Screening of antibacterial producing endophytic bacteria isolated from Medicinal plant *Coleus amboinicus*

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Abstract

The objective of the current study was isolation of endophytic bacteria from Coleus amboinicus L. leaves and to evaluate its metabolite efficacy. Totally 5 bacterial colonies were isolated by direct plating method of which three strains are Gram positive in nature. Among 5, three were oxidase positive and all are catalase positive in nature. Isolate strain (CA2) is endospore producing rod bacterium identified as Bacillus sp showing positive antibacterial effect on test pathogens. The antimicrobial potential of endophytes reveals that isolate Bacillus sp has potent promising antibacterial agent production against Escherichia coli, Proteus vulgaris, K.pneumoniae and Candida albicans. The strain antibiotic production is largely regulated by dextrose, moderately by fructose and lactose and not by sucrose and sodium acetate. The compound is isolated and metabolite profile is identified by GCMS revealing 45 peak and 40 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 antimycobactrial drug screening by in silico against 3-Oxoacyl-[acyl-Carrier protein] Reductase. Molecular docking reveals that acebutolol and guanethidine are strong antimycobaterial agents. In conclusion, this study demonstrated that medicinal plants had a wide variety of cultivable endophytic bacteria and serve as a promising source for the production of industrially important organic acids.

Keywords: Herbal plant, Endophyte, Antimycobacterial, Indian borage, Docking.

Introduction

The World Health Organization (WHO) has stated that 80% of the developing world still benefits from the use of traditional medicines derived from medicinal plants[10]. The total estimated number of plants is approximately 374,000[5] in comparison to 28,187 medicinal species used by humans (Medicinal Plant Names Services, 2021). WHO has also recorded the names of over 20,000 species of medicinal plants and described medicinal plants as one of the potential sources of new drugs[19]. More than 100 countries have developed regulations for medicinal plants. There are over 1340 plants with defined antimicrobial activity and over 30,000 antimicrobial compounds have been isolated from plants.

Moreover, it has been estimated that 14–28% of higher plant species are medicinal and that 74% of bioactive plant-derived compounds were discovered based on ethnomedicinal uses.[12] The herb *Coleus aromaticus*(CA) has got multiple potentials and is used for variety of reasons in different pockets of the world. The herb *Coleus aromaticus/amboinicus* belongs to the botanical family Lamiaceae (Labiatae) and is now referred as Plectranthus genus[15]. The plant is distributed throughout India, cultivated in the gardens. The majority of these substances have a volatile character and are effective in treating a wide range of illnesses. These plant's volatile components play a vital role in its many medicinal qualities. Asthma, constipation, fever, cold, cough, headache and skin problems are among the ailments that can be treated using *C. aromaticus* leaves[1].

These bacteria have been used as biological weapons to prevent human diseases because they proliferate in various plant components and can stop plant disease. Numerous compounds generated by these bacteria including novobiocins, kakadumycins, celastramycins A–B and munumbicins A–D, show antibacterial properties. These metabolites are isolated from enddophytic actinobacteria[14]. Thymol and carvacrol were reported as major constituents of essential oil extracted from leaf extract[16]. The ability of strains of the *Bacillus subtilis* group is to create a broad range of secondary metabolites including lipopeptides, polyketides, class I and II bacteriocins, bacitracin and small AMPs[4]

Material and Methods

Source and isolation of endophytic bacteria: Plant materials *Coleus amboinicus* was collected from local area of Thambaram, Chennai on January 2023. The leaves were washed with running tap water for 10 minutes and surface-sterilized using 70% ethanol for 1 minute and rinsed with autoclaved water and surface-dried with sterile filter paper.

After surface sterilization, the samples were cut aseptically into 1 cm long segments. The surface sterilized leaves are placed onto tryptic soy agar plates for the bacterial growth. The plates were incubated at 25°C for 3 days. Colony morphology, Gram staining, catalase and oxidase test for morphologically distinct colonies were carried out.

