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BIOACTIVE COMPOUND ANALYSIS AND BIOACTIVITIES OF ENDOPHYTIC BACTERIA FROM *CISSUS QUADRANGULARIS*

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ABSTRACT: Endophytic bacteria were isolated from the stem of *Cissus quadrangularis* on Nutrient agar medium. The bacterial isolates were cultured in Nutrient broth. Ethyl acetate extracts of endophytic bacteria were evaluated for the presence of bioactive compounds and total phenolic content. The phytochemical screening of extracellular ethyl acetate extracts showed the presence of phenols. The total phenol content in the extracellular extract of a bacterium labeled as CqB9 was found to be 7.05 ± 0.13 mg/g. The extracellular crude extract of CqB9 displayed considerable antibacterial activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Klebsiella pneumoniae*. GC-MS analysis of CqB9 extract revealed several peaks indicating the presence of different secondary metabolites having bioactive potential. The extract of CqB9 was found to possess antioxidant activity with an IC_{50} value of 157.5 ± 0.4 µg. The cytotoxic activity using MTT assay showed that the crude extract inhibited the proliferation of the MCF-7 cell line in a dose-dependent manner. CqB9 was found to be similar to *Achromobacter anxifer* by 16S rRNA sequencing. The bioactive components such as phenols and those predicted by GC-MS may contribute to the bioactivities of the isolate. The results reveal that the endophytic bacterium isolated from *Cissus quadrangularis* has the potential to produce bioactive compounds. The endophytic bacteria and the metabolites produced by them need to be explored further for potential source and novel natural bioactive compounds.

INTRODUCTION: There is an emerging need for bioactive compounds derived from natural sources for the development of drugs [1]. Endophytes are a specific group of microorganisms that are found in internal tissues of healthy plant and do not cause visible damage to their hosts.

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Exploring the untapped natural products from the endophytes increases the chances of finding novel compounds. Attention has been directed towards endophytes of medicinal plants as these plants are being traditionally used for generations to treat symptoms of numerous diseases.

Exploring bioactive compounds of endophytic bacteria isolated from an ethnomedicinal plant may provide an alternative source for the extraction of potential metabolites on a large scale [2]. *Cissus quadrangularis* is a perennial plant of the family Vitaceae. It is also known as an adamant creeper, square stalked vine, veldt grape, devil's backbone,

asthisamharaka, hadjod, pirandai and mangara valli [3]. *Cissus quadrangularis* has been investigated scientifically in animal models based on the folk and traditional uses to validate its potential to cure a variety of ailments [4]. *Cissus quadrangularis* has potent fracture healing property and also possesses antimicrobial, antiulcer, antioxidative, antiosteo-porotic and gastroprotective activities [5]. Stem and root extracts of this plant are important in the management of various ailments. Some other reports on *Cissus quadrangularis* justify its effectiveness in antioxidant and free radical scavenging activity *in-vitro* [6,7]. *Cissus quadrangularis* also has anticancerous potential [8, 9].

Most of the literature covers work on bioactivities of endophytic fungi, but endophytic bacteria remain unexplored. Meagre reports are available on the endophytic bacteria of *Cissus quadrangularis* and their bioactivities. As a part of our ongoing efforts towards finding novel bioactive agents from natural resources, we investigated bioactive components, antibacterial, antioxidant, and cyto-toxic efficacy of endophytic bacterium isolated from the stem of *Cissus quadrangularis*.

MATERIALS AND METHODS:

Isolation of Endophytic Bacteria from Stem of *Cissus quadrangularis*: The stems of plant *Cissus quadrangularis* were collected from the Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women, Chennai, Tamil Nadu. The plant sample was identified and authenticated at Botanical Survey of India (BSI) Coimbatore, Tamil Nadu (Confirmation I.D No: BSI/SRC/5/23/2018/Tech/1884). Fresh and healthy stems were washed thoroughly under running tap water and were surface sterilized. Finally, they were washed thrice with sterile distilled water. Thereafter, the outer part of the stem was excised off and the remaining part of the stem was sliced into thin sections. The sections of stems were placed over the Nutrient Agar plate and incubated for 2-4 days. Aseptic conditions were maintained throughout the steps. The endophytic bacteria emerging from the sections of stem were isolated and purified by continuous subculturing [10].

Preparation of Crude Extract of Endophytic Bacteria: Each bacterial culture was inoculated in Nutrient Broth and incubated for 10 days. Supernatants were collected after centrifugation. The supernatants were subjected to solvent extraction using ethyl acetate. The concentrated residue of each extract was regarded as a crude sample and stored under refrigeration for further use.

