International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 20s (2025)
https://theaspd.com/index.php/ijes

Environmentally Friendly Exploitation of Endophytic Bacteria from Evolvulus alsinoides and its Bio-molecular Characterization

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Abstract
In the microbial world, endophytic microbial communities have come to be a lot of attention in the recent few decades in a variety of sectors of biology. Antimicrobial substances are produced by endophytes in a variety of ways. Endophytes can be a valuable source of bioactive compounds, and they might be recovered, described, and studied in order to find new lead medicinal compounds. The aforementioned study is to spot out endophytic bacteria from Evolvulus alsinoides L. leaves. The plant samples were taken in the Yercaud hill station. Using Tryptic soy Agar, colonies were recovered from surface pre - sterilized leaves of Evolvulus alsinoides L. Screening of Asparaginase, amylase, galactosidase, amino acid and organic acid production by isolates were qualitatively performed. Antimicrobial secondary metabolite extracted from submerged fermentation medium and evaluated by well diffusion method. Antibacterial activity reveals isolate Micrococcus endophyticus, Gram positive Cocci showed antibacterial effect and the extracted metabolite were identified by GCMS. Totally 45 different compounds were identified and Acebutolol, Guanethidine, 2-[2- (Benzoyloxy) ethoxy] ethyl benzoate, Photocitral B and Strychane, 1-acetyl-20.alpha.-hydroxy- 16-methylene are found to be significant compounds and demonstrated a greater capacity for the 3-Oxoacyl ACP reductase enzyme inhibition. In silico antimycobacterial data reveals that these compounds are interacted and acted as novel antimycobacterial agents. By partial 16srRNA sequencing E8 isolate is determined and closely related as Micrococcus endophyticus.
Keywords: *endophytes, antibacterial, metabolites, molecular docking, sequencing.*

1. INTRODUCTION

The usage of plants as medicines has a extensive history in the treatment of numerous diseases. The plant derived metabolites or compounds have a role in clinical use, improved patient lenience and acceptance[1]. To date, 35,000-70,000 species of plant have been screened for their medicinal use. Plants particularly those with ethnopharmacological uses have been the primary sources of medici ne for early drug discovery[2,3]. Some bacterial species like endophytic bacteria are the useful bacteria of plants that bloom inside tissues of plants and can progress the plant growth under normal and stress conditions[4]. They are beneficial to host plants unswervingly by improving nutrient uptake and by controlling growth and stress connected phytohormones[5]. Ramblingly, endophytic bacteria can improve plant well-being by directing pests and pathogens with secondary metabolites antibiotics and hydrolytic enzymes[6-8]. Medicinal plants like Evolvulus alsinoides are particularly rich reservoirs of these endophytes, which offer a diverse array of secondary metabolites with therapeutic potential[9]. The plant *E. alsinoides*, known for its neuroprotective and adaptogenic properties, contains various phytochemicals such as alkaloids, flavonoids, and phenolic compounds[10]. *Evolvulus alsinoides* is a prostrate many branched plant with a

small woody rootstock that blooms in blue, white, and pinkish colours. Slender miniature morning glory is the common name for this plant[11].

Fig 1. Leaves with flower of *Evolvulus alsinoides* plant

Plants containing endophytic microorganisms have been found to be an antecedent of commodity amidst medicinal prospect when collate to plant solely.

Endophytes are harmless microorganisms that reside inside plant tissue. Nearly every plant species on the globe contains endophytic bacteria[12]. These endophytic bacteria have been used as biological agents to regulate human diseases because they colonize diverse areas of plants and can stop plant sickness[13]. Plants benefit from the presence of endophytes as well as these endophytes emit antibiotics or hydrolytic enzymes that inhibit microbial plant diseases from colonising the host plant in conjunction with insect nematodes from contagioning the host plant[14-15].

A microorganism, such as fungus or bacteria, that spends all or an element of its life process enclosed by healthy tissues of a thriving plant, usually without instigating ailments signs is called an endophyte[16]. Endophytic enter tissues through germination radicles, secondary roots, stomata or the damage site and inside a plant can be found in the cells, the intercellular gaps or the vascular system, legume nodules, stems, leaves, roots, seeds, fruits, ovules, tubers, and ovules[17,18]. It emit chemicals that instigate the host defence system upon other infections through the process of induced systemic resistance[19]. Antimicrobials, anti-angiogenic, anti-viral, anti-diabetic and other biologically active chemicals are used in the pharmaceutical sector[20]. Endophytic bacteria-derived compounds have a wide range of uses in medicine and as biocontrol agents[21]. The goal of this work is to establish and check the bioactive chemicals from bacterium joined to *Evolvulus alsinoides*. The study also aimed to isolate the endophytes from the leaves of *Evolvulus alsinoides* and to evaluate the antimicrobial constituents and biomolecular characterization by GC-MS.