Submerged Fermentation: All the isolates were selected for antibacterial compound screening. Strains were allowed to grow on inorganic salt medium composed of KH_2PO_4 2.38 g^{-1}; $MgSO_4.7H_2O$ 1.00 g^{-1}; $CuSO_4.5H2O$ 0.0064 g^{-1}; $FeSO_4.7H_2O$ 0.0011 g^{-1}; $MnCl_2.4H_2O$ 0.0079 g^{-1}; $ZnSO_4.7H_2O$ 0.0015 g^{-1} at pH 7. Erlenmeyer flask of 250 ml containing 100 ml of basal medium was sterilized. 1% glucose and 0.1% yeast extract used as carbon/nitrogen source. Fermentation was carried out under 37°C for 72h under 150 rpm.

Extraction and antibacterial activity: Antimicrobial activity was determined by well diffusion plate assay. Briefly, cell free culture filtrate of fermented medium was collected by centrifugation at 10000 rpm for 30 min and filtered through Whatmann filter paper. The filtrate pH was tested and then 100µL was loaded onto Muller Hinton agar plate pre loaded with bacterial and yeast test pathogens. The testing plates are kept under 10°C to diffuse and then incubated for 24 hours at 37°C. Antimicrobial activity was determined by the presence of inhibition zone around well.

Effect of carbon: To determine the ability of active strain growth on inorganic salt medium with different carbon, a 250 ml Erlenmeyer flask containing 100 ml of basal medium was used. 1%, yeast extract used as nitrogen source. 2% of different carbon source like glucose, lactose, fructose, sucrose and sodium acetate were amended and sterlized. 10% inoculum was introduced to the heat sterilized cool medium and kept incubation under 100rpm for 48h. The cell free culture filtrate was extracted with equal volume of ethyl acetate and concentrated (10 mg/mL). Antibacterial activity was done by well diffusion method.

Metabolite Profiling: Cell free extracts were suspended on ethyl acetate and heated at 40 °C for 2 h under magnetic stirrer. After mixing, the extract derivatives from solvent phase were collected and used for secondary screening. GC-MS analysis was performed on a Clarus 680 gas chromatograph coupled to a Clarus SQ8 quadrupole mass spectrometer (Perkin Elmer Inc.,). Gas chromatography was carried out on a dimethyl polysiloxane fused-silica capillary column with helium as a carrier gas at a constant flow of 1 mL/min. The oven temperature program was as follows: 80 °C for 2 min, increase from 80 to 190 °C at a rate of 10 °C/min, increase from 190 to 280 °C at a rate of 15 °C/min with hold for 5 min, then 10 °C/min until 300 °C and hold for 14 min.

Molecular docking: The PubChem website (http://pubchem.ncbi.nlm.nih.gov) provides a download for the ligand structure of the generated molecule. Using Autodock Tools, the target protein MabA from Mycobacterium tuberculosis (pdb:1UZM) was fetched and docking file preparation was carried out. While the receptor preparation was being conducted by adding hydrogen polar, the grid box was set to know the position of the binding site and the format was changed to .pdbqt. This file was saved in a single folder in the C: drive on the computer. Molecular docking process was conducted using AutodockVina.

Ligands and receptor that were already in drive C, were copied and converted in the form of notepad and saved with a conf.txt name, AutodockVina was executed with command prompt program. Molecular docking analysis was done by looking at the free energy value of binding docking results, viewed at the output in log.txt format.

Results and Discussion

The relative colony forming of the bacterial isolates from the surface sterilized leaf was observed and five morphologically different bacterial strains were isolated from *Coleus amboinicus* L. The colony morphology of isolated strains was different among isolates and showed mostly white, creamy and opaque; some are irregular, rhizoid small, smooth, raised and opaque. The colonial morphology and physiology characters of isolated bacteria are presented in table 1. Out of 5 endophytic bacteria, 60% were Gram negative and oxidase positive and 100% were catalase positive. The bacterial strains were named CA1-CA5 and CA2 was found to be Gram positive rod Bacillus sp.