Analysis of Bioactive Compounds: The crude extracts of the endophytic bacteria were subjected to phytochemical analysis for the presence of secondary metabolites, namely carbohydrates, tannins, phenols, flavonoids, and terpenoids by the standard methods [11, 12].

Determination of Total Phenolic Content: The amount of phenol in each of the extracellular extracts was determined by the Folin-Ciocalteu's colorimetric method and calculated from a calibration curve obtained with gallic acid as standard (10 mg/10 ml). From the standard solution, 20 to 100 µl was taken and added to different test tubes. The extract was added in a separate test tube at a concentration of 10 mg/ml and 5ml of Folin-Ciocalteu's reagent (1:10 dilution) was added, and the contents were mixed thoroughly. 4ml of 0.7 M sodium carbonate was added, and the mixture was incubated for 30 min. The absorbance was measured at 765 nm in a UV-Visible spectrophotometer. The results were expressed in gallic acid equivalence of the samples (GE) mg/g of the extract [13].

Antimicrobial Activity: The crude extracts of the endophytic bacteria were screened for antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Klebsiella pneumoniae.* This assay was carried out using standard agar well diffusion method [14]. Nutrient agar medium was used for the assay. The plates were incubated for 24 h at 37 °C. Zones of inhibition were observed and measured.

Identification of Bioactive Compounds using GC-MS Analysis: The extracellular extract of CqB9 was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) to analyze the bioactive compounds. GC-MS analysis was performed at the SAIF, IIT-Madras, Chennai, Tamil Nadu. The endophytic extract was subjected to GC and MS, JOEL GC mate equipped with secondary electron multiplier (Agilent Technologies 6890N Network

GC system for Gas chromatography). The column (HP5) was fused with silica 50 m × 0.25 mm I.D. The experimental conditions were maintained at 20 min at 100 °C, column temperature was 235 °C for 3 min; injector temperature was 240 °C; carrier gas: helium; and split ratio was 5:4. The sample (1µl) was evaporated in a splitless injector at 300 °C, and the run time was 30 min. The components were identified by gas chromatography coupled with mass spectrometry. The spectrum of GC-MS was analyzed using the NIST library. The name, molecular weight and molecular formula of the compounds were ascertained.

DPPH Free Radical Scavenging Activity: The ability of the samples to reduce the DPPH radical (1, 1-diphenyl-2-picrylhydrazyl) was investigated by the method described by Blois [15]. A stock solution of the compound was prepared for the concentration of 10 mg/ml. Different concentrations of the extract (200, 400, 600, 800, 1000 µg) were added at an equal volume to the methanolic solution of DPPH (0.1 mM). The reaction mixture was incubated for 30 min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated three times. Ascorbic acid was used as a standard control. The annihilation activity of free radicals was calculated in percent inhibition according to the formula

% Inhibition = {(A of control – A of Test) / A of control} × 100

Cytotoxic Activity: Breast cancer cell line MCF-7, obtained from National Centre for Cell Science (NCCS), Pune, India, was used to determine the cytotoxic activity. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin in 5 % CO_2 at 37 ºC. Cytotoxicity of the extract at various concentrations was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described by Mosmann [16] with modification. The viable cells were determined by the absorbance at 570 nm by a microplate reader. All readings were taken in triplicates.

% Inhibition = (Control - Test / Control) × 100

16S rRNA Sequencing: The endophytic bacterium CqB9 was identified by 16S rRNA gene sequence. The genomic DNA of endophytic bacteria was extracted, and 16S rDNA was amplified in Polymerase Chain Reaction (PCR) using the genomic DNA as a template and bacterial universal primers. The sequence was analyzed using a bioinformatics tool, and a phylogenetic tree was constructed using Mega X and bootstrap algorithm [17].

RESULTS AND DISCUSSION:

Preliminary Qualitative Analysis of Bioactive Compounds in the Endophytic Bacterial Extracts: From the present study, it can be inferred that all the endophytic bacterial extract showed the presence of carbohydrates and phenols in **Table 1**. Tannins and flavonoids were found to be present in CqB9, Cq11, and CqB12. Several secondary metabolites are alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, and phenols. These compounds also have an important role in therapeutic applications such as anticancer, antioxidant, antimicrobial, anti-inflammatory, and immunosuppressive agents [18]. The presence of these metabolites indicates that the extracts of these endophytic bacteria may possess bioactivities.