2.MATERIALS AND METHODS

2.1. Isolation of endophytes

The whole plant of *Evolvulus alsinoides* was collected from yercaud hill on January 2022. Surface sterilization of leaves were done by washed with running tap water for 10 minutes, 70% ethanol twice for 1 minute and rinsed with autoclaved water that dried with sterile filter paper. The samples are aseptically cut to 1 cm long segments and punched with sterile pin after surface sterilization. The surface sterilized physically damaged leaves are placed onto Tryptic soy agar plates for the growth of bacteria and incubated at 35°C for 2 to 3 days. Colonies were selected after 48 h and sub cultured on nutrient agar.

2.2. KOH string test of isolated culture

A loopful of culture is mixed with a drop of 3 percent KOH and placed in a glass slide. The released cellular DNA makes the liquid thick or "stringy" if the cells lysis by gram- negative bacterium is indicated positive string test. Gram positive bacteria are unaffected by KOH because their cell walls contain a stronger layer of peptidoglycan.

2.3.Secondary screening of active endophyte

To determine the ability of active strain allowed to produce active metabolites during growth on inorganic salt medium used as basal media composed of KH2PO4 - 2.38 g/l; FeSO4.7H2O - 0.0011 g/l; MgSO4.7H2O - 1.00 g/l; MnCl2.4H2O - 0.0079 g/l; ZnSO4.7H2O - 0.0015 g/l; CuSO4.5H2O - 0.0064 g/l at pH 7. 100 ml of basal medium in 250 ml of Erlenmeyer flask was sterilized using autoclave. 5% glucose used as carbon source. 1% Peptone, yeast extract and potassium nitrate used as nitrogen source. 10% inoculum was introduced and fermentation carried out at 100rpm for 48h. Cell free culture filtrate was taken and extracted with equal volume of ethyl acetate, concentrated (10mg/mL) and antimicrobial activity was done by well diffusion method.

2.4.Metabolite profiling of active endophyte

Cell Dried extracts were resuspended on ethyl acetate that heated for 12 h at 50 °C. Following evaporation of the derivatizing reagent and extract derivatives, they were solubilized in ethyl acetate and transferred for gas chromatography analysis. The GC-MS analysis was carried out using a Clarus 680 gas chromatograph linked to a Clarus SQ8 quadrupole mass spectrometer (Perkin Elmer Inc.,). Gas chromatography was performed on a 5 percent diphenyl 95 percent Dimethyl Polysiloxane fused-silica capillary column using helium as a carrier gas at a constant flow rate of 1 mL/min. The gas chromatograph has an electrically controlled split/splitless injection port. The injection (1 L) was carried out in splitless mode at 250 °C. The oven temperature schedule was as follows: 80 °C for 2 **minutes, 10 °C/min rise from 80 to 190 °C, 15 °C/min increase from 190 to 280 °C and hold for 5 minutes, then 10 °C/min till 300 °C and hold for 14 minutes.**

3.RESULTS AND DISCUSSION

In *Evolvulus alsinoides* leaf tissues, endophytic bacterium were analysed microbiologically to determine how frequently they colonised the tissues. Evolvulus alsinoides leaves samples are inoculated on Tryptic soy agar medium showed different colonies with different morphology developed between 24-48h. The number of CFU of surface sterilised leaf was examined and that leaves exhibited 18 CFU. The relative colony forming of the bacterial isolates from surface sterilised leaf is shown in Fig 2. Eight different kinds of bacterial strains were observed from *Evolvulus alsinoides* L. The bacterial strains were named as E1-E7 and *Micrococcus endophyticus*. The frequency of cell wall, catalase and oxidase is given in figure 3. The colonies morphology of isolated strains were white, pink, creamy, red-, circular or irregular, Rhizoidal small, smooth, raised, opaque and mucilaginous with entire edge(Fig. 4).The colonial morphology and physiology characters of isolated bacteria were presented in Table 1. The cell wall of isolates characterised by KOH mount and Catalase and oxidase were used to distinguish the strains. Out of 8 endophytic bacteria 75% were Gram positive and oxidase positive were 25% were catalase positive. Isolation and Identification of Bacterial endophytes from *Evolvulus nummularius L* leaves were previously reported by some authors[22].