The discovery that endophytes inhabit healthy plant tissues and may serve as a substitute source of metabolites, attracts increased attention from researchers who are looking for unique biochemical substances with potential therapeutic value but little ecological consequence. It was reported that endophytic fungi isolated from *Coleus amboinicus* Lour exhibited antimicrobial activity by Astuti et al. [2] Similarly, 45 bacterial endophytes with the majority of *Bacillus* spp. were isolated from *Coleus aromaticus* parts by Sowmya and Krishna[17].

Following submerged fermentation on minimal broth, the secondary metabolite production by endophytic Bacillus sp was principally tested for antimicrobial activity towards four pathogenic test strains using the well diffusion method on Mueller-Hinton agar plates, which showed positive results. Among the carbon sources, lactose, dextrose and fructose media showed antibacterial effects on both Gram-positive and gram-negative bacteria. High activity was recorded in dextrose followed by fructose and lactose (Figure 1). No activity was noted on the other two carbon enriched media. The secondary antimicrobial activity (Figure 2) of ethyl acetate extract from production mediums showed 14, 16, 12, 10 mm respectively among *E.coli*, *K.pneumoniae*, *P.vulgaris* and *C.albicans* whereas the standard antibiotic showed 18, 22, 16, 16 mm.

The data is given in table 2. Research on nutritional parameters revealed that using glucose as a carbon source resulted in the maximum antibacterial activity. An inorganic nitrogen source increased antibiotic synthesis by *Bacillus* sp[18]. Antimicrobial activity of entophytic bacterial populations isolated from medical plants was previously reported by many researchers[3,13].

Figure 1: Effect of carbon on antibacterial production

Figure 2: Antimicrobial activity of ethylacetate extract from *Bacillus* sp

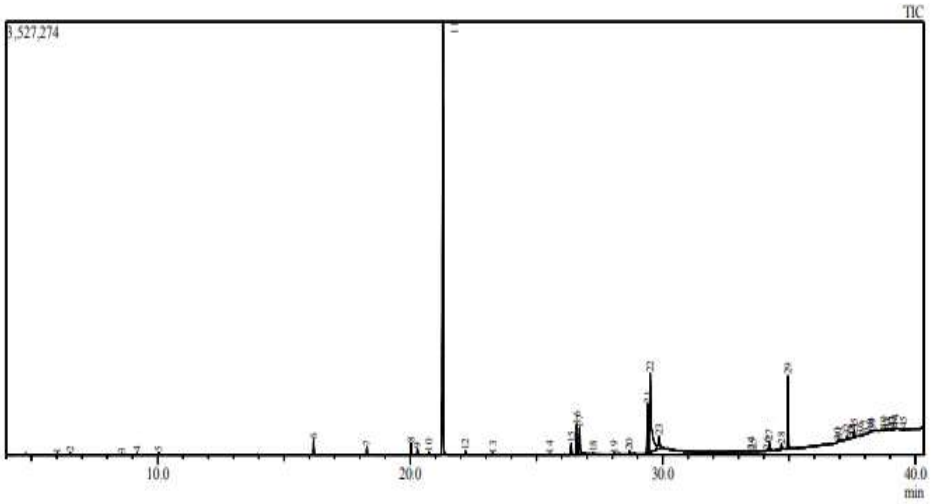

Figure 3: GC MS spectrum of secondary metabolite

Table 1
Colony morphology tabulation

Strain code	Colony morphology	Grams stain	Oxidase test	Catalase test
1	Irregular white opaque smooth colony, flat	Gram positive cocci	Negative	Positive
2	Circular, glistening creamy white ,convex colony	Gram positive rod	positive	Positive
3	Circular, pink color flat translucent raised colony.	Gram negative rod	Negative	Positive
4	Irregular, glistening mucoid ,creamy white colony	Gram negative rod	Positive	Positive
5	Circular, smooth white umbonate colony	Gram negative cocci	positive	Positive

Table 2
Secondary screening of antimicrobial effect

Strains	Primary screening Culture filtrate	Secondary screening	Ofloxacin /Amphotericin
E.coli	+	14	18
P.vulgaris	+	16	22
K.pneumoniae	+	12	16
C.albicans	+	10	16