TABLE 1: PRELIMINARY QUALITATIVE ANALYSIS OF BIOACTIVE COMPOUNDS IN THE ENDOPHYTIC BACTERIAL EXTRACTS

Tests	CqB1	CqB2	CqB3	CqB4	CqB5	CqB6	CqB7	CqB8	CqB9	CqB10	CqB11	CqB12	CqB13
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	-	-	-	-	-	-	-	-	+	-	+	+	-
Flavonoids	-	-	-	-	-	+	+	-	+	-	+	+	-
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	-	-	-	+	+	+	-	-	+	-	+	-	-
Steroids	-	-	-	-	-	-	-	-	+	-	-	-	-

+ indicates presence, - indicates the absence

Determination of Total Phenolic Content: Total phenolic content of extracellular ethyl acetate extracts of 13 endophytic bacteria, estimated using the Folin-Ciocalteu's colorimetric method, is shown in **Fig. 1**. Among the 13 extracellular extracts of the isolates, the highest phenolic content of 7.05 ± 0.13 mg/g was estimated in the extract of CqB9. Phenols are secondary plant metabolites that

are ubiquitously present in plants and are also produced by plant-associated bacteria. The presence of such metabolites enhance the level of antioxidant [19, 20,] and antagonistic activities [21, 22] of endophytic bacteria, making them effective strains for the isolation and characterization of bioactive compounds.

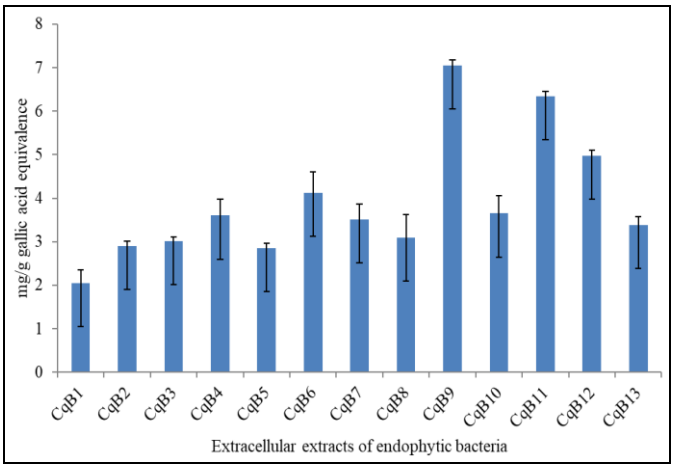

FIG. 1: DETERMINATION OF TOTAL PHENOLIC CONTENT

Phenolic compounds are a class of antioxidant agents that act as free radical terminators, and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals [23]. Antibacterial activity of phenolic compounds may be attributed to different mode of action like inhibition of synthesis of nucleic acid, destabilization, and permeabilization of cytoplasmic membrane [24].

Antimicrobial Activity: The extracellular ethyl acetate crude extracts of all the isolates were screened for antibacterial activities against *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli*, and *Klesbsiella pneumoniae*. The results **Table 2** revealed that the extract CqB9 possesses considerable antibacterial activity. These activities may be due to the presence of bioactive compounds. Antibacterial activities of ethyl acetate extracts of *Cissus quadrangularis* against *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* has been reported [25].

TABLE 2: SCREENING FOR ANTIBACTERIAL ACTIVITIES OF EXTRACELLULAR CRUDE EXTRACTS

Test Bacteria	Cq B1	Cq B2	Cq B3	Cq B4	Cq B5	Cq B6	Cq B7	Cq B8	Cq B9	Cq B10	Cq B11	Cq B12	Cq B13	Control
1	++	+++	-	-	-	-	+++	+	+++	++	++	-	+++	++++
2	+	++	-	-	-	+++	++	-	+++	-	-	-	++	++++
3	+++	++	++	++	+++	++	+	-	+	++	-	+	+	++++
4	+++	-	-	++	++	++	-	-	+++	-	++	++	-	++++

+: Diameter of zone of inhibition </=10mm, ++: Zone of inhibition >10mm< /=15mm, +++: Zone of inhibition > 15mm, ++++: >20mm, -: no activity. 1. *Bacillus subtilis*, 2. *Staphylococcus aureus*, 3. *Escherichia coli*, 4. *Pseudomonas aeruginosa*

GC-MS: The utilization of GC-MS for the screening of different compounds is common today [26]. The efficiency of separation and identification of compounds from complex biological mixtures is very high by GC-MS [27]. The GC-MS of extracellular crude extract of CqB9 showed presence of 10 major compounds as indicated by some highest peaks **Fig. 2**. The predicted compounds and their biological activities are discussed in **Table 3**. GC-MS analysis indicated that the bacterium CqB9 produced bioactive compounds having antioxidant, antimicrobial, and anticancerous activities.

