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Isolation, identification and bioactive potential of bacterial endophytes from *Coleus*

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Coleus (Lamiaceae) is a large and widespread genus comprising of species with diverse ethnobotanical uses. In the present study, bacterial endophytes were isolated from *Coleus forskohlii* and *Coleus aromaticus*. Endophytes are the microorganisms which reside within the plants without showing any harmful effect on its host. Diverse types of endophytes live symbiotically within almost all plants and in turn help the plant in a number of ways such as imparting resistance against biotic and abiotic stresses, producing compounds involved in attraction of pollinators, inducing the plant defense mechanisms, *etc.* The bacterial endophytes isolated in this study, were characterized by microscopic examination (using gram staining) and molecularly identified by sequencing the 16S rRNA. Extracts were prepared from endophytic biomass using solvents of different polarities (methanol, ethyl acetate and butanol) and were screened for their bioactive potential (*in vitro* cytotoxicity anti-microbial, and anti-oxidant activity). Scale-up of endophytes showing promising results is under process, which will help in isolation of pure compounds.

Keywords: *Coleus aromaticus*, *Coleus forskohlii*, Cytotoxicity, Dimethyl Sulfoxide (DMSO), Lactones

Coleus is a member of the Lamiaceae family (mint family). The name *Coleus* is derived from Koleos, which is a Greek word that means sheath around the style. *Coleus* genus is distributed in tropical to subtropical regions and in warm temperate climatic zone on mountains of India, Nepal, Burma, Sri Lanka, Thailand, and Africa[1]. Many plants which belong to the genus *Coleus* have also been used in traditional medicine. *C. aromaticus* extracts show ameliorative effects in cough and other respiratory ailments[2]. Secondary metabolites produced by medicinal herbs are responsible for their curative properties[3]. *C. forskohlii*, for instance is known for production of forskolin, which is primarily responsible for the antihypertensive and anti-obesity properties of the herb[4,5].

Plant tissues harbor live microbes within them, which are known as endophytes. Endophytes are cosmopolitan and show interactions like mutualism or sometimes antagonism with their host plants[6]. Endophytes are also known to exert numerous advantageous effects on host plants, such as induction of resistance to drought, herbivory, parasitism[7], stimulation of plant growth[8], nitrogen fixation[9,10] and enhancement of abiotic and biotic stress tolerance[11], *etc.* Extensive studies indicate that endophytes comprise a large variety of microorganisms including fungi, bacteria and viruses[12]. Endophytic bacteria mostly belong to the phyla Actinobacteria, Proteobacteria, and Firmicutes[13] and may be either gram-negative or gram-positive. Several endophytic bacteria belonging to the genus *Achromobacter*, *Acinetobacter*, *Bacillus*, *Brevibacterium*, *Pseudomonas*, *Xanthomonas*, *etc.* have been isolated from a large number of plants[14]. Endophytes could also be explored for new bioactive molecules possessing various therapeutic activities such as antibacterial, anticancer, antiviral, anti-oxidant and anti-diabetic[15]. Diverse classes of natural products like alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, *etc.* have been isolated from endophytic microbes.

Materials and Methods

Sample collection

Authentic plant material of *C. aromaticus* and *C. forskohlii*, cultivated at the experimental fields of CSIR-IIIM (Jammu, India), was used for endophyte isolation.

Endophyte isolation and identification

Isolation of endophytes was carried out from stem tissue of two different species of *Coleus* by using the

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protocol described by LEO Costa, with minor amendment[16]. Fresh plant material was collected and washed with autoclaved distilled water followed by surface sterilization using successive treatments of 70% ethanol and 2% sodium hypochlorite. Surface sterilized plant tissues were then placed on sterile tryptone soya agar plates for isolation of endophytic bacteria. The plates were incubated at 37°C for 3-4 days. The petri-plates were regularly checked for the appearance of endophytic bacterial colonies which were eventually purified by sub culturing. Control plates on which no plant tissue was placed, did not show any growth of bacteria.

Morphological and microscopic features of pure bacterial cultures were recorded by noting colony characteristics and carrying out Gram staining. For molecular identification, genomic DNA was extracted from the isolated endophytes. The variable region of 16S rDNA region of the bacterial isolates was amplified by using universal primers (Table 1) in a polymerase chain reaction (PCR). The quality of amplified products was checked on 1% (*w/v*) agarose gel and eluted using Qiaex® II gel extraction kit (Qiagen, Germany). The quantity and purity of the eluted PCR product was checked through spectrophotometer, and the samples were sequenced as described earlier[17].