Table 3
NIST matched compounds

Peak	Retention Time	Area %	Compound Name
1	6.031	0.09	Cyclopropane, 1,2-Bis(1-Methylethyl)
2	6.53	0.23	2,5-Bis(Trimethylsiloxy)-, Trimethylsilyl Ester
3	8.577	0.1	Tetradecane
4	9.183	0.14	3-Diethoxy-2-Butanone
5	10.043	0.16	Phosphorous Acid, Triphenyl Ester
6	16.174	1.23	Propane, 1,1-Diethoxy-
7	18.283	0.77	Octadecane
8	20.029	1.07	Benzenedicarboxylic Acid, Diethyl Ester
9	20.284	0.57	Octadecane
10	20.74	0.22	Triethyl Ester Of 1-Propene-1,2,3-Tricarboxylic Acid
11	21.0	48.42	Propanetricarboxylic Acid,
12	22.183	0.33	Octadecane
13	23.263	0.17	Decanoic Acid
14	25.531	0.11	2-Pyrrolidinone-5-D, (S)-
15	26.37	1.16	2-(1,3-Benzothiazol-2-Ylsulfanyl)Ethanol #
16	26.59	2.9	Dibutyl Phthalate
17	26.704	3.03	N-Hexadecanoic Acid
18	27.241	0.09	(E)-2,3-Epoxy-1-(Methoxymethoxy)Tetradecane
19	28.072	0.07	1-Hexanol, 5-Methyl-
20	28.672	0.35	1-Hexadecanol
21	29.401	7.21	9,12-Octadecadienoic Acid (Z,Z)-
22	29.5	17.61	Oleic Acid
23	29.85	2.45	Octadecanoic Acid
24	33.485	0.1	Photocitral B
25	33.58	0.08	1-Nonene, 2-Ethyl-3- (Methoxymethoxy)
26	34.13	0.09	5-Decyne
27	34.218	1.02	2-[2-(Benzoyloxy) Ethoxy]Ethyl Benzoate
28	34.696	0.55	1h-Indole, 2-Methyl-5-Nitro-
29	34.955	7.41	Bis(2-Ethylhexyl) Phthalate
30	36.925	0.13	Octadeca-9,12,15-Trienoate
31	36.988	0.12	2-Hydroxy-3-[(9e)-9-Octadecenoyloxy]Propyl (9e)-9-
32	37.297	0.31	Octadecanoic Acid, 2,3-Dihydroxypropyl Ester
33	37.455	0.07	Tetradecanedioic Acid, 2tbdms Derivative
34	37.53	0.04	Propylphosphonic Acid, Fluoroanhydride, 4-Methylcyclohexyl
35	37.577	0.69	Benzenedicarboxylic Acid, Bis(2-Ethylhexyl) Ester
36	37.81	0.13	Tetraoxatetradecane-1,14-Diyl Dibenzoate
37	38.009	0.11	Methyl 11-Methyl-Dodecanoate
38	38.225	0.05	2-Exo-Acetoxybicyclo[2.2.1]Heptane
39	38.279	0.14	Xi-Methyl-17-Isocholest-16-En-3.Beta.-Ol
40	38.746	0.17	3-Nonanone
41	38.854	0.03	Guanethidine
42	39.06	0.04	Acebutolol
43	39.09	0.18	Strychane, 1-Acetyl-20.Alpha.-Hydroxy-16-Methylene-
44	39.214	0.04	Stearic Acid,
45	39.533	0.03	Silane

GCMS analysis of active metabolite (Fig. 3) shows the presence of 45 different peaks and the peaks matched with NIST and identified compounds are enlisted in table 3. The major identified compound was propanetricarboxylic acid (48.42%; RT 21.0 min), followed by oleic acid (17.61%; RT 29.5 min). In addition to that, N-Hexadecanoic Acid, Tetradecane Phosphorous Acid, Triphenyl Ester, Propane, Cyclopropane, Butanone, Oleic , Octadecane, Decanoic,

