version 12) for phase and base-line correction.

2.9. Antibacterial evaluation of ethyl acetate extracts and 1-undecene

The ethyl acetate extract of certain endophytic bacterial isolates (GST-8, GST-5, GST-2, GST-4, GSS-7 and GSL-5) and isolated compound (1-undecene) were studied for potential antibacterial activity using disc diffusion method against standard human pathogenic bacterial strains such as *E. coli* ATCC 25922^T, *P. aeruginosa* ATCC 27853^T and S. *aureus* ATCC 25923^T. A 1000 μg/mL of each ethyl acetate extract and five serial dilutions (50, 100, 300, 500 and 1000 μg/mL) of 1-undecene compound were tested against these individual type culture strains. Chloramphenicol disc (30 μg/disc) and DMSO impregnated paper disc (6 mm in diameter) were served as positive and negative controls, respectively. Each concentration (100 μL) was then loaded onto a 6 mm diameter. Whatman filter paper disc and impregnated discs were placed on Petri dishes which contained MHA medium and previously inoculated with bacterial solution equivalent with 0.5 McFarland standards. After incubating for 18–24 h, the activity of each sample against each bacterium was monitored by measuring the diameter of the cleared area observed around each disc with a caliper (in mm).

3. Results

3.1. Morphological, microscopic and biochemical characterization

Of the total segments (15) of each plant part applied onto TSA medium, the leaves and stems segments supported maximum endophytic bacterial emergence (13, 86.7 % each) and the minimum bacterial count was observed in the tuber segments (11, 73.3 %). From the emerged 30 pure isolates of endophytic bacterial cells (Additional File: Fig. S_2), 10 of them obtained from leaves, 12 of them isolated from stems and 8 of them obtained from tubers (Fig. 1 and Additional File: Fig. S_2). These isolates were selected as potential endophytic bacterial isolates with distinct morphological and colony color. Out of the 30 obtained endophytic bacterial isolates, 15 exhibited a white color (GSL-1, GSL-2, GSL3, GSL-4, GSS-1, GSS-2, GSS-3, GSS-4, GSS-5, GSS-6, GSS-7, GSS-8, GSS-9, GST-1 and GST-3), 4 a white brown (GST-4, GST-5, GST-6 and GST-7), 3 a brown (GSS-10, GSS-12 and GST-8), 2 a white purple (GSL-5 and GSL-6), another 2 a light orange (GSL-10 and GST-2) and the remaining 3 were observed as white green (GSL-7), orange (GSL-8) and white (GSS-11) (Table 1).

The Gram-staining test result (Table 1, Fig. 2 a-d and additional File: Fig. S_3) showed that 21 of the 30 isolates were confirmed as Gram-positive while the remaining nine were found to be Gram-negative isolates. All the endophytic bacterial isolates exhibited a rode-shape except GSL-7 and GSS-11 which were observed as *cocci*. Besides, 19 isolates (GSL-1, GSL-2, GSL-3, GSL-4, GSL-5, GSS-2, GSS-3, GSS-4, GSS-5, GSS-6, GSS-7, GSS-8, GSS-9, GSS-12, GST-1, GST-3, GST-6, GST-7 and GST-8) were found to be spore formers (Table 1, Fig. 3 a & b and Additional File: Fig. S_3).

Following the Bergey's manual methods of identification, 19 were found to be spore forming bacterial isolates, 17 (GSL-1, GSL-2, GSL-4, GSS-1, GSS-3, GSS-4, GSS-5, GSS-6, GSS-7, GSS-8, GSS9, GSS-12, GST-1, GST-3, GST-6, GST-7 and GST-8) of them were found to be positive for VP test while 2 (GSL-3 and GL-5) of them were negative for VP test. All the VP positive spore forming isolates exhibited a cell with diameter of < 1 μm. Those VP negative spore formers were examined under microscope for the presence of swollen cells and none of them showed any colony with exceptional size. Besides, the entire spore forming bacterial isolates showed a positive response to the citrate test and grew at 6.5 % NaCl concentration (w/v). Regarding the Gram-negative bacterial isolates, they provided different responses toward various biochemical tests (Table 1). All the isolates were found with a negative response toward oxidase, Vogues-Proskauer, citrate, lysine decarboxylase, phenylalanine and urea biochemical tests except the GSS-1 isolate which reacted positively to citrate and phenylalanine tests. Besides, all the Gram-negative isolates were found to be motile and showed a positive response to methyl red (MR), indole and ornithine biochemical tests. Furthermore, all isolates, except GSS-1, were found to be lactose and glucose fermenters, and gas/acid producers.

