Research Article

Bioactivity of the Endophytic Bacteria Inhabiting the Egyptian Medicinal Plant *Hyoscyamus muticus*

Noura Sh. A. Hagaggi[1*], Marwa E.A. Khalaf[2] and Eman A. El Rady[2]

[1]Botany Department, Faculty of Science, Aswan University, Aswan 81528, Egypt; [2]Chemistry Department, Faculty of Science, Aswan University, Aswan 81528, Egypt.

Abstract | Although medicinal plants provide various biochemicals for pharmaceutical applications, overharvesting may cause their extinction. Therefore, to preserve plant resources, the researchers must consider the microbial endophyte cultures as an alternative route for drug discovery. The aim of this study was to isolate and identify the endophytic bacteria from the medicinal *Hyoscyamus muticus* (L.) (Egyptian henbane) plant, and investigate their bioactivities. According to the sequences of their 16S rRNA genes, the isolated bacteria from root, stem, leaf, and flower were respectively identified as *Bacillus pumilus, Bacillus mojavensis, Bacillus australimaris*, and *Psychrobacter pulmonis*. The ethyl acetate extracts of all bacterial isolates were rich in phenolics, flavonoids, and hydrocarbons such as pentacosane, eicosane, hexadecane, heneicosane, pentadecane, and tetracosane. The extracts expressed an anti-inflammatory potential against the inhibition of protein denaturation by 77–95 % at 100 µg/ ml. Moreover, these extracts displayed strong antibacterial efficacy against *Salmonella typhi, Staphylococcus aureus*, and *Klebsiella pneumoniae*. Furthermore, the antioxidant activity of the extracts ranged from 47.12± 1.68 to 103.6± 3.8 µM Trolox equivalent/ µg extract, scavenging the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals by 43.66-90.21 %. This study highlights the potential of the endophytic bacteria associated with *H. muticus* as substitute producers of plant-related bioactive chemicals with anti-inflammatory, antibacterial, and antioxidant properties. To our knowledge, this is the first study concerning the endophytic bacteria from *H. muticus*.

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***Correspondence** | Noura Sh. A. Hagaggi, Botany Department, Faculty of Science, Aswan University, Aswan 81528, Egypt; **Email:** nourasharkawi@sci.aswu.edu.eg
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Introduction

Hyoscyamus muticus (L.) is a *Solanaceae* family medicinal shrub that grows naturally in desert areas (Täckholm, 1974), which has been used in medicine since ancient history due to its abundant phytochemicals and medicinal properties (Lekmine *et al.*, 2025). Despite plants being potential sources for new drugs, their uses have some restrictions; as plants are found in a variety of environments, have different geographic distributions, and their chemical compositions also varied, in addition, the excessive

harvesting of these plants threatens their biodiversity and places them at risk of extinction (Mishra *et al.*, 2023). As a result, it is urgent to find new eco-friendly and sustainable sources of plant bioactive products. Endophytic bacteria that inhabit the inner tissues of plants can build a symbiotic relationship with their host plants and produce a similar array of bioactive metabolic compounds as those of their hosts (Wu *et al.*, 2021). Endophytic bacteria are now being used more frequently as an alternative approach for producing bioactive secondary metabolites because of their self-sustainability and controllable growth conditions (Cabello, 2020). Therefore, the most highly regarded alternative for the synthesis of a variety of bioactive phytocompounds is the study of endophytes; particularly the endophytic bacteria (Drożdżyński *et al.*, 2024).

According to previous studies, diverse endophytic bacterial members related to the genera *Bacillus, Pseudomonas, Brevibacterium, Acinetobacter*, and *Agrobacterium* were documented to produce a wide range of biologically active compounds that could be used in many industries such as food, cosmetics, pharmaceuticals, and agriculture (Strobel, 2003; Feng *et al.*, 2022). The secondary metabolites produced by the endophytic bacteria are categorized based on their functional groups into various classes, including alkaloids, flavonoids, phenolics, saponins, tannins, chinones, steroids, and others (Kumari *et al.*, 2023). A previous study hypothesized that as *H. muticus* lives in a harsh desert environment and is best known for its numerous pharmaceutical activities (Abd El-Hafeez *et al.*, 2022); it may contain secondary metabolites-producing endophytic bacteria with significant bioactivities. Thus, the objectives of the current study were to isolate and identify the endophytic bacteria from the different parts of *H. muticus*, analyze the secondary metabolites existing in their extracts, and evaluate their anti-inflammatory, antibacterial, and antioxidant potentials.

Materials and Methods

Plant samples

Fresh healthy *H. muticus* plants were obtained from the campus of Aswan University, Egypt, and instantly transported into the bacteriology laboratory for further study.

Isolation and identification of the endophytic bacteria

Tap water was used to remove debris from plants, subjected to surface sterilization using NaOCl (5%) for 1 min., followed by ethanol (70%) for 1 min., and finally rinsed three times with sterilized distilled water (Vincent, 1970). Using a sterile scalpel, the samples were separated into parts, including roots, stems, leaves, and flowers. Each part was mashed in a sterilized solution containing 0.85% NaCl and then filtered. On plates of nutrient agar (NA), 1 ml of each filtrate was spread using a glass spreader and incubated at 37 °C for 3 d. After incubation, the developing bacterial colonies were selected based on their phenotypic differences. After preliminary screening of secondary metabolites produced by each isolate, the four most potent isolates coded as Hm-R, Hm-S, Hm-L, and Hm-F were selected for further study as representative models of root, stem, leaf, and flower-associated bacteria, respectively.