The endophytes isolated in this study were submitted to Col. Sir RN Chopra Microbial Resource Center Jammu (MRCJ), which is a national microbial repository registered at World Data Centre for Microorganisms, with registration number 1117. The MRCJ identification numbers for the submitted endophytes are mentioned in (Table 2).

Preparation of extracts

Pure bacterial cultures were grown in Tryptone Soya Broth (TSB) at 37°C for 5 days. After cultivating endophytes for five days, the culture broth was centrifuged at room temperature, for 5 m at 7000 rpm. The supernatant was decanted into a new vessel and the cell pellet was separated. Then, cell pellet and broth were extracted with solvents of different polarity (methanol, ethyl acetate and butanol). The cell pellet was extracted with methanol while broth was extracted twice with ethyl acetate and butanol.

Table 1 — Primer sequence of 16s rRNA used for molecular identification

Primer	Primer code	Sequence	*T*m (°C)
16s rRNA forward primer	27F	5′-AGAGTTTGAT CMTGGCTCAG-3′	55
16s rRNA reverse primer	1525R	5′-AAGGAGGT GWTCCARCC-3′	55

Bioactive potential of endophytes

Anti-microbial activity

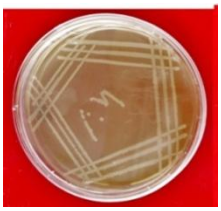
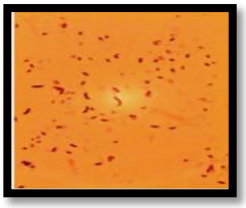
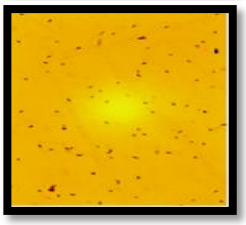
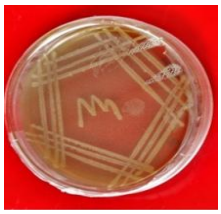
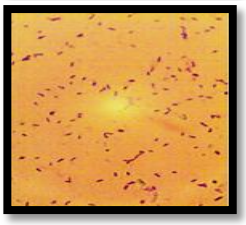
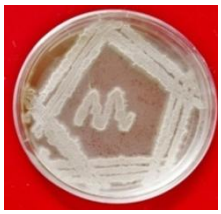
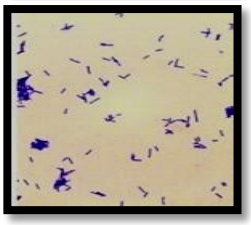
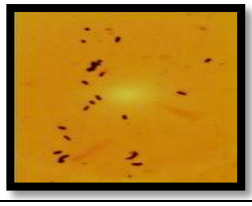
The endophytic extracts were assayed for various activities to estimate the bioactive potential of these isolates. 10 mg of the crude extract was dissolved in 1 mL of methanol for carrying out antimicrobial activity assay using the agar well diffusion method[18]. For assessment of antimicrobial activity a panel of test microbes comprising of *Escherichia coli* (MTCC 730), *Klebsiella pneumoniae* (ATCC 75388), *Bacillus cereus* (IIIM 25), *Bacillus subtilis* (MTCC 121), *Micrococcus luteus* (MTCC 2470), *Staphylococcus aureus* (MTCC 96), *Streptococcus pyogenes* (MTCC 442), *Candida albicans* (ATCC 90028) and *Sacchromyces cerevisiae* (MRCJ 92), were used. Actively growing cultures of test microbes were spread on appropriate medium to achieve lawn growth. Wells were prepared by scooping out the media and they were filled then with 40 µL of extract (10 mg/mL in methanol). Zone of inhibition was recorded after an incubation of 24 h.

Anti-cancer/in vitro cytotoxic activity

10 mg/mL of crude extract in Dimethyl Sulfoxide (DMSO) was used for carrying out cytotoxicity assays. Human cancer cell lines-A549 (Lung), HCT-116 (Colon) and MCF-7 (Breast) were obtained from U.S. National Cancer Institute (NCI). These cell lines were grown in tissue culture flasks and maintained in complete growth medium (RPMI-1640) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin and 100 units/mL penicillin in incubator at 37°C, 5% CO_2 and 98% relative humidity (RH).