Table 2
^1H,^{13}C and DEPT-135 NMR data ($CDCl_3$) of 1-undecene.

1-undecene			
C/H	δ^1H (multiplicity)	$\delta^{13}C$	DEPT-135
1	4.99 (*trans*, d, $J = 17.33$), 4.93 (*cis*, d, $J = 9.76$)	114.1	$= CH_2$
2	5.82 (m)	139.3	= CH
3	2.04 (br q or m)	33.8	$-CH_2-$
4	1.26 (m)	29.2	$-CH_2-$
5	1.26 (m)	29.6	$-CH_2-$
6	1.26 (m)	29.5	$-CH_2-$
7	1.26 (m)	29.4	$-CH_2-$
8	1.26 (m)	28.9	$-CH_2-$
9	1.26 (m)	31.9	$-CH_2-$
10	1.26 (m)	22.7	$-CH_2-$
11	0.88 (t, $J = 13.52$)	14.1	$-CH_3$

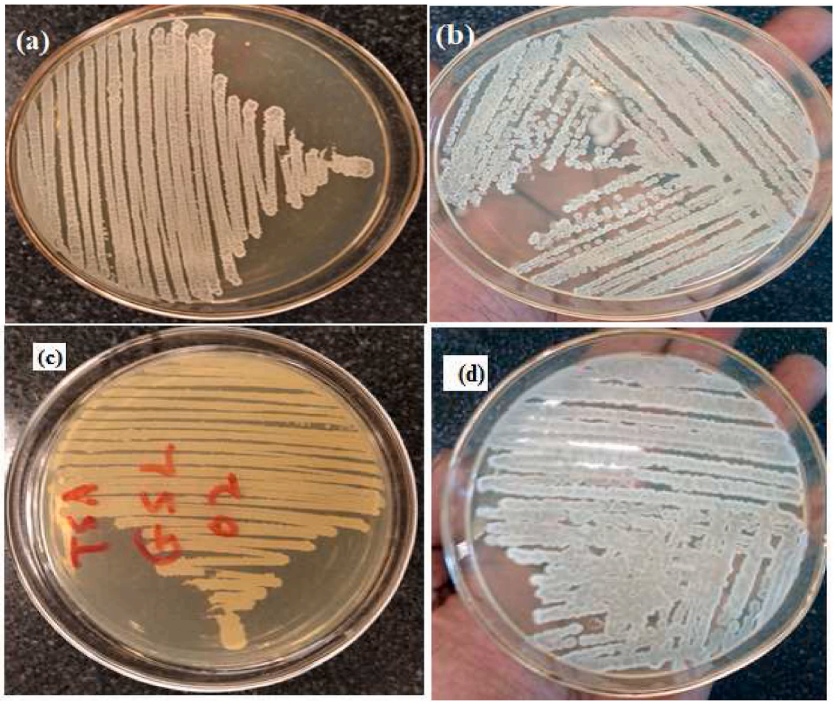


Fig. 1. Pure colonies for certain endophytic bacterial isolates obtained from *Gloriosa superba.* **(a)** Indicates pure colony for GSL-1. **(b)** Indicates pure colony for (GSL-3 with gel colony that obtained from. **(c)** Indicates for *Bacillus subtilis* (GSL2) and **(d)** indicates *B. amyloliquefaciens* (GSS7).