The bacterial isolates were sent to the applied biotechnology company, Ismailia, Egypt, for DNA extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. For each bacterial isolate, the extracted DNA was checked for purity before subsequent molecular analysis. DNA samples were shipped to SolGent Company, Daejeon, South Korea for polymerase chain reaction (PCR) and 16S gene sequencing. PCR was performed using two universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3') (Frank *et al.*, 2008). A nucleotide marker of 100 base pairs was used to reconfirm the purified PCR amplicons using agarose gel (1 %). Sense and antisense directions sequences of the amplicons were performed using the 27F and 1492R primers and dideoxynucleotides (dd NTPs). The percent identity of the isolates was determined using BLAST results of the present sequences against the NCBI database. Phylogenetic analysis was performed using the neighbor-joining method in MEGA X (10.1.7) (Kumar *et al.*, 2018).

Extraction of secondary metabolites

The secondary metabolites were extracted from the culture filtrates using ethyl acetate according to the procedure described by Seerangaraj *et al.* (2017). Each bacterial isolate was grown for 72 h in 5 L of nutrient broth (NB) at 37 °C and 150 rpm. The growing cultures were collected after inscubation, the cells were eliminated by centrifugation for 15 min.

at 10.000 rpm and 4 °C, and the supernatants were filtered using sterile cheesecloth. The culture filtrates were thoroughly vortexed with equal volumes of ethyl acetate for 2 h. Solvent fractions were separated using a separatory funnel, evaporated at room temperature, and the extracts were stored at 4 °C for further studies.

Evaluation of the bioactivities of the bacterial extracts

Anti-inflammatory assay: The anti-inflammatory activity of the bacterial extracts against protein denaturation was evaluated *in vitro* using Padmanabhan and Jangle (2012) procedure. 1 ml of each extract at various concentrations (10-100 µg/ ml) was mixed with 1 ml of bovine serum albumin solution (1 %). In control tubes, 1 ml of the extract was mixed with 1 ml of dist. water. The pH of the reaction mixtures was adjusted to 6.3 and then incubated at 37 °C for 20 min. Protein denaturation was carried out by heating at 70 °C for 10 min. After that, the reaction mixtures were cooled, the optical densities (OD) were measured at 660 nm using a spectrophotometer (T60U UV-Vis, PG Instruments Ltd, England). The percent of denaturation inhibition (%) was calculated according to the following equation described by Fayez *et al.* (2023):

$$\% \text{ Inhibition} = \frac{\text{OD660 of control} - \text{OD660 of extract}}{\text{OD660 of control}} \times 100$$

Antibacterial assay: The CLSI guidelines (2010) were followed to evaluate the antibacterial efficacy of the extracts against *Salmonella typhi* ATCC27870, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Proteus mirabilis* ATCC29906, and *Klebsiella pneumoniae* ATCC4352, which were obtained from the bacteriology laboratory, Faculty of Science, Aswan University. Briefly, three wells (6 mm) were made aseptically in plates of inoculated Mueller-Hinton agar using a sterile cork borer. 100 µg/ ml of each extract was prepared using dimethyl sulfoxide (10 %). In each well, 50 µl of each extract was individually inoculated into each well. 50 µL of dimethyl sulfoxide (10 %) and 50 µl of chloramphenicol (100 µg/ mL) were used as negative and positive controls, respectively. After incubation at 37 °C for 24 h, the inhibition zones around the wells were measured using a calibrated ruler.

Antioxidant assay: The antioxidant activity of the extracts was determined using the DPPH assay according to the method conducted by Boly *et al.*, (2016). Extracts were prepared at a final concentration of 100 µg/ ml in methanol-DMSO (ratio 75:25). A reaction mixture containing 100 µl fresh DPPH (0.1 %) and 100 µl of the extract were added to a 96-well plate. The reaction was carried out in darkness at room temperature for 30 min. The optical density was measured at 540 nm using a microplate reader (FLUOstar® Omega, Germany). Trolox was used as an antioxidant standard. The total antioxidant activity of each extract was expressed as µM Trolox equivalent/ µg extract using the Trolox standard curve, and the respective DPPH scavenging (%) was calculated according to the following formula reported by Baliyan *et al.* (2022):

$$\% \text{ Scavenging} = \frac{\text{OD 540 Blank} - \text{OD 540 Extract}}{\text{OD 540 Blank}} \times 100$$

Determination of chemical constituents of the endophyte's extracts

Quantification of total phenolics: As stated by Singleton *et al.* (1999), the total phenolic content of each extract was spectrophotometrically assessed using the Folin-Ciocalteu reagent. The bacterial extract (1 ml) and the Folin-Ciocalteu reagent (1 ml) were added to a volumetric flask (5 ml) and left to react. Then, 7 % sodium carbonate solution (1 ml) was added; the volume was completed to 5 ml with deionized water and left in darkness. After 120 min., the absorbance was measured at 700 nm. Gallic acid was used as a reference phenolic compound. The total phenolic content of each extract was calculated using the gallic acid calibration curve as µg gallic acid equivalent per mg extract.

Quantification of total flavonoids: Based on the aluminum-flavonoid complex formation, the total flavonoid contents of the extracts were spectrophotometrically evaluated according to Chang *et al.* (2002). The bacterial extract (1 ml) was mixed with methanol (1 ml) and 10 % $AlCl_3$ solution (200 µl). The reaction mixture was incubated for 3 min at room temperature, and then 200 µl of 1 M CH_3COONa were added. The reaction mixture was incubated at room temperature in darkness for 40 min. The absorbance was measured at 510 nm. Quercetin was used to construct the standard curve. Per each mg of the extract, the total flavonoid content was quantified as µg quercetin equivalent.