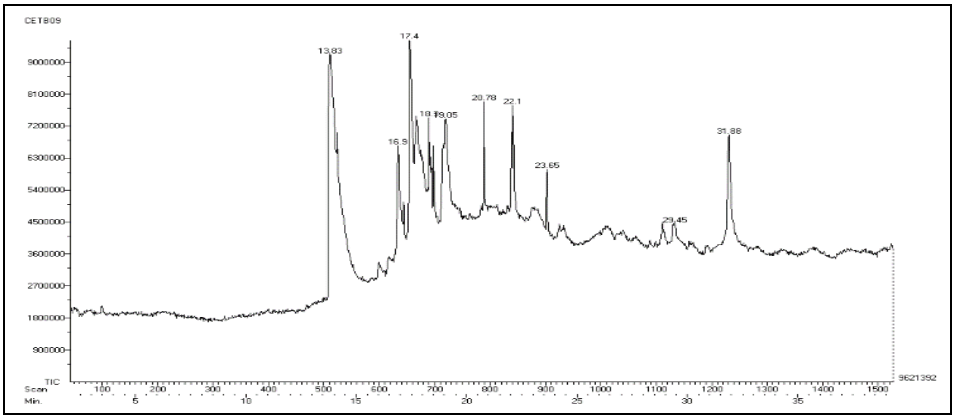

FIG. 2: GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF EXTRACELLULAR EXTRACT OF CQB9

TABLE 3: GC-MS ANALYSIS OF EXTRACELLULAR EXTRACTS OF CQB9

S. no.	Compound	Retention time in minutes	Molecular weight g/mol	Molecular Formula	Nature of the compound	Activity found
1	Diethyl Pthalate	13.83	222	$C_{12}H_{14}O_4$	Aromatic diester	Antimicrobial, Antioxidant, Anticancer [28]
2	n-Hexadecanoic acid	17.4	256	$C_{16}H_{32}O_2$	Saturated fatty acid	Antioxidant, Pesticide, Hypocholesterolemic, Nematicide, Anti-androgenic Flavor, Hemolytic, 5-Alpha Reductase Inhibitor, cytotoxic [28, 29]
3	Oleic acid	18.3	282	$C_{18}H_{34}O_2$	Unsaturated fatty acid	Antihypertensive, Increase HDL and Decrease LDL Cholesterol, Antibacterial [28, 30]
4	9-Octadecenoic acid, [E]-	19.05	282	$C_{18}H_{34}O_2$	Elaidic acid/ trans-Oleic acid	Antidiarrhoeal [31-33]
5	2,6-Bis(1,1dimethylethyl)-4phenylmethylene cyclohexa-2,5-dien-1-one	20.78	294	$C_{21}H_{26}O$	Ketoenol	Not found
6	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	22.1	279	$C_{22}H_{34}O_4$	Plasticizer compound	Antimicrobial [34]
7	3,4-Dihydroxy-1,6-bis(3-methoxy-phenyl)-hexa-2,4-diene-1,6-dione	31.88	354	$C_{18}H_{12}O_6$	ketoenol	Not found
8	Isopropyl stearate	29.45	326	$C_{21}H_{42}O_2$	Stearic acid	An emollient, skin conditioning agent, binder and humectant activities [35]
9	But-2-endiamide, N, N'-bis(4-methoxyphenyl)-	23.65	326	$C_{16}H_{12}N_2O_4$	amide	Not found

DPPH Free Radical Scavenging Activity: DPPH radical is widely used to estimate antioxidant activity due to its ability to tap hydrogen atoms [37]. The extracellular ethyl acetate extract of CqB9 exhibited significant antioxidant activity in a dose-dependent manner, as depicted in **Fig. 3**. Fifty percent inhibition (IC_{50}) was achieved when the concentration of CqB9 extract was 157.5 µg. The IC_{50} value of 334.9 µg and 427.1 µg was reported for the endophytic bacteria *Bacillus tequilensis* and *Bacillus subtilis*, respectively isolated from a medicinal plant *Fagonia indica* [38]. IC_{50} value is a widely used parameter for the measurement of free radical scavenging activity. Low IC_{50} indicates significant activities as compared to high IC_{50} value [39].