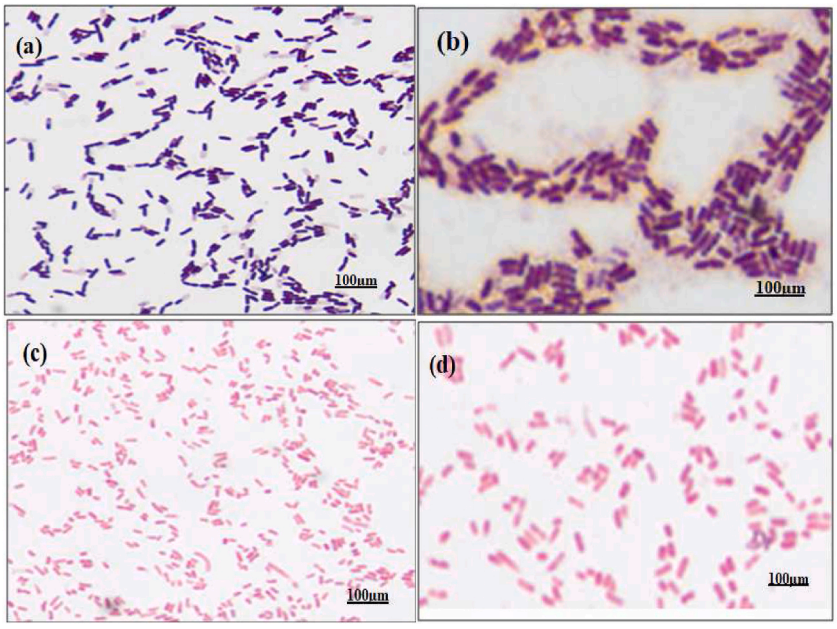


Fig. 2. Gram staining for certain endophytic bacteria isolated from *Gloriosa superba*. **(a)** GSL-1, **(b)** GSL-3, **(c)** GST-5 and **(d)** GSS-1.

3.2. Identification of isolated bacterial endophytes based on protein profile using MALDI-TOF MS analysis

Based on the MALDI-TOF MS analysis (Table 1), 26 out of 30 endophytic bacterial isolates were identified in which 20 were found to be with score values of ≥2.0 and 6 were identified with score values of ≥1.7 (Additional File: Table S_1). And they were generally classified into *Bacillus* spp., *Escherichia* spp. and *Providencia* spp. Among them, 17 isolates (*B. subtilis* GSL-1, *B. subtilis* GSL-2, *B. subtilis* GSL-4, *B. subtilis* GSL-5, *B. subtilis* GSL-7, *B. subtilis* GSS-2, *B. subtilis* GSS-4, *B. subtilis* GSS-5, *B. subtilis* GSS-6, *B. amyloliquefaciens* GSS-7,

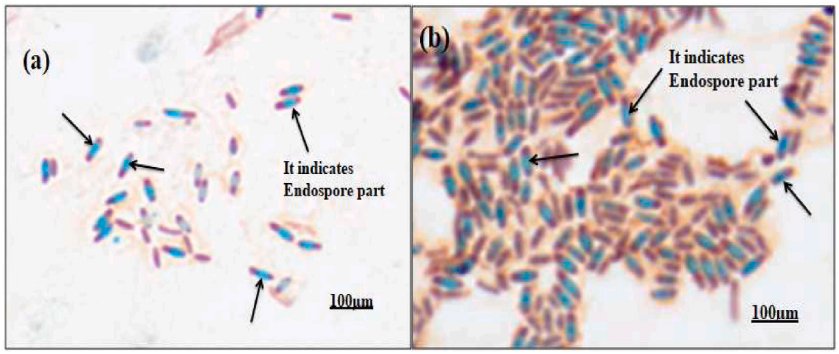


Fig. 3. Endospore test for Gram positive isolates obtained from *Gloriosa superba.* **(a)** indicates endospore test for GSL-1**. (b)** Indicates endospore test for GSL-3 with long rod- Shaped.