In this assay, cell suspension of appropriate cell seeding density was added in 96 well flat bottom plates. After 24 h of incubation under culture conditions, the cells were treated with 100 µg of test extracts or known cytotoxic agents (as positive control). Assay plates were incubated for another 48 h at 37°C. Upon completion of incubation, cells were fixed with ice cold TCA (trichloroacetic acid) for 1 h at 4°C. After 1 h, the plates were rinsed three times with water and allowed to air dry. Thereafter, 100 µL of 0.4% Sulforhodamine B (SRB) dye was added and plates were incubated for 30 min at room temperature. Plates were washed 3 times with 1% (*v/v*) acetic acid

Table 2 — Morphology, microscopy and molecular identification of bacterial endophytes

Code	Plant name	Morphology	Microscopy	Sequencing results	*MRCJ Ids*
CFM1	*C. forskholii*			*Bacillus nealsonii*	MRCJ-897
CFM2	*C. forskholii*			*Bacillus subtilis*	MRCJ-898
CFM3	*C. forskholii*			*Bacillus vallismortis*	MRCJ-896
CAM1	*C. aromaticus*			*Bacillus cereus*	MRCJ-899
CAM2	*C. aromaticus*			*Bacillus subtilis*	MRCJ-900
CAM3	*C. aromaticus*			*Bacillus subtilis*	MRCJ-901

to remove the unbound SRB dye. After drying at room temperature, the bound dye was solubilized by adding 100 µL of 10 mM TRIS buffer (pH-10.4) to each well and incubation on a rotary shaker for 5 min. Finally, OD was taken at 540 nm in a microplate reader (Thermo Fisher Scientific, USA). The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental test value. As described earlier, the percentage growth in presence of test material was calculated considering the growth in absence of any test material as 100%[19].

Antioxidant activity

The radical scavenging activity of extracts was measured by using method described by Blois (1958)[20]. It's based on the principle that DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical on

accepting a hydrogen (H) atom from the scavenger molecule *i.e*. antioxidant, reduces to diphenylpicryl hydrazine, which is yellow in color and results in the decrease in absorbance at 515 nm. Ascorbic acid was used as positive control and per cent inhibition was determined as previously described[21].

Results and Discussion

Endophytes are widely distributed in all plant species. Endophytes are known to impart certain physiological and ecological benefits to the host plant as well as produce valuable secondary metabolites which may be exploited for pharmaceutical and industrial uses[22]. Recently some studies have been conducted to catalogue the diversity of endophytic bacteria in medicinal plants and analyze their bioactive potential[23]. With this rationale, we carried out isolation of endophytes from *Coleus* sp.

Surface sterilized stem tissue of *C. forskohlii* and *C. aromaticus* were cut into pieces of appropriate size and incubated for several days on sterile TSA medium, for isolation of endophytic bacteria. In order to confirm the effectiveness of surface sterilization procedure, after surface sterilization, plant tissues were rinsed with sterile water and a sample of the flow-through water was also plated on TSA medium and incubated at 37°C for 10 days. As expected there was no bacterial growth observed in this negative control. This is important so as to ensure that epiphytic microbes are not isolated as false endophytes. It is pertinent to note here that the method for surface sterilization, the procedure of endophyte isolation, the medium used and temperature at which the plates are incubated may affect the diversity of isolated endophytes[24]. Total six bacterial endophytes (Table 2) were isolated from stem of two species of *Coleus*, which were then examined morphologically and microscopically. Identification of bacteria isolates was performed using molecular approach based on sequence analysis of the variable region of 16S rRNA[25]. 16S rRNA gene is found in all prokaryotic organisms, and consists of conserved and varied sequences. The conserved sequences are used as binding sites for universal primers while the sequence analysis of the varied region helps to identify the bacterial species. The sequence of variable region of 16S rRNA is conserved within members of the same species and differs in representatives from other bacterial species. The species is determined from the percentage of sequence similarity with the 16S rRNA sequences which are available in NCBI GenBank (https://www.ncbi.nlm.nih.gov). 16S rRNA sequences of same species exhibit ≥ 99% identity, while an identity of ≥ 97% is exhibited by members of same genus[26]. In our study, all the isolated endophytes were gram positive bacteria that belong to the genus *Bacillus. Bacillus* species have been identified as endophytes in the medicinal plant *Echinacea*[27], while *Bacillus subtilis* and *B. megaterium* were isolated as endophytes from the root of *Chlorophytum borivilianum*[28].