Detection of volatile metabolites by Gas chromatography/ Mass spectrometry (GC/MS) analysis

In the TR-5MS GC column (Thermo Scientific),

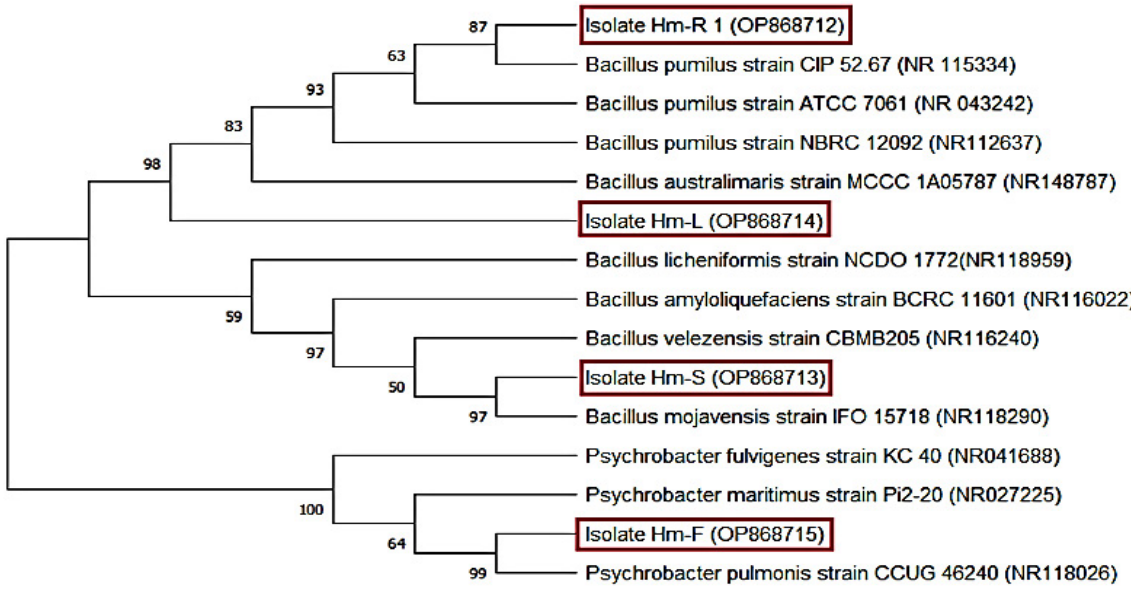

Figure 1: *Neighbor-joining phylogenetic tree of H. muticus- associated endophytic bacteria.*

1 µl of each diluted extract in hexane (1:10) was injected. The column temperature was increased at a rate of 4.0 °C/ min. from 60 to 240 °C. Helium (1 ml/ min) was used as a carrier gas. By comparing the mass spectra and retention indices of the samples with the reference database of the NIST library, the unknown constituents in the samples were identified (Koilybayeva *et al*., 2023).

Statistical analysis

All experiments were repeated twice with three biological replicates. The obtained data were analyzed by one-way analysis of variance (ANOVA) at a level of $P \leq 0.05$, using the Minitab (version 18.1) software. Values are the means ± standard errors (SEs).

Results

Identification of the endophytic bacteria

Based on the percentage of 16S rRNA gene sequence similarity, the present isolates Hm-R, Hm-S, Hm-L, and Hm-F were identified as *Bacillus pumilus, Bacillus mojavensis, Bacillus australimaris*, and *Psychrobacter pulmonis*, respectively, and their respective NCBI accession numbers were OP868712, OP868713, OP868714, and OP868715. The relationships between the isolates and the most closely related species from NCBI were illustrated in a neighbor-joining phylogenetic tree (Figure 1).

Anti-inflammatory activity

The statistical analysis showed that, throughout the tested concentrations of all extracts, the inhibition of protein denaturation was concentration-independent (f-ratio = 0.52347, p-value = 0.718895). The most potent isolates demonstrating ant-inflammatory potential were Hm-R and Hm-F, which inhibited the denaturation of protein by 95 ±1.52 and 90 ± 0.57 % at 100 µg/ ml, respectively (Figure 2).

Antibacterial potential

Interestingly, all the extracts exhibited inhibition against the tested pathogenic bacteria (Figure 3). The broadest spectrum of antibacterial potency of all the extracts was against *S. typhi, Staphylococcus aureus*, and *K. pneumonia*, respectively, which displayed inhibition zones diameters ranging from 22±0.57 to 31±0.14 mm in a percentage of 88 to 96.8 %, compared to the antibacterial activity of chloramphenicol as a standard antibiotic. On the other hand, the extracts exhibited moderate antibacterial activity against *E. coli* and *P. mirabilis*, with inhibition diameters ranging from 14± 1.52– 26± 0.15 mm representing 56–83.9 % compared to chloramphenicol activity.

Antioxidant activity

All extracts possessed antioxidant activity. According to their potency, the extracts were arranged in the following order: Hm-R<Hm-F<Hm-S<Hm-L as represented in Figure 4. The antioxidant activity of Hm-R, Hm-F, Hm-S, and Hm-L extracts was 103.6± 3.8, 70.29± 2.5, 54.48± 2.19, and 47.12± 1.68 µM Trolox equivalent/µg extract, respectively, which exhibited DPPH scavenging activities of 90.21 %, 66.16 %, 50.81 %, and 43.66 %, respectively.