Cytotoxic Activity: MTT assay is a well-established *in-vitro* method for assessing cytotoxicity against cancer cell lines. Extracts of *Cissus quadrangularis* have shown to inhibit the proliferation of MCF-7 cell lines [40]. In order to understand the effect of extracellular ethyl acetate extract of the endophytic bacterium CqB9 isolated from *Cissus quadrangularis* on human breast cancer cells, experiments were conducted using cultured MCF-7 cell lines. The cytotoxic potential of the extract on MCF-7 cell lines are given in **Fig. 4**. MTT assay results revealed that as the concentration of CqB9 extract was increased, the percent inhibition of MCF-7 cell lines also increased.

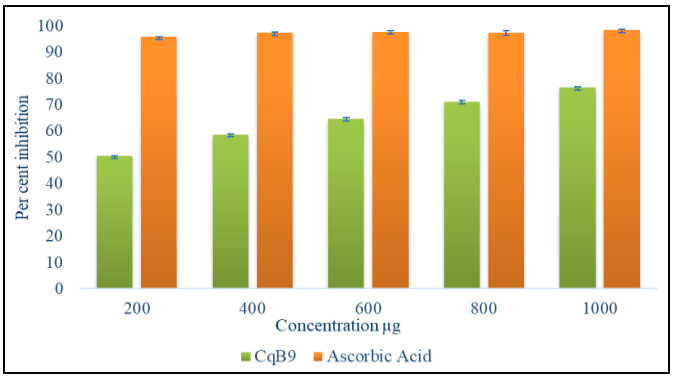

FIG. 3: DPPH FREE RADICAL SCAVENGING ASSAY OF EXTRACELLULAR EXTRACT OF ENDOPHYTE CQB9

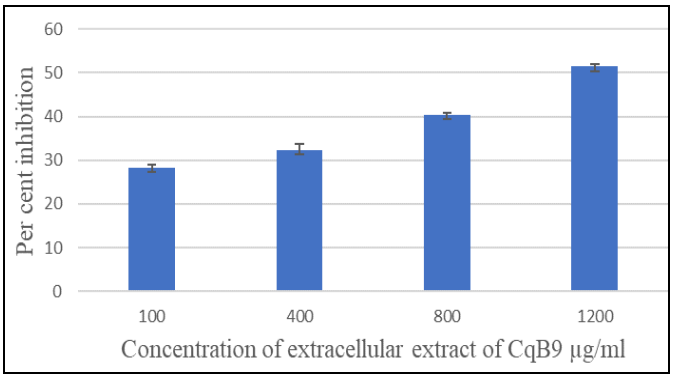

FIG. 4: CYTOTOXIC ACTIVITY OF EXTRACELLULAR EXTRACT OF CQB9 AGAINST MCF-7 CELL LINE

The extract showed a percent inhibition of 28.33 ± 0.6 at 100 µg/ml concentration, and the percent inhibition increased to 51.42 ± 0.63 at a concentration of 1200 µg/ml. Results of cytotoxic assays showed that the extracellular ethyl acetate extract of endophytic bacterium CqB9 inhibited MCF-7 cancer cell proliferation.

16S rRNA Sequencing: The endophytic bacterium CqB9 was characterized by 16S rRNA sequencing, and a phylogenetic tree was constructed using MEGA X software and bootstrap algorithm **Fig. 5**. CqB9 was found to be similar to *Achromobacter anxifer*.

To the best of our knowledge, this is the first paper to report the isolation of endophytic bacterium from *Cissus quadrangularis* identified to be similar to *Achromobacter anxifer*.