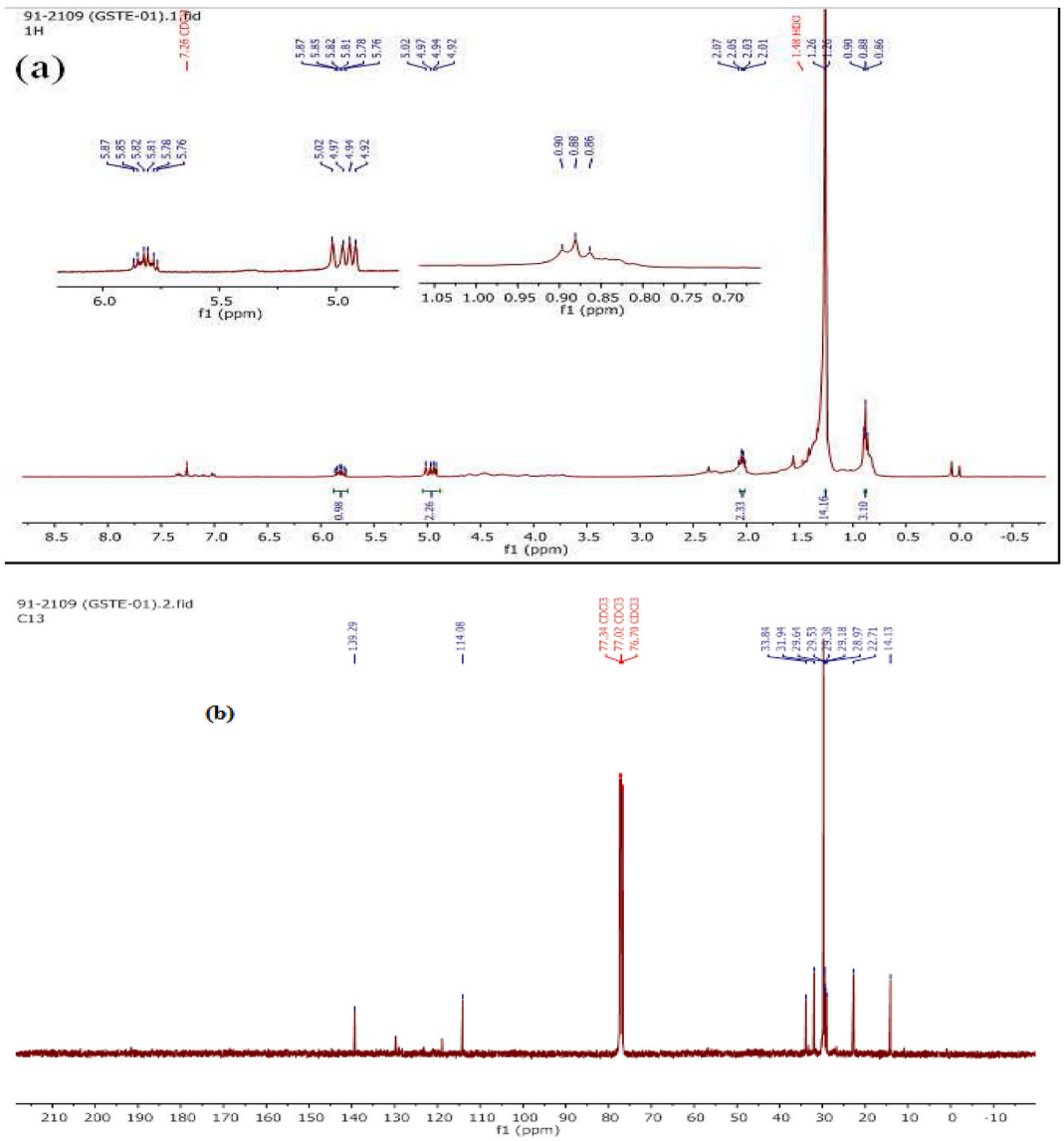


Fig. 4. ^{1}H **(a)** ^{13}C **(b)** and DEPT-135 **(c)** NMR spectra ($CDCl_3$) of **1-undecene**.