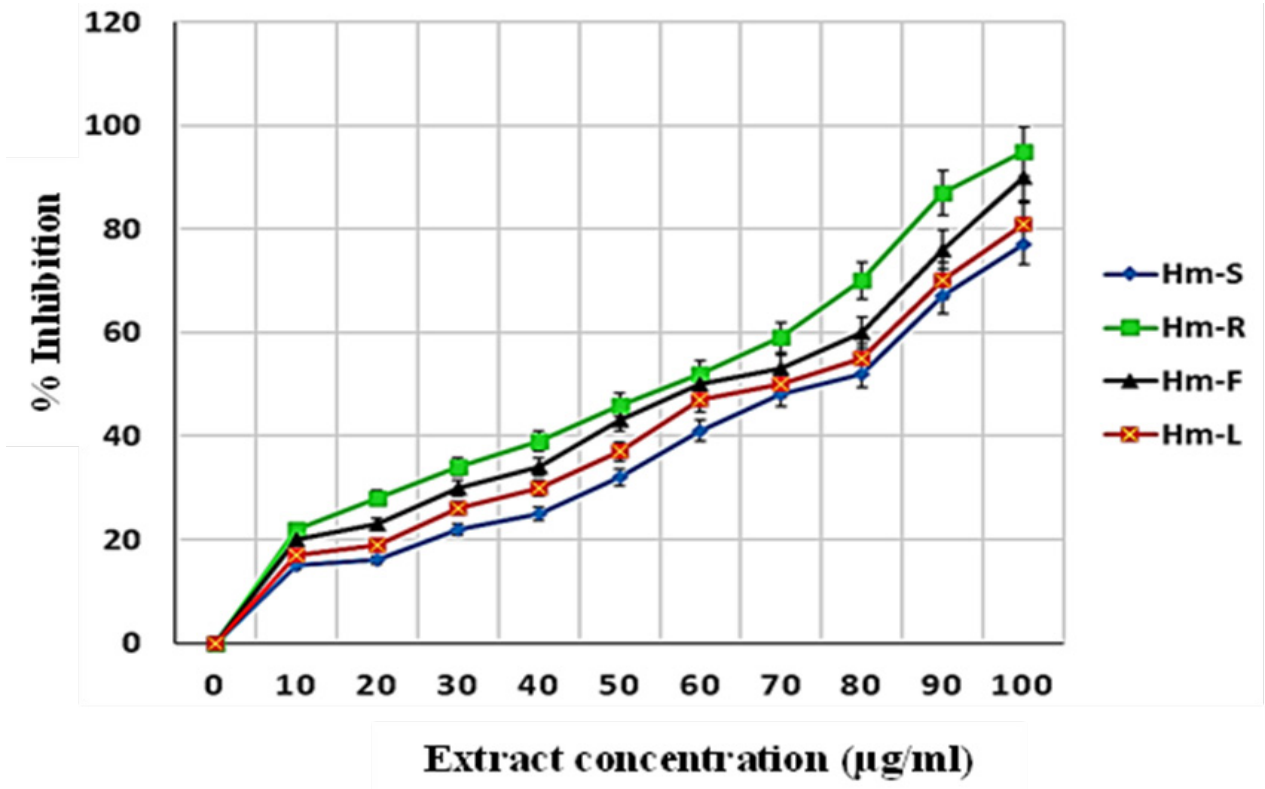

Figure 2: *Inhibition percentage of protein denaturation by ethyl acetate extracts of H. muticus- associated endophytic bacteria.*