The molecular and physiological interactions between the host and its endophytic microbes may influence the secondary metabolites produced by either. The endophytic microbes have been reported to produce secondary metabolites that are beneficial as antitumor, antifungal, antibacterial or anti-diabetic compounds[29,30]. Preliminary screening of isolated endophytes was done in order to assess their bioactive potential. Crude extracts were prepared using solvents of different polarities. 10 mg of crude extract was dissolved in 1 mL of methanol. All the extracts of isolated endophytes were evaluated for their anti-microbial activity. The diameter of inhibition zone was recorded. Table 3 shows the results of

Table 3 — Antimicrobial activity of endophytic extracts

Sample code	Inhibition zone (diameter of zone in mm)								
	ML	SP	BC	CA	SA	KP	EC	SC	BS
CFM1-E	13	10	15	-	15	-	-	-	10
CFM2-E	-	-	10	-	-	-	-	-	-
CFM3-E	-	-	-	-	-	-	-	-	-
CAM1-E	-	-	-	-	-	-	-	-	-
CAM2-E	-	-	-	-	-	-	-	-	-
CAM3-E	-	-	-	-	-	-	-	-	-
CFM1-M	-	-	9	-	5	-	-	8	-
CFM2-M	-	-	-	-	-	-	-	-	-
CFM3-M	-	-	-	-	8	5	12	-	-
CAM1-M	-	-	-	-	-	-	-	-	-
CAM2-M	-	-	-	-	-	-	-	-	-
CAM3-M	-	-	-	-	-	-	-	-	-
CFM1-B	-	-	-	8	5	-	-	-	-
CFM2-B	-	-	-	-	-	-	-	-	-
CFM3-B	-	-	-	-	-	-	-	-	-
CAM1-B	12	-	6	-	-	10	4	-	2
CAM2-B	-	5	7	-	8	-	-	-	-
CAM3-B	12	8	14	12	4	8	5	8	4

ML-*Micrococcus luteus,* SP-*Streptococcus pyogenes,* BC- *Bacillus cereus,* CA-*Candida albicans,* SA-*Staphylococcus aureus,* KP-*Klebsiella pneumoniae,* EC-*Escherichia coli,* SC-*Sacchromyces cerevisiae,* BS-*Bacillus subtilis,* E after sample code denotes the ethyl acetate extract, M for methanolic extracts, and B for butanolic extracts.

preliminary anti-microbial screening against the panel of nine microbes. Earlier endophytes belonging to *Bacillus* sp. and *B. subtilis* have been shown to produce antibiotics[31,32]. Another endophytic *Bacillus* sp. exhibited antifungal activity against *C. albicans*[32].

Endophytic extracts were also evaluated for their *in vitro* cytotoxic potential using cytotoxicity assays against three human cancer cell lines (Lung: A549, Colon: HCT-116, Breast: MCF-7). From the preliminary screening at a concentration of 100 µg/mL, ethyl acetate and butanolic extracts of endophytes showed better activity as compared to the methanolic extracts. Ethyl acetate and butanolic extract of CAM1 showed good cytotoxic activity (>95%) against all the tested cancer cell lines (Table 4). Ethyl acetate extract of CAM3 also showed ≥80% cytotoxicity in all the cell lines. While butanolic extract of CAM2 showed ≥68% cytotoxicity in all cell lines. Incidentally, extracts of endophytes isolated from *C. aromaticus* showed better cytotoxicity as compared to those isolated from *C. forskohlii*. In other studies as well, endophytic bacterial belonging to the genus *Bacillus* have been shown to exhibit *in vitro* cytotoxicity/anti-cancer activity. For instance, *Bacillus amyloliquefaciens* sp. endophytic bacteria isolated from *Ophiopogon japonicas* was reported to show antitumor activity[33]. Similarly, protein extract of endophytic bacteria *Bacillus subtilis* isolated from *Dioscorea zingiberensis* showed significant anticancer activity against human pulmonary adenocarcinoma cell lines[34].