Figure.2 Surface sterilized leaves

International Journal of Environmental Sciences
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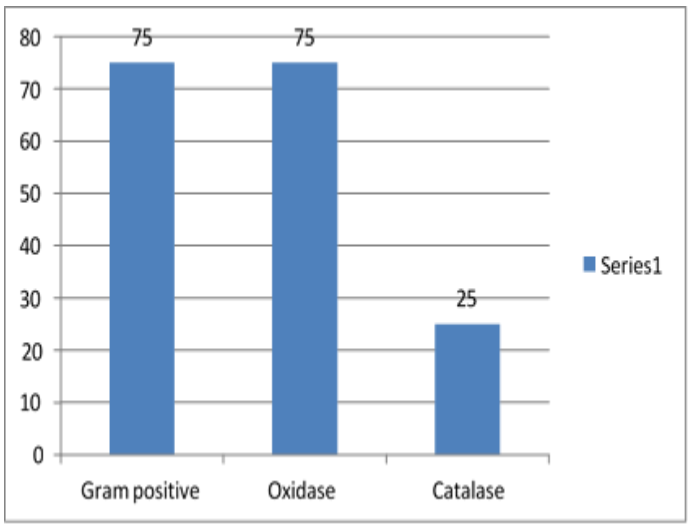

Figure.3 Frequency of cell wall catalase and oxidase

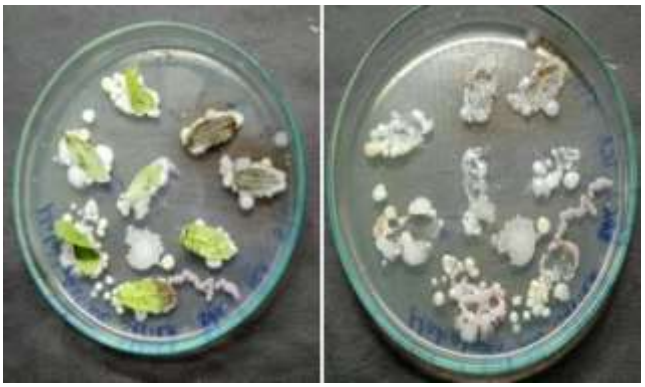

Figure.4 Isolated endophytic cultures

Table.1 Colony morphology of isolated strains

S.no	Strain code	Colony morphology	KOH identification	oxidase test	catalase test
1	E-1	Irregular white opaque smooth colony, flat	Gram positive	Negative	Positive
2	E-2	Circular, glistening creamy white ,convex colony	Gram positive	Negative	Negative
3	E-3	Circular, pink color flat translucent raised colony.	Gram positive	Negative	Positive
4	E-4	Irregular, glistening mucoid ,creamy white colony	Gram negative	Positive	Positive
5	E-5	Circular, smooth white umbonate colony	Gram positive	Negative	Positive

6	E-6	Circular, flat smooth yellowish colony	Gram positive	Negative	Negative
7	E-7	Irregular white translucent colony	Gram negative	Positive	Positive
8	*Micrococcus endophyticus*	Rhizoidal curled pink colony.	Gram positive	Negative	Positive

Consequently, eight plant-isolated cultures of endophytic bacteria were screened to determine their suitability as a source of microorganisms for the manufacture of organic acids and amino acids. Primary metabolites were produced when an isolated strain was grown utilising submerged state fermentation (SF) and minimal medium. The frequency of primary metabolite producer is given in figure 2. 0.01% Bromothymol blue with Minimal media is a dye of pH indicator, shows production of organic acid. Pinkish purple production is a result of reaction between amino acid and Ninhydrin and an amino acid which is considered as positive. All the isolates shows amino acid positive (n=8) and 37.5% were organic acid positive (n=3). Congo red agar method for exopolysaccharides (EPSs) production, reveals that all the isolated strain failed to grow on the agar for 48hours and colonies developed after 48hours failed to produce EPS. Assessments of bio film formation of the endophytic microorganisms are grown in Congo red agar with 5% sucrose is recommended qualitatively[23].Studies on primary metabolite of endophytic bacteria were less explored. This study qualitatively evaluated the production of organic, amino acid and EPS production among isolates and the result is summarised on table.2.