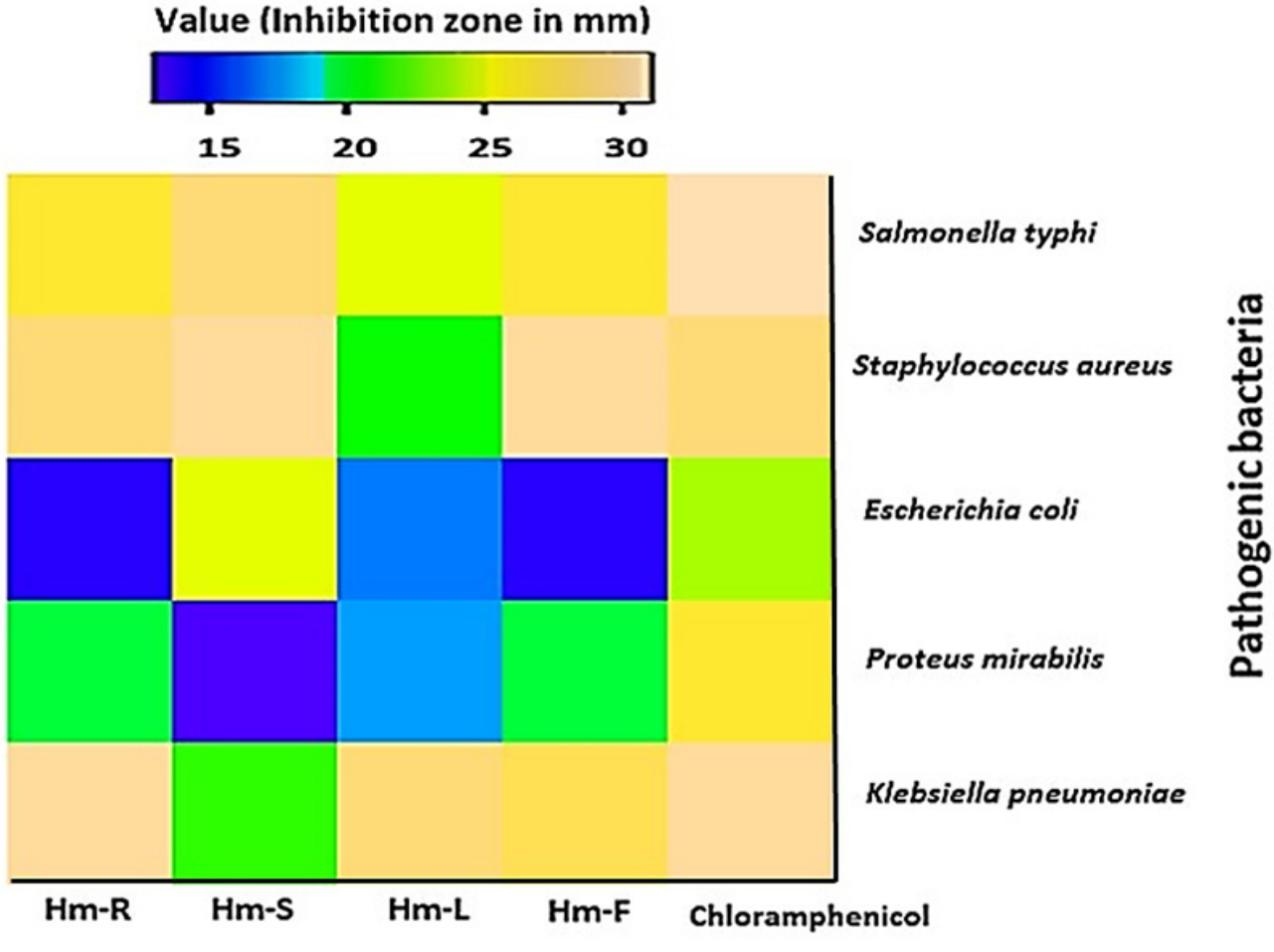

Figure 3: *Inhibition zone-based heat map displaying the antibacterial activity of ethyl acetate extracts against the tested pathogenic bacterial strains.*

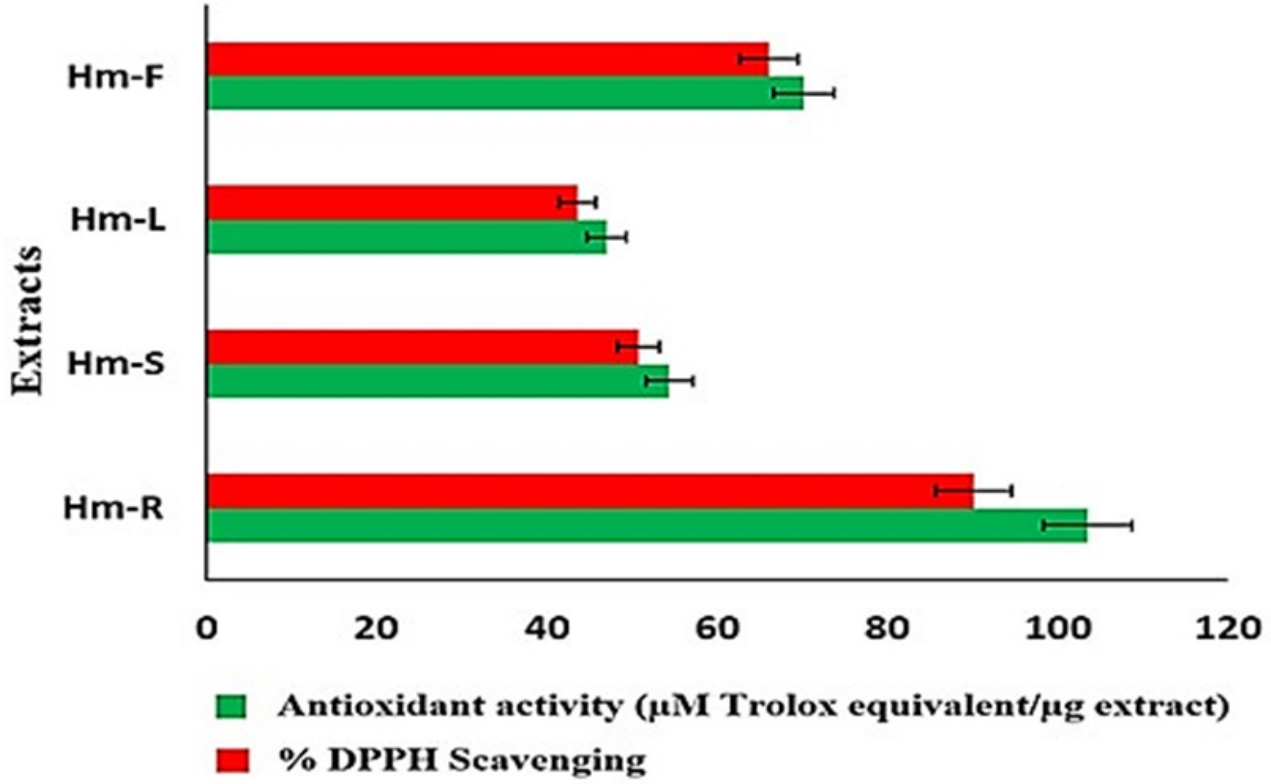

Figure 4: *The antioxidant and scavenging activities of ethyl acetate extracts of H. muticus- associated endophytic bacteria.*