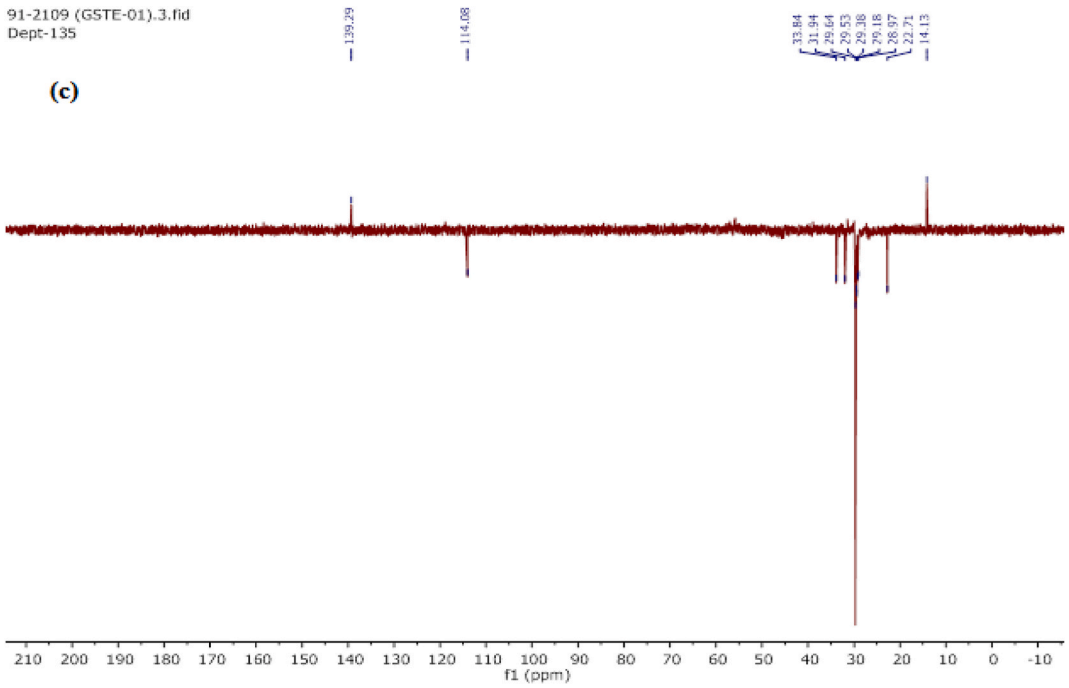


Fig. 4. (*continued*).

B. subtilis GSS-8, *B.* subtilis GSS-9, *B. atrophaeus* GSS-12, *B. subtilis* GST-1, B. amyloliquefaciens subsp. *plantarum* GST-3, *B. subtilis* GST-6 and *B. subtilis* GST-7) were from *Bacillus* spp., 8 (*E. coli* GSL-6, *E. coli* GSL-8, *E. coli* GSL-9, *E. coli* GSL-10, *E. coli* GSS-11, *E. coli* GST-2, *E. coli* GST-4 and GST-5) were identified as *Escherichia* strains. And 1 (*Providencia rettgeri* GSS-1) was confirmed as *Providencia* strain. Whereas two isolates (*B. megaterium* GSL-3 and *B. subtilis* GSS-3) were not totally detected (with zero score value) and two other isolates (*Corynebacterium* sp GSS-10 and *B. subtilis* GST-8) were found with no possibility of organism identification (with score values of <1.7). These isolates might be noble endophytic bacterial isolates. Majority of the identified endophytic bacterial isolates (65.4 %) were found to be *Bacillus* spp. and then followed by the *Escherichia* spp. (30.77 %) (Additional File: Fig. S_2).

3.3. Structural elucidation of 1-undecene

In the ^{1}H NMR spectrum (Fig. 4), a multiplet signal was distinguished at the downfield chemical shift value of δ_H 5.82 which indicates the presence of an olefinic methine proton (=CH-2). Besides, two doublet signals were indicated in the acquired ^{1}H spectrum at δ_H 4.99 (1H, H-1, d, $J = 17.33$ Hz) and 4.93 (1H, H-1, d, $J = 9.76$ Hz) attributed to a *trans* and *cis* protons, respectively, of an olefinic methylene group. Furthermore, the spectrum showed three signals of aliphatic methylene protons and a methyl group at the aliphatic region which appeared at the chemical shift values of δ_H 2.04 (2H, H-3, br q or m), 1.26 (14H, H-4, H-5, H-6, H-7, H-8, H-9 and H-10, m) and 0.88 (3H, H-11, t, $J = 13.5$ Hz). This information suggests that the isolated compound contained a terminal olefinic bond which was also supported by the presence of two carbon signals at δ_C 139.3 (C-2) and 114.1 (C-1) in the ^{13}C and DEPT-135 spectra (Fig. 4), which ascribed to the methine and methylene carbons of the alkene group. Moreover, the ^{13}C spectrum demonstrated additional nine carbon signals which represented a total of nine carbon atoms belonging to eight different methylene at δ_C 33.8 (C-3), 31.9 (C-9), 29.6 (C-5), 29.5 (C-6), 29.4 (C-7), 29.2 (C-4), 28.9 (C-8) and 22.7 (C-10) and one methyl at δ_C 14.1 (C- 11) groups (Table 2).