Table.2 Screening of Primary metabolites of isolated strains

S.no	**Strain code**	**Amino acid Test**	**Organic acid Test**	**EPS production**
1	E-1	Positive	Negative	Negative
2	E-2	Positive	Positive	Negative
3	E-3	Positive	Negative	Negative
4	E-4	Positive	Positive	Negative
5	E-5	Positive	Negative	Negative
6	E-6	Positive	Negative	Negative
7	E-7	Positive	Positive	Negative
8	*M.endophyticus*	Positive	Negative	Negative
9	N	8	3	0
10	%	100	37.5	0

GCMS analysis of active metabolite (Fig.5) shows presence of 28 different peaks and the peaks matched with NIST and identified compounds are enlisted on table 3. The major identified compound is 1,2,3-Propanetricarboxylic Acid, 2-Hydroxy-, Triethyl Ester (48.42%; RT 21.296 min) followed by Oleic Acid(17.61% RT 29.5 min). Addition to that N- Hexadecanoic Acid, Tetradecane Phosphorous Acid,

Triphenyl Ester, Propane , Cyclopropane, Butanone, Oleic Acid, Octadecane, Decanoic Acid, Octadecanoic Acid and other ester of fatty acids were detected. Pharamcologically active Compounds like Strychane, 1-acetyl-20.alpha.-hydroxy-16-methylene, Photocitral B, 2-[2-(Benzoyloxy) ethoxy]ethyl benzoate, 3-Nonanone, Guanethidine and Acebutolol were also identified. PubChem reveals Cardioselective beta-blocker like Acebutolol is used in the medication of high blood pressure, angina pectoris and cardiac arrhythmias[24]. Guanethidine is an antihypertensive drug that reduces the release of Catecholamines such as nor epinephrine[25].