Octadecanoic and other esters 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 revealed that compound acebutolol is used in the treatment of hypertension, angina pectoris and cardiac arrhythmias. Guanethidine is an antihypertensive drug that reduces the release of catecholamines, such as norepinephrine. The ADME properties of the selected compounds are given in table 4. Previous reports says that 69 volatile organic compounds were identified from five Bacillus species and all five were found to share different chemical classes. [8]

SwissADME is used to predict models for physicochemical properties, pharmacokinetics and drug-likeness of compounds. Due to the limited characteristics of absorption, distribution, metabolism, excretion and toxicity (ADMET), a promising drug's potential could be ruined. It is interesting to note that 5 molecules were found to comply with Lipinski's rule of five, with the majority receiving a favorable bioavailability rating. Strychane, 1-acetyl-20.alpha. -hydroxy-16-methylene, photocitral B, 2-[2-(benzoyloxy)ethoxy] ethyl benzoate, 3-nonanone, guanethidine and acebutolol were pharmacologically active.

The solubility, which was defined by the aqueous solubility value, is another crucial characteristic for the compound's absorption and distribution in the body. A computer-based drug design strategy called ADMET analysis could contribute to the very first phase of drug development. and screening of 1,2-benzenedicarboxylic acid isolated from Bacillus sp. exhibiting anticancer activity.[11] Using selected compounds to determine the inhibitor activity against the MabA (PDB 1UZM) chain from Mycobacterium TB, docking tests were conducted. The docking experiment was carried out with the help of the molecular docking tool AutoDockVina (version 1.1.2). Each compound's docked positions were analyzed and the pose with the lowest binding free energy was selected. Fig. 4 shows the hydrogen bond interactions of the target proteins between the active ligand and certain amino acid residues. Arg, Ser, Phe, Lys and Asp are the residues that interacted with compounds. The anticipated binding free energies in kcal/mol were used to determine the molecular docking values, which recorded the strongest score of -7.2 along with five hydrophobic bonds between the ligand and Phe,Asp residues (table 5).

The strongest ligand/protein affinity was indicated by the lowest binding free energy, often known as the best docking score. Antihypertensive and antimicrobial activity of acebutolol were previously reported by Kruszewska et al.[9] The docking showed the formation of hydrogen bond interactions of the target proteins between the active ligand and certain amino acid residues.

Table 6 shows amino acids interacting with guanethidine. Ser222 is the only residue that interacts with ligand. The predicted binding free energies in kcal/mol were used to determine the molecular docking values recorded with the strongest score of -6.5 kcal/mol.

In addition, the phe residue plays a crucial role in the interaction between ligand and receptor by forming hydrophobic interactions. The strongest ligand/protein affinity was indicated by the lowest binding free energy, often known as the best docking score recorded between ligand and receptor (fig. 5).

Table 4
ADME properties of selected compounds

Compound	MOL Weight g/mol	H Donor	H acceptor	LOG P	LOG S	Solubility	GI Absorption	BBB	CYP1A2 Inhibition	Lipinski
Strychane, 1-acetyl-20.alpha.-hydroxy-16-methylene-	338	1	3	2.36	-3.06	S	HIGH	YES	NO	YES,0 Violation
Photocitral B	152.23	0	1	2.35	-2.24	S	HIGH	YES	NO	YES,0 Violation
2-[2-(benzoyloxy)ethoxy]ethyl benzoate #	314.33	0	5	3.14	-3.65	S	HIGH	YES	YES	YES,0 Violation
3-nonanone	142.24	0	1	2.71	-2.12	S	HIGH	YES	NO	YES,0 Violation
Guanethidine	198.31	2	2	0.83	-1.16	VS	HIGH	NO	NO	YES,0 Violation
Acebutolol	336.43	3	5	2.14	-2.46	S	HIGH	NO	NO	YES,0 Violation