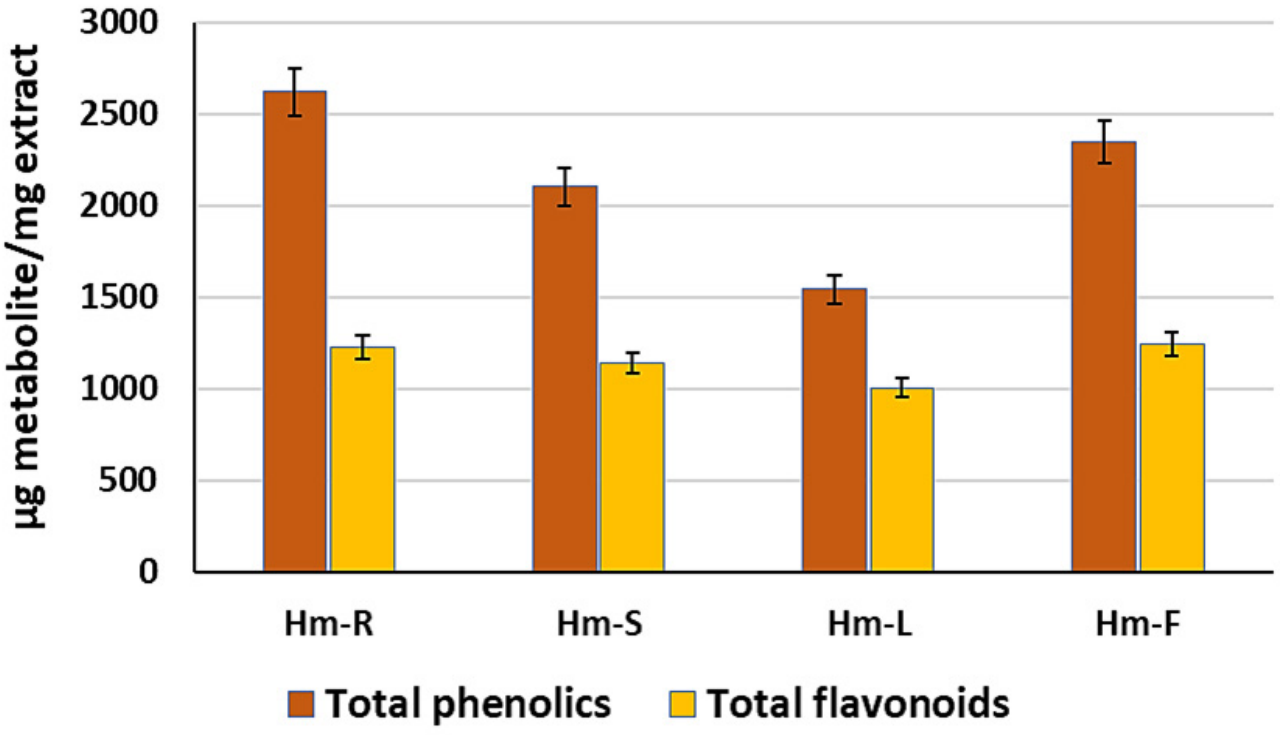

Figure 5: *Total phenolic and flavonoid contents of ethyl acetate extracts of H. muticus- associated endophytic bacteria.*

Total phenolics and flavonoids

The total phenolic contents in the ethyl acetate extracts of Hm-R, Hm-S, Hm-L, and Hm-F were 2620± 7.07, 2103± 2.80, 1543±2.12, and 2350± 4.24 µg gallic acid equivalent/ mg extract, respectively (Figure 5). On the other hand, the extracts of Hm-R, Hm-S, Hm-L, and Hm-F contained total flavonoid quantities of 1226± 3.65, 1140± 1.41, 1005± 0.70, and 1245± 2.12 µg quercetin equivalent/ mg extract, respectively (Figure 5).

Gas chromatography/Mass spectrometry (GC/MS) analysis

Based on the mass spectra and the retention indices, the bioactive compounds in the extracts were identified as presented in Tables 1, 2, 3, and 4. In the extract of Hm-R, the most abundant compounds were Heptadecane- 2-methyl- (17.894 %), Pentacosane (13.336 %), Disulfide- di-tert-dodecyl (11.470 %), Nonane- 2,2,3-trimethyl- (11.321 %), Eicosane (10.701 %), and Tridecanol- 2-ethyl-2-methyl- (8.112 %). Among twenty-four identified compounds in Hm-S extract, the most prevalent were Octane- 2,5,6-trimethyl- (19.092 %), Butyl aldoxime- 2-methyl- anti- (18.321 %), Dibutyl phthalate

Table 1: *Gas chromatography/Mass spectrometry (GC/MS) profile showing the major bioactive compounds in Hm-R extract.*

No.	Compound name	MF	MW	RT	Area %	Peak area	Peak height
1	Nonane, 2,2,3-trimethyl-	$C_{12}H_{26}$	170	4.426	11.321	1709131	66867
2	Formic acid, 2-methylpentyl ester	$C_7H_{14}O_2$	130	5.213	4.379	661119	34707
3	Octane, 5-ethyl-2-methyl-	$C_{11}H_{24}$	156	7.376	5.987	903832	69700
4	Octadecane	$C_{18}H_{38}$	254	7.736	5.787	873751	58814
5	Heptadecane, 2-methyl-	$C_{18}H_{38}$	254	9.089	17.894	2701569	145307
6	Pentacosane	$C_{25}H_{52}$	352	9.497	13.336	2013320	115811
7	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	402	11.378	11.470	1731725	89849
8	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	242	11.940	8.112	1224642	56867
9	Eicosane	$C_{20}H_{42}$	282	14.532	10.701	1615566	40634
10	Octane, 2-methyl-	C_9H_{20}	128	15.242	11.014	1662776	52631

Where: MF: Molecular formula, MW: Molecular weight, RT: Retention time

Table 2: *Gas chromatography/Mass spectrometry* **(***GC/MS) profile showing the major compounds in Hm-S extract.*