DPPH technique is a simple and quick assay used for measuring the antioxidant activity of a compound or extract[35]. Hydroxyl free radical is one of the reactive oxygen species causing damage to the cell membrane[36]. Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initialization or propagation of oxidizing chain reactions. The IC_{50} values for all the extracts of endophytes isolated in our study are given in (Table 5). Butanolic extract of endophyte CAM2 isolated from *C. aromaticus* showed potent DPPH radical scavenging activity. Antioxidant compounds are often added to anti-ageing and other food supplements, especially for the geriatric population[37-39]. Antioxidant compounds also exhibit anti-inflammatory, antitumor, antimutagenic, anticarcinogenic and antibacterial activities[40,41]. Exopolysaccharides (EPS) from an endophytic bacterium *Bacillus cereus* isolated from *Artemisia annua* was reported to show

Table 4 — Percentage cytotoxicity of endophytic extracts

Tissue		Lung	Colon	Breast
Cell Line		A549	HCT-116	MCF-7
CONC. (ug/mL)	CODE	% Cytotoxicity		
100	CFM1-E	25	32	68
100	CFM2-E	39	8	41
100	CFM3-E	48	63	37
100	CAM1-E	98	100	95
100	CAM2-E	48	53	50
100	CAM3-E	80	100	99
100	CFM1-M	32	0	14
100	CFM2-M	24	0	36
100	CFM3-M	27	24	5
100	CAM1-M	29	1	60
100	CAM2-M	43	19	40
100	CAM3-M	42	16	41
100	CFM1-B	36	59	50
100	CFM2-B	42	28	59
100	CFM3-B	0	46	65
100	CAM1-B	76	72	81
100	CAM2-B	73	68	76
100	CAM3-B	18	55	38
1 µM	PACLITAXEL	72	-	-
20 µM	5-FU	-	-	52
1 µM	DOXORUBICIN	-	64	-

E after sample code denotes the ethyl acetate extract, M for methanolic extracts and B for butanolic extracts.

Table 5 — Measurement of DPPH radical scavenging activity of endophytes

Sample code	IC_{50} values
CFM1-E	247.784945 ± 0.207389042765052
CFM2-E	240.716453333333 ± 0.226942438810671
CFM3-E	415.295966666667 ± 0.488553797788267
CAM1-E	269.166666666667 ± 0.351188458428418
CAM2-E	255.649659862225 ± 0.285045378527962
CAM3-E	270.679245031444 ± 0.181955400828583
CFM1-M	137.263133333333 ± 0.24296125891454
CFM2-M	247.505223333333 ± 0.419507787333362
CFM3-M	301.188109121061 ± 0.766098818078713
CAM1-M	286.636862176968 ± 0.694544676547266
CAM2-M	155.489480913856 ± 0.221465422376804
CAM3-M	273.84263098277 ± 1.45127416060675
CFM1-B	193.584866666667 ± 0.220858718037868
CFM2-B	251.80264921781 ± 0.471293215670691
CFM3-B	91.4782186666667 ± 0.3714356986626
CAM1-B	237.214433333333 ± 0.220461342038317
CAM2-B	2.51791333333333 ± 0.464814946224122
CAM3-B	281.359059 ± 0.462004030548421
Ascorbic acid	25.5895858082193 ± 0.0747814920820673

IC_{50}, the concentration of extracts (µg/mL) causing 50% inhibition of DPPH radical.

E after sample code denotes the ethyl acetate extract, M for methanolic extracts and B for butanolic extracts.

antioxidant and DNA damage protecting activity[42]. Similarly, EPS of an endophytic bacteria *Paenibacillus polymyxa* showed strong scavenging activities on superoxide and hydroxyl radicals[43]. Endophytes represent a microbial resource that is yet to be fully explored and exploited for bioactive compounds with therapeutic potential[44].

Conclusion

In this study, we isolated endophytes from *Coleus forskohlii and Coleus aromaticus.* The endophytes were assessed for their bioactive potential through preliminary assays for *in vitro* cytotoxicity, anti-microbial, and anti-oxidant activities. Some of the crude extracts exhibited promising activity and further investigations are needed for scaling up of the pure endophytic cultures, activity guided fractionation and isolation of pure active compounds.

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