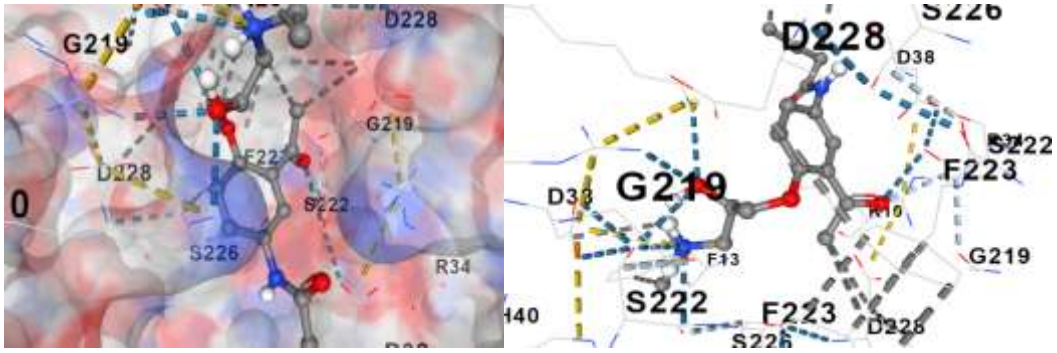

Figure 4: Formationof Hydrogen bond by Acebutolol

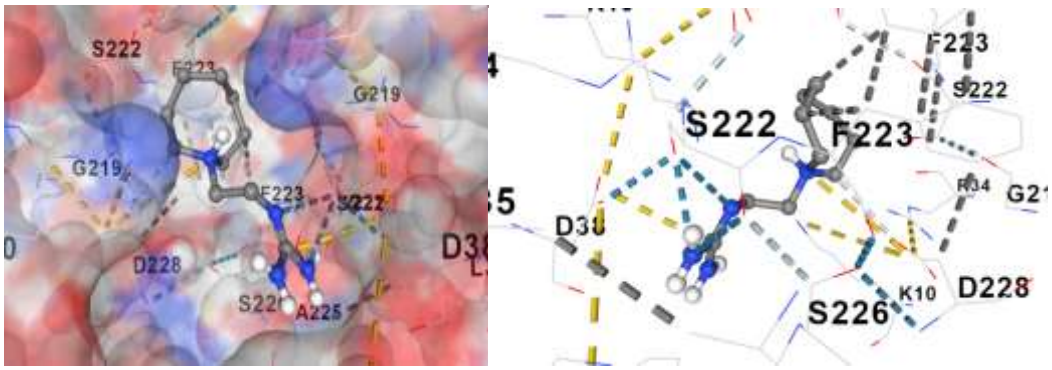

Figure 5: Formationof Hydrogen bond by Guanethidine

Table 5
Docking score of Acebutolol

Cavity size	Score	No of H bonds	Amino acid Vs Atoms	Other interaction
495	-7.1	6	Lys, Arg, Ser,Asp Atom O1 and N1	Hydrophobic bonds 5:Phe,Asp

Table 6
Docking score of Guanethidine

Cavity size A°	Score	No of H bonds	Amino acid Vs Atoms	Other interaction
728	-6.5	2	Ser222 Atom N1	Hydrophobic bonds 1 Phe

Previous reports state that guanethidine has been used to treat chronic pain caused by complex regional pain syndrome.[6] It has a role as an antihypertensive agent, an adrenergic antagonist and a sympatholytic agent (Pubchem). Crushed leaves of *Coleus aromaticus* have been reported to exhibit bronchodilator effects and are employed for treating tuberculosis in addition to being utilized as a burn remedy due to their anti-inflammatory qualities. Hence, the bacteria adopted from the leaf may also have anti tubercle activity. Further *in vitro* studies on *Mycobacterium* sp are needed to explore the potential of the compound.

Conclusion

Endophytic isolate identified as *Bacillus* sp. adopted from the leaves of *C. amboinicus* produced antimicrobial agents. Acebutolol and guanethidine compounds produced by Bacillus sp. showed antimycobacterial activity and interesting drug-designing properties against the TB, according to *in silico* screening. The study emphasizes the importance of bacterial metabolites in the synthesis of biological antibacterial agents and the possibility of *Bacillus* sp. as a useful target for tuberculosis drug research.

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