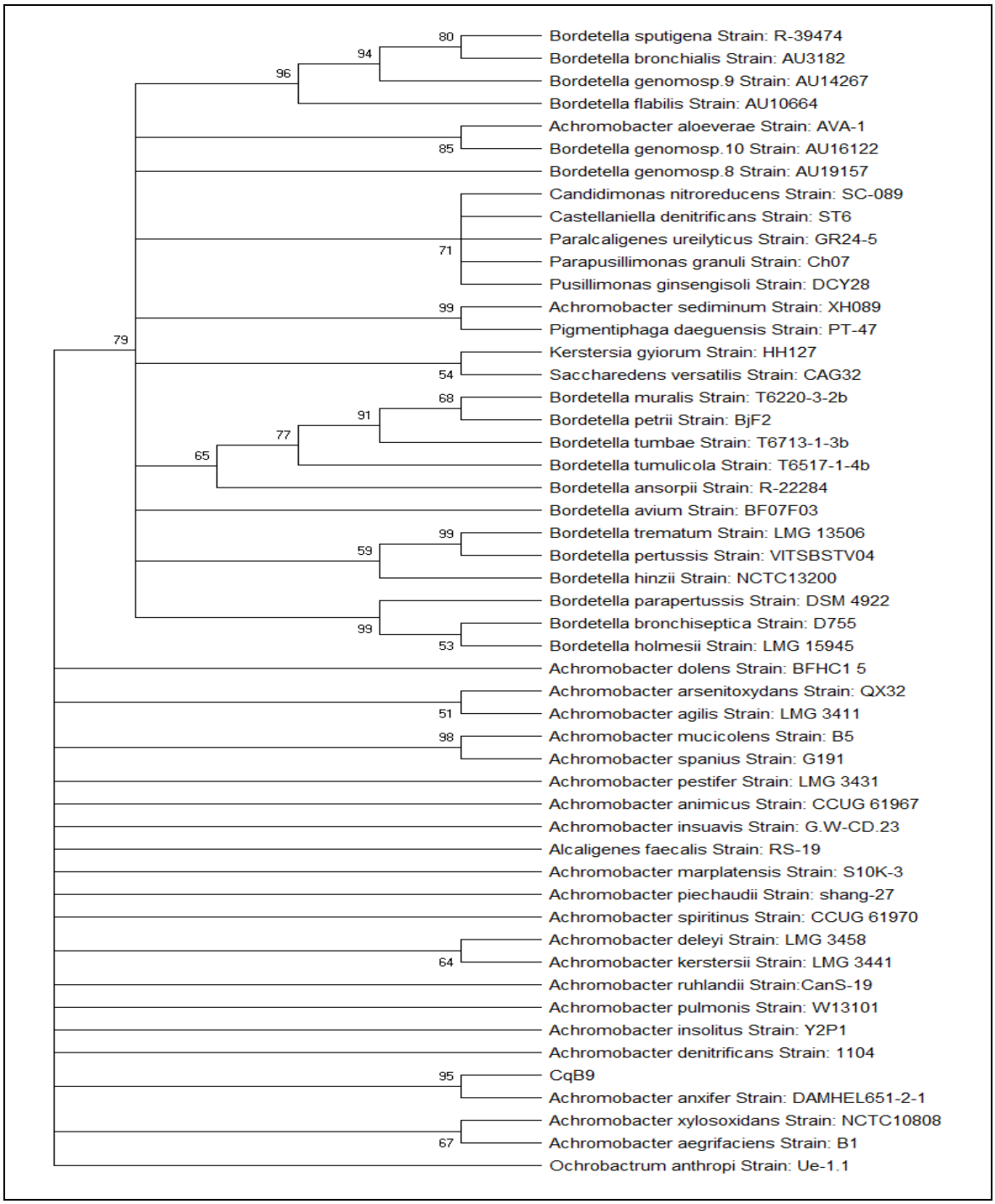

FIG. 5: PHYLOGENETIC TREE OF THE ENDOPHYTE CQB9 BASED ON 16S rRNA SEQUENCING CONSTRUCTED USING MEGA X AND BOOTSTRAP ALGORITHM

CONCLUSION: Endophytes are considered to be potent sources of bioactive compounds. In this study, endophytic bacteria were isolated from a medicinal plant *Cissus quadrangularis,* and one of them was identified to be similar to *Achromobacter anxifer* by 16S rRNA analysis. The study shows the antibacterial and antioxidant potential of the endophytic bacteria isolated from *Cissus quadrangularis*. The bioactive compounds were predicted by using GCMS. The results of this study represent that endophytic bacteria may serve as a potential source of natural antibacterial, antioxidant and anticancerous compounds. The cytotoxic activity using MTT assay showed that the crude extract inhibited the proliferation of MCF-7 cell line in a dose dependent manner. However, this needs to be further confirmed by staining methods, supporting parameters like DNA fragmentation experiments and comparison with cytotoxic activity on normal cell lines. Further, the bioactive components having antibacterial, antioxidant and cytotoxic property need to be purified and studied. This study is focused on exploring endophytic bacteria from the stem of *Cissus quadrangularis* for their novel bioactive compounds.

In conclusion, the identification of the metabolites from the extracellular extracts of CqB9 identified as *Achromobacter anxifer,* predicts that this endophyte is capable of producing bioactive compounds. Further work is to be carried out to confirm the bioactivities of these metabolites. This study will provide an introduction to more comprehensive work on bioactive compounds produced by the bacterial endophytes from this important medicinal plant.

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