No.	Compound name	MF	MW	RT	Area %	Peak area	Peak height
1	Nonane, 2,2,3 trimethyl-	$C_{12}H_{26}$	170	4.421	3.354	1262471	89074
2	Heptane, 4-methylene-	C_8H_{16}	112	5.208	1.662	625644	44012
3	Ethanamine, N-pentylidene-	$C_7H_{15}N$	113	7.374	2.767	1041370	80576
4	Furan, tetrahydro-2,5-dimethyl-, trans-(±)-	$C_6H_{12}O$	100	7.734	1.910	718958	52403
5	Dodecane	$C_{12}H_{26}$	170	9.090	9.694	3648878	224188
6	Eicosane	$C_{20}H_{42}$	282	9.171	1.654	622549	44920
7	Heptadecane, 8-methyl-	$C_{18}H_{38}$	254	9.497	9.233	3475442	201959
8	Heptadecane, 2-methyl-	$C_{18}H_{38}$	254	9.591	2.461	926196	51620
9	2H-Pyran-2-one, tetrahydro-3,6-dimethyl-	$C_7H_{12}O_2$	128	9.684	1.857	699152	42812
10	Hexadecane	$C_{16}H_{34}$	226	10.059	2.869	1079883	64560
11	Chloroacetic acid, hexyl ester	$C_8H_{15}ClO_2$	178	10.786	2.260	850776	36547
12	2,2-Dimethyleicosane	$C_{22}H_{46}$	310	11.203	2.273	855681	38609
13	Octacosane	$C_{28}H_{58}$	394	11.379	11.024	4149590	191434
14	Eicosane, 9-octyl-	$C_{28}H_{58}$	394	11.489	2.197	826775	41947
15	Heneicosane	C_21H_{44}	296	11.939	8.961	3373007	144178
16	Hexadecane, 8-hexyl-8-pentyl-	$C_{27}H_{56}$	380	12.060	2.098	789803	39175
17	Heptacosane, 1-chloro-	$C_{27}H_{55}Cl$	414	12.268	2.418	910277	36294
18	1-Undecene, 5-methyl-	$C_{12}H_{24}$	168	12.546	2.025	762319	36582
19	Didodecyl phthalate	$C_{32}H_{54}O_4$	502	13.701	2.148	808381	35205
20	5-Ethyl-5-methylnonadecane	$C_{22}H_{46}$	310	14.531	7.423	2794080	107674
21	Cyclobutane, 1,2-diethyl-, trans-	C_8H_{16}	112	14.571	4.467	1681555	76577
22	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	15.239	11.393	4288395	170138
23	Butyl aldoxime, 2-methyl-, anti-	$C_5H_{11}NO$	101	18.321	2.270	854523	26685
24	Octane, 2,5,6-trimethyl-	$C_{11}H_{24}$	156	19.092	1.579	594154	23209

Where: MF: Molecular formula, MW: Molecular weight, RT: Retention time.

Table 3: *Gas chromatography/Mass spectrometry (GC/MS) profile showing the major compounds in Hm-L extract.*

No.	Compound name	MF	MW	RT	Area %	Peak area	Peak height
1	Heptane, 2,2,4,6,6-pentamethyl-	$C_{12}H_{26}$	170	4.431	17.923	1863723	88149
2	Decane, 3,6-dimethyl-	$C_{12}H_{26}$	170	5.215	6.015	625432	39894
3	Hexadecane, 3-methyl-	$C_{17}H_{36}$	240	7.376	6.058	629978	48577
4	Heptadecane, 2-methyl-	$C_{18}H_{38}$	254	9.089	11.252	1170008	70347
5	Decane, 3,8-dimethyl-	$C_{12}H_{26}$	170	9.496	8.025	834492	52157
6	1,3,5,7,9-Pentaethyl-1,9-dibutoxypentasiloxane	$C_{18}H_{48}O_6Si_5$	500	10.790	6.947	722371	30387
7	Hentriacontane	$C_{31}H_{64}$	436	11.379	9.110	947253	44039
8	Eicosane	$C_{20}H_{42}$	282	11.938	5.750	597876	28847
9	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	14.528	12.937	1345218	36211
10	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	15.245	15.983	1661912	56329

Where: MF: Molecular formula, MW: Molecular weight, RT: Retention time.

Table 4: *Gas chromatography/Mass spectrometry (GC/MS) profile showing the major compounds in Hm-F extract.*

No.	Compound name	MF	MW	RT	Area %	Peak area	Peak height
1	Heptane, 2,2,4,6,6-pentamethyl-	$C_{12}H_{26}$	170	4.429	7.257	1397729	74639
2	Decane, 3,8-dimethyl-	$C_{12}H_{26}$	170	5.214	3.043	586155	37512
3	Octane, 2,4,6-trimethyl-	$C_{11}H_{24}$	156	7.376	4.135	796408	62495
4	Hexacosane	$C_{26}H_{54}$	366	7.736	2.985	574953	42100
5	Pentadecane	$C_{15}H_{32}$	212	9.090	11.808	2274449	141610
6	Tetracosane	$C_{24}H_{50}$	338	9.498	10.443	2011445	112059
7	Hexadecane	$C_{16}H_{34}$	226	10.061	3.496	673346	40043
8	Noradrenaline tetraTMS	$C_{20}H_{43}NO_3Si_4$	457	10.788	4.212	811314	32554
9	Eicosane	$C_{20}H_{42}$	282	11.204	3.058	588982	29848
10	3,5-Dimethyldodecane	$C_{14}H_{30}$	198	11.380	10.571	2036079	106983
11	Pentacosane	$C_{25}H_{52}$	352	11.941	6.850	1319359	65469
12	Didodecyl phthalate	$C_{32}H_{54}O_4$	502	13.706	2.916	561611	25886
13	2-methyloctacosane	$C_{29}H_{60}$	408	14.531	13.557	2611310	69945
14	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	15.241	15.670	3018358	118457

Where: MF: Molecular formula, MW: Molecular weight, RT: Retention t.

(15.239 %), Cyclobutane- 1,2-diethyl- trans- (14.571 %), 5-Ethyl-5-methylnonadecane (14.531%), and Didodecyl phthalate (13.701 %). Meanwhile, Heptane- 2,2,4,6,6-pentamethyl- (17.923 %), Dibutyl phthalate (15.983 %), Hexadecanoic acid-methyl ester (12.937 %), Heptadecane- 2-methyl- (11.252 %), Hentriacontane (9.110 %) and Decane- 3,8-dimethyl- (8.025 %) represented the main components of Hm-L extract. Furthermore, fourteen compounds were found in the extract of Hm-F, among which Dibutyl phthalatehad (15.241 %), 2-methyloctacosane (14.531 %), Didodecyl phthalate (13.706 %), Pentacosane (11.941 %), 3,5-Dimethyldodecane (11.380 %), Eicosane (11.204 %), Noradrenaline tetra TMS (10.788 %), and Hexadecane (10.061 %) had the highest percentages.