3.4. In-vitro antibacterial activity of ethyl acetate extracts and 1-undecene

The ethyl acetate extracts (1000 μg/mL) of *E. coli* GST-2, E. coli GST-4, *E. coli* GST-5, *B. subtilis* GST-8, *B. subtilis* GSL-5 and *B. amyloliquefaciens* GSS-7 bacterial isolates, and the 1-undecene (at 50, 100, 300, 500 and 1000 μg/mL concentration) were found effective against *E. coli* ATCC 25922^T, *S. aureus* ATCC 25923^T and *P. aeruginosa* ATCC 27853^T bacterial strains as indicated in Table 3 which showed mean and standard deviation of obtained inhibition zone.

Table 3 shows that all the tested ethyl acetate extracts indicated activity (≥7 mm) against all bacterial strains, except *E. coli* GST-2 and *E. coli* GST-5 which were found inactive (<7 mm) against *E. coli* ATCC 25922^T and *S. aureus* ATCC 25923^T, respectively. The higher inhibition zone (9.4 ± 0.6 mm) against *E. coli* ATCC 25922^T was recorded by the ethyl acetate extract of *B. subtilis* GSL-5 culture filtrate and then followed by *B. amyloliquefaciens* GSS-7 (8.6 ± 0.1 mm) and *E. coli* GST-4 (8.4 ± 0.1 mm) isolates. The ethyl acetate extracts of *E. coli* GST-5 and *B. subtilis* GST-8 showed a slight inhibitory effect against the same bacterium with 7.1 ± 0.1 mm and 7.0 ± 0.0 mm inhibition zone, respectively. Whereas the ethyl acetate extract of *E. coli* GST-2 culture filtrate showed higher inhibitory (8.4 ± 0.8

Table 3
Antibacterial activity of isolated 1-undecene compound and ethyl acetate extracts of certain endophytic bacterial isolates against *E. coli* ATCC 25922^{T}, *S. aureus* ATCC 25923^{T} and *P. aeruginosa* ATCC 27853^{T}.

S. N.	Bacterial stains	Concentrations (μg/mL) and respective inhibition zone in diameter (mean ± SD, in mm) against 1-undecene					Chloramphenicol (30 μg/disc)	Inhibition zone in diameter (mean ± SD, in mm) at 1000 μg/mL against certain endophytic bacterial isolates						
								GST-8	GST-5	GST-2	GST-4	GSS-7	GSL-5	Chloramphenicol (30 μg/disc)
1	*E. coli* ATCC25922^{T}	50 7.3 ± 0.5	100 8.1 ± 0.1	300 8.3 ± 0.0	500 8.8 ± 0.1	1000 9.1 ± 0.1	25.3 ± 0.8	7.0 ± 0.0	7.1 ± 0.1	6.7 ± 0.0	8.4 ± 0.1	8.6 ± 0.1	9.4 ± 0.6	25.3 ± 0.8
2	*S. aureus* ATCC 25923^{T}	50 6.6 ± 0.1	100 6.7 ± 0.1	300 7.1 ± 0.1	500 7.5 ± 0.3	1000 7.6 ± 0.3	19.2 ± 1.6	7.9 ± 0.6	6.9 ± 0.0	8.4 ± 0.8	8.3 ± 0.3	7.1 ± 0.2	7.1 ± 0.2	19.2 ± 1.6
3	*P. aeruginosa* ATCC 27853^{T}	50 7.5 ± 0.0	100 8.3 ± 0.5	300 9.1 ± 0.0	500 9.4 ± 0.1	1000 9.5 ± 0.0	7.3 ± 0.5	8.0 ± 1.2	8.2 ± 0.9	8.4 ± 0.0	8.8 ± 0.2	7.6 ± 0.3	7.6 ± 0.1	7.3 ± 0.5