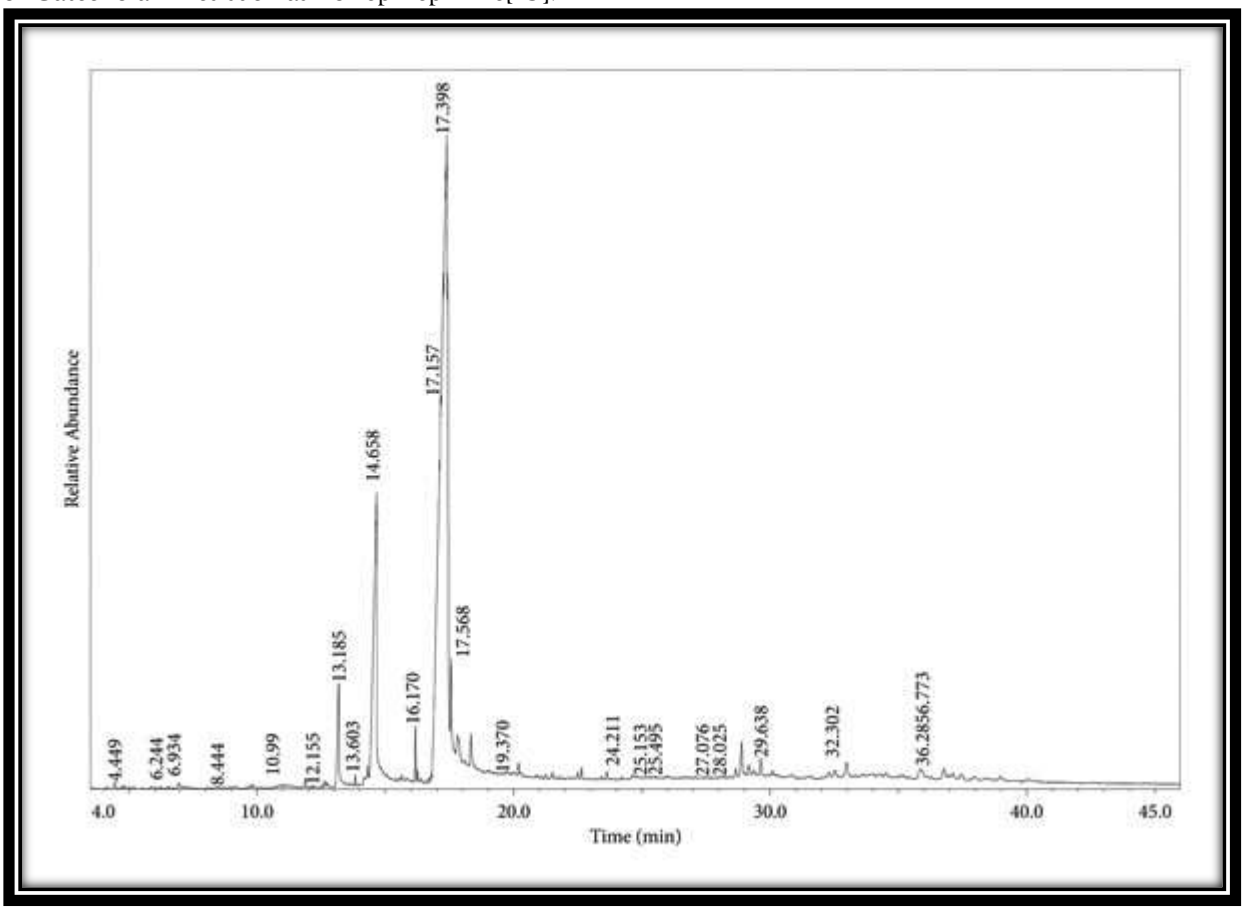

Figure 5. GC-MS analysis of biomolecular metabolites

Table. 3 Compounds matched with NIST library

S.no	Peak	Retention	Area %	Compound Name
1	1	6.031	0.09	3,3-Diethoxy-2-Butanone
2	2	6.53	0.23	Phosphorous Acid, Triphenyl Ester
3	3	8.577	0.1	Propane, 1,1-Diethoxy-
4	4	9.183	0.14	Cyclopropane, 1,2-Bis(1-Methylethyl)-, Cis-
5	5	10.043	0.16	Benzoic Acid, 2,5-Bis(Trimethylsiloxy)-,
6	6	16.174	1.23	Tetradecane
7	7	18.283	0.77	Octadecane
8	8	20.029	1.07	1,2-Benzenedicarboxylic Acid, Diethyl Ester
9	9	20.284	0.57	Octadecane
10	10	20.74	0.22	Triethyl Ester Of 1-Propene-1,2,3-Tricarboxylic
11	11	21.296	48.42	1,2,3-Propanetricarboxylic Acid, 2-Hydroxy-,
12	12	22.183	0.33	Octadecane
13	13	23.263	0.17	Decanoic Acid
14	14	25.531	0.11	2-Pyrrolidinone-5-D, (S)-

15	15	26.37	1.16	2-(1,3-Benzothiazol-2-Ylsulfanyl)Ethanol #
16	16	26.59	2.9	Dibutyl Phthalate
17	17	26.704	3.03	N-Hexadecanoic Acid
18	18	27.241	0.09	(E)-2,3-Epoxy-1-(Methoxymethoxy)Tetradecane
19	19	28.072	0.07	1-Hexanol, 5-Methyl-
20	20	28.672	0.35	1-Hexadecanol
21	21	29.401	7.21	9,12-Octadecadienoic Acid (Z,Z)-
22	22	29.5	17.61	Oleic Acid
23	23	29.85	2.45	Octadecanoic Acid
24	24	33.485	0.1	Photocitral B
25	25	33.58	0.08	1-Nonene, 2-Ethyl-3-
26	26	34.13	0.09	5-Decyne
27	27	34.218	1.02	2-[2-(Benzoyloxy)Ethoxy]Ethyl Benzoate #
28	28	34.696	0.55	1h-Indole, 2-Methyl-5-Nitro-

Totally 8 bacterial colonies were isolated by Direct plating method of which 75% are Gram positive and oxidase positive where as 25% catalase positive. Strains are designated as E1to E7 and *Micrococcus endophyticus*. The resulted bacterial endophyte isolates have confirmed for screening of primary and secondary metabolites of their potential with enzyme. Out of 8, three strains (E2, E4 and E7) are organic acid positive and all are positive including *Micrococcus endophyticus* on amino acid and negative on EPS production. Likewise all the isolate and *Micrococcus endophyticus* were positive on amylase production[26]. Out 8 isolates, six including *Micrococcus endophyticus* were Galactosidase positive and *Micrococcus endophyticus* is L- Asparginase producer[27]. The biomolecular metabolite profile identified by GCMS reveals 45 different compounds with important pharmacologically known active molecules. Strychane, 1-acetyl-20.alpha.-hydroxy-16-methylene,photocitral B, 2- [2-(Benzoyloxy)ethoxy]ethyl benzoate,3-nonanone, Guanethidine and Acebutolol were used for antimycobacterial drug screening by in silico against fatty acid synthesizing of 3-Oxoacyl-[acyl- Carrier protein] Reductase. Molecular docking reveals that Acebutolol, 2-[2-(benzoyloxy) ethoxy] ethyl benzoate, Guanethidine and 1-acetyl-20.alpha.-hydroxy-16-methylene are strong anti-microbial agents.

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