Discussion

A diverse range of unique secondary metabolites were derived from living organisms, where most of these compounds were detected in the plants and their endophytes (Gouda *et al.*, 2016). Even though bacteria occupy a significant position among these endophytes; however, their secondary metabolites have not been sufficiently studied (Singh *et al.*, 2017). Despite this, several previous studies revealed that *H. muticus*-associated fungi have been explored (El-Zayat *et al.*, 2008; Abdel-Motaal *et al.*, 2010); however, to the best of our knowledge, no report is available for the *H. muticus*-associated bacteria. Consequently, the current study is the first report concerning the isolation, the identification, and the bioactivities of the bacterial

endophytes inhabiting the *H. muticus*.

In this study, *B. pumilus* Hm-R, *B. mojavensis* Hm-S, *B. australimaris* Hm-L, and *Psychrobacter pulmonis* Hm-F were isolated from *H. muticus* root, stem, leaf, and flower, respectively. It has been reported that the members of the genus *Bacillus* were the most predominant among the medicinal plants associated with the bacterial endophytes (Ek-Ramos *et al*., 2019).

In the present study, the extractions were prepared from the isolated endophytic bacteria using ethyl acetate as a solvent due to its low cost, low toxicity, high extraction efficiency, and agreeable odor. The ethyl acetate extracts were investigated *in vitro* for their bioactivities, including anti-inflammatory, antibacterial, and antioxidant potencies.

The obtained results revealed that the anti-inflammatory activity of the extracts was strongly associated with their phenolic and flavonoid concentrations. This was in line with the results of the others, who revealed a positive association between the quantities of phenolic and flavonoid components in the extracts and their anti-inflammatory properties (Khalifa *et al*., 2021; Rosero *et al*., 2022; Gao *et al*., 2025). Moreover, it was observed that the extracts of Hm-R and Hm-F expressed the strongest anti-inflammatory activity, which may be attributed to their phenolic contents (Figure 5) and their eicosane and pentadecane contents (Tables 1 and 4). The high anti-inflammatory activity of eicosane and pentadecane was previously documented (Okechukwu, 2020).

It is interesting to note that all the extracts showed an antibacterial efficacy against every tested pathogenic strain (Figure 3). The antibacterial potential of the extracts may be related to their higher contents of hydrocarbons such as pentacosane, eicosane, hexadecane, heneicosane, pentadecane, and tetracosane, as revealed by GC/MS analysis (Tables 1–4). The antibacterial properties of hydrocarbons have previously been reported (Faleye *et al*., 2024). Furthermore, it was observed that the antibacterial potency of the extracts with high phenolic contents was particularly strong. This is consistent with the results of previous studies that indicated a positive relationship between the contents of phenolics in the extracts and their antibacterial properties, which may relate to the fact that phenolics can inhibit the biosynthesis of nucleic acid and the metabolic processes of the bacterial cell (Babaa and Malikb, 2014; Takó *et al*., 2020). Several bacterial endophytes such as *B. subtilis, B. amyloliquefaciens, B. velezensis, B. altitudinis, B. licheniformis, Paenibacillus terrae,* and *Pseudomonas thivervalensis* have been reported to possess antibacterial properties (Christina *et al*., 2013; Hnamte *et al*., 2024).

Our human bodies can produce free radicals due to internal factors such as diseases and metabolism, and external factors including irradiation, pollution, and food (Hassan *et al*., 2024). Overproducing these free radicals causes oxidative damage and subsequent damage to lipids, proteins, and DNA, triggering several human diseases such as cancer, diabetes, and cardiovascular disease (Yang *et al*., 2024). The application of external antioxidants can help to mitigate this oxidative damage. Therefore, the research for natural substances with anti-oxidative action has been more focused recently (Muhtari *et al*., 2024). In the current study, the total antioxidant activity of the extracts was evaluated using Trolox as a standard antioxidant. Interestingly, it was found that the extract of Hm-R exhibited the highest antioxidant and scavenging activities, followed by Hm-F, Hm-S, and Hm-L extracts. The antioxidant activity of the extracts was proportional to their phenolic contents. The positive correlation between antioxidant activity and phenolic content was recently documented (Ouamnina *et al*., 2024). On the other hand, the extracts displayed the ability to scavenge DPPH radicals, which may be related to their ability to donate hydrogen (Soares *et al*., 1997; Mahlangu *et al*., 2024).

Conclusions and Recommendations

In this study, the endophytic bacteria associated with the medicinal plant *H. muticus* were isolated and identified. Extracts from these isolates were prepared using ethyl acetate. The extracts were rich in phenolics, flavonoids, and hydrocarbons. They had significant bioactivities, including anti-inflammatory, antibacterial, and antioxidant properties, making them promising natural alternatives for the future development of drugs. We recommend exploiting these endophytic bacteria as a natural source of medicines after conducting the necessary *in vivo* assays on human cells to ensure safety of their application for human use.

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Novelty Statement

This is the first report of studying the endophytic bacteria inhabiting the Egyptian medicinal plant *Hyoscyamus muticus* and their bioactivities.

Author's Contribution

N.Sh.A.H: Supervision, Research design, Data analysis and writing the original draft. M.E.A.K: Methodology and Investigations. E.A.El-R: Supervision, Research design, Review and editing. All authors approved the final manuscript.

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