mm) effect against *S. aureus* ATCC 25923^T and then followed by *E. coli* GST-4 (8.3 ± 0.3 mm) and *B. subtilis* GST-8 (7.9 ± 0.6 mm) ethyl acetate extracts. *P. aeruginosa* ATCC 27853^T was found to be vulnerable to all ethyl acetate extracts with better inhibitory values shown by *E. coli* GST-4 (8.8 ± 0.2 mm) followed by *E. coli* GST-2 (8.4 ± 0.0 mm) and *E. coli* GST-5 (8.2 ± 0.9 mm) ethyl acetate extracts.

As it can be seen in Table 3, the isolated compound, 1-undecene, established an activity (>7 mm inhibition zone value) against *E. coli* ATCC 25922^T and *P. aeruginosa* ATCC 27853^T, at all tested concentrations with the higher diameter of zone of inhibition values of 9.1 ± 0.1 mm and 9.5 ± 0.0 mm, respectively. The highest inhibition zone was recorded at the maximum concentration (1000 μg/mL). At the remaining concentrations of 50, 100, 300 and 500 μg/mL, the compound was able to inhibit the growth of *E. coli* ATCC 25922^T (7.3 ± 0.5 to 8.8 ± 0.1 mm) and *P. aeruginosa* ATCC 27853^T (7.5 ± 0.0 to 9.4 ± 0.1 mm). This 1-undecene compound also tried to inhibit the growth of *S. aureus* ATCC 25923^T at 300, 500 and 1000 μg/mL (>7 mm) concentrations with 7.1 ± 0.1, 7.5 ± 0.3 and 7.6 ± 0.3 mm zone of inhibition, respectively.

4. Discussion

Gram-positive and endo-spore forming endophytic bacterial isolates were obtained from *Gloriosa superba*. According to Bergey's manual [16], those spore bearing isolates were recognized as *Bacillus* spp. In the present study, various biochemical reactions indicated that all the Gram-positive isolates were found to be positive for the presence of catalase and amylase enzymes with the strongest response (+++) for GSL-4 and GSS-6 isolates, and moderate response (++) for GSL-2 isolate particularly against the catalase enzyme.

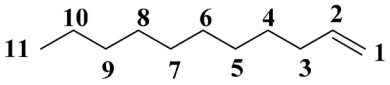
The endophytic bacterial isolates were generally identified as *Bacillus*, *Corynebacterium, Escherichia*, and *Providencia* species based on the MALDI-TOF MS analysis. In line with this study, certain endophytic bacterial species were identified as *Bacillus*, *Escherichia, Enterococcus, Enterobacter, Paenibacillus, Pantoea,* and *Staphylococcus* using MALDI-TOF MS techniques from the Great Nettle (*Urtica dioica* L.) that grown in Algeria [15]. The same authors further reported that the dominant endophytic bacterial isolate was identified as *Bacillus pumilus*-ME which is a similar finding to the recent our report. Besides, several human pathogenic bacteria isolates such as *B. cereus*, *Enterobacter cloacae*, *Kosakonia cowanii*, *Klebsiella pneumoniae*, *Pantoea agglomerans*, *P. aeruginosa*, *P. fulva*, *P. mendocina*, *P. mosselii*, and *S. epidermidis* were reported from plant roots and suggested as plant growth promoters [17]. Certain endophytic bacterial species such as *Bacillus, Neobacillus, Peribacillus, Pseudomonas*, and *Terribacillus* were also identified using MALDI-TOF MS from Canola crops with bio-fertilizing efficiency. However, the most dominant isolate belonged to the *Paenibacillus* and *Pseudomonas* [18]. Using the same techniques, potential endophytic bacterial species such as B. subtilis and B amyloliquefaciens *were obtained from* aerial roots of banyan (Ficus benghalensis) [19].

In the present study, the overall NMR spectral information (Table 2) inferred that the structure of the isolated compound was found to be 1-undecene (Fig. 5). Previous research works claimed that 1-undecene was found to be produced naturally by certain *Pseudomonas* species that obtained from various types of natural sources. For instance, Tagele et al. (2019) [20] reported that 1-undecene was isolated as the dominant semi-volatile compound from *P. chlororaphis* subsp. *Aurantiaca,* obtained from the maize rhizosphere. Zhou et al. (2014) [21] also reported the same compound from *P. fluorescens* originated from *Atractylodes Lancea* seedlings. The fatty acid biosynthesis pathway is the means for the production of 1-undecene by the *Pseudomonas* species with the help of an *undA* gene that existed in bacteria genomics [18,22]. It was also reported that [23], 1-undecene that served as an olfactory signal had been obtained from *P. aeruginosa*. The same authors stated that 1-undecene was induced to employ as fighting responses in worms like *Caenorhabditis elegans* thereby enabling them to defend from bacterial infections.

The ethyl acetate extracts (1000 μg/mL) of *E. coli* GST-2, *E. coli* GST-4, *E. coli* GST-5, *B. subtilis* GST-8, *B. subtilis* GSL-5 and *B. amyloliquefaciens* GSS-7 bacterial isolates were found to be effective against the tested standard human pathogenic bacterial strains evaluated in this study. Similarly, it was reported that ethyl acetate extracts of *Bacillus* sp. SS_4 play a significant role in the reduction of biofilm producing *P. aeruginosa* PAO1 [24]. The same pathogen (*P. aeruginosa*) was inhibited by the ethyl acetate extracts of *B. subtilis* BR_4 isolated from mangrove roots and associated soil samples in India [25]. Antibacterial and antioxidant activities were also reported from ethyl acetate extract of *Streptomyces* AIA12 and AIA17 isolates obtained from gut of *Chanos chanos* [26]. The same authors stated that the crude extract of extracellular secondary metabolites that obtained by ethyl acetate extraction was able to inhibit certain pathogenic strains such as *B.* cereus ATCC 10876, E. coli ATCC 25922, Listeria monocytogenes, P. aeruginosa InaCC B52, S. *aureus* ATCC 25923, and Salmonella Typhimurium ATCC 14028 with minimum inhibitory and minimum bactericidal concentrations of 2.5 to 0.31 and 5.0 to 0.31 mg/mL, respectively.

In our case also, the ethyl acetate extracts of *B. subtilis* GSL-5 and *B. subtilis* GSS-7 showed comparable inhibitory effect on the growth of *E. coli* ATCC 25922^T with inhibitory values of 9.4 ± 0.6 and 8.6 ± 0.1 mm, respectively. Similarly, the crude butanol and ethyl acetate extract of *B. subtilis* CU_{12} culture filtrate showed an inhibiting effect against *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, and *Pythium sulcatum* [27].

The isolated 1-undecene compound was also found with considerable activities against *E. coli* ATCC 25922^T and *P. aeruginosa* ATCC 27853^T. In line with this study, it has been reported that 1-undecene is found to be effective and strong antagonistic activities against *R. solani* [28] and *Phytophthora infestans* [29] which are medically important pathogens at high concentration. It has also been confirmed that 1-undecene is employed to inhibit the growth of *S. aureus* ATCC 25923^T at various concentrations. Similarly, it has been also reported that *Pseudomonas fluorescens* derived 1-undecene [30] is able to inhibit *Legionella pneumophila* and employed as novel strategies to fight legionellosis. In conclusion, the present finding may shed light on similar and related works to be conducted in Ethiopia as it was reported herein for the first time. The present work also recommends the molecular characterization of the isolated endophytic bacteria to fully identify them in strain level and further investigations on whole parts of *Gloriosa superba* to explore other endophytic microbes including fungi and associated bioactive compounds.



1-undecene

Fig. 5. Suggested structure of compound 1-undecene isolated from *Gloriosa superba* associated *Escherichia coli*endophytic bacterial isolate.

Ethics approval and consent to participate

- Review and/or approval by an ethics committee was not needed for this study because it did not involve human and animal sample and experiments.
- Informed consent was not required for this study because no participants/patients were included.

Consent for publication

Not applicable.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22104.

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