

## Endophytic Bacteria from *Cissus quadrangularis*, A Promising Source of Bioactive Compounds

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The medicinal plant *Cissus quadrangularis* has been extensively explored with respect to the presence of phytochemicals and their bioactivities. The available research data on endophytic bacteria of this plant is very limited. The main objective of this study is to isolate endophytic bacteria from the stem of *Cissus quadrangularis* and to determine the presence of bioactive compounds, antioxidant and antiosteosarcoma potential of the extracellular ethyl acetate crude extracts of endophytic bacteria. One of the isolates CqB14 was identified to be similar to *Pseudomonas* sp. as analyzed by 16S rRNA sequencing. Qualitative phytochemical screening and HPTLC analysis of the extracellular ethyl acetate crude extract of CqB14 showed the presence of alkaloids in addition to other secondary metabolites. GCMS analysis predicted the presence of antimicrobial and anticancerous compounds. This study also reveals the antioxidant and antiosteosarcoma potential of endophytic bacteria from *Cissus quadrangularis*.

**Keywords:** Antiosteosarcoma, Antioxidant, *Cissus quadrangularis*,  
Endophytic bacteria, GCMS analysis, HPTLC.

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Bacteria or fungi that are associated with plant tissues either within or between the plant cells and those that do not cause any diseases are known as endophytes <sup>1</sup>. Endophytic bacteria have been isolated from different parts of the plant and inhabit most of the plant species <sup>2</sup>. Bioactive metabolites produced by endophytes have been documented and are found to have antibiotic, antiviral, anticancer, antiinflammatory and antioxidant activities. Compared to soil related

microbes, endophytes closely associated with their host plant produce biological molecules greater in number and diversity. Therefore, there is greater possibility to screen unique and active substances from endophytes and to discover new bioactive compounds. Compared to plants, endophytes are sustainable sources of natural products and can be used for large scale production of biologically active compounds to meet the industrial demands as it is easier to scale up the fermentation process

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of microbes. Although all plants seem to harbour endophytes with some bioactive content and activities, the plants that have medicinal value are of particular interest. There lies a great opportunity to discover distinct and fascinating microorganism among countless plants in various locations and environs. One of the rationales for selecting a plant to isolate endophytes and to discover natural product is to opt plants that have an ethnobotanical history and those that have specific uses or interesting values. Sometimes, the curative abilities of the herbal plants may be attributed to the endophytes of the plant than the natural products of the host plant. Ultimately, plants with ethnobotanical history are reasonable candidates for study, since the therapeutic uses for which the plant may have been chosen relates more to the microflora inhabiting the plant than to the compounds present in plant itself<sup>3</sup>.

*Cissus quadrangularis* is a traditional medicinal plant widely used in India and has varied therapeutic claims. The use of the plant to heal fractures and to set bones is stressed in the ayurvedic literature, the Nigathus. The plant possesses noteworthy phytoconstituents that back its several ameliorative activities besides bone remineralization. Today's pharmacological study over *Hadjod* proves the ancient classical references of the plant and reestablishes the plant's potentiality to cure several diseases. The extracts of *Cissus* possess antioxidant and anticancerous potential<sup>4</sup>.

The stem of *Cissus quadrangularis* comprises excellent antioxidants such as vitamin C, carotenoids, calcium and steroids. In ayurvedic and modern drug development areas the plant is treated as a versatile medicinal plant for its worthy therapeutic uses. It does not produce any poisonous effects when consumed<sup>5</sup>.

Numerous reports are available on diversity of endophytic bacteria and fungi in medicinal plants, but there are meagre reports on endophytic bacteria from *Cissus quadrangularis*. Endophytic bacteria from *Cissus quadrangularis* may produce similar compounds as that of the host plant or exhibit bioactivity. Therefore, the present study aims to identify and analyze the bioactive potential of *endophytic* bacteria isolate CqB14 associated with medicinal plant *Cissus quadrangularis*.

## MATERIAL AND METHODS

### Isolation of endophytic bacteria and extraction of extracellular compounds

The plant was collected from Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), 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) as *Cissus quadrangularis* L. Fresh and healthy stem was surface sterilized by standard methods<sup>3</sup>. The stem was washed thrice with sterile distilled water. After surface sterilization the outer part of the stem was excised off and the remaining part of the stem was sliced into thin sections. The sections of stem were placed over Nutrient Agar plates and were incubated for 2-4 days. Aseptic conditions were maintained throughout. The endophytic bacteria emerging from the sections of stem were selected and purified by subculturing. Each pure 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 crude sample and stored under refrigeration for further use.

### Preliminary qualitative phytochemical screening

The extracellular crude extracts of the endophytic bacteria were analysed for the presence of secondary metabolites, namely carbohydrates, phenols, flavonoids, alkaloids and terpenoids by the standard methods<sup>6,7</sup>.

### HPTLC analysis

The High Performance Thin Layer Chromatography (HPTLC) analysis was performed using Hamilton syringe and CAMAG LINOMAT 5 instrument at Anchrom, Mumbai. The extracellular ethyl acetate extract (1:2) of bacteria labelled as CqB14 was subjected to HPTLC fingerprinting and was checked for alkaloids in HPTLC analysis. Increasing volume of the extract was loaded on silica gel 60 F 254 coated TLC plate. Chloroform: Toluene: Ethanol (4:4:1) was used as the mobile phase for HPTLC fingerprinting and Toluene: ethyl acetate: methanol: Ammonia 25 % (30:30:15:1) for alkaloids. Images of the developed TLC plates were

taken at white light, UV 254 nm and UV 366 nm in a photo documentation chamber. The developed TLC plates were derivatized with anisaldehyde sulfuric acid reagent and dragendorff's reagent for fingerprinting and detection of alkaloids respectively. The TLC plates were fixed in scanner stage (CAMAG TLC SCANNER 4) and scanned. The peak table and peak densitogram were noted.

#### Antioxidant assay

The antioxidant activity of the extracellular ethyl acetate extract of the endophytic bacteria

CqB14 was tested by DPPH assay. The method described by Blois<sup>8</sup> was used to check the ability of the extracts to reduce the DPPH radical (1, 1-diphenyl-2-picrylhydrazyl). A stock solution of 10 mg/ml concentration was prepared using the extract. Different concentrations of the extract (200, 400, 600, 800, 1000  $\mu$ g) were added to methanolic solution of DPPH. The reaction mixture was incubated and the absorbance was recorded at 517 nm. The experiment was repeated three times. Ascorbic acid was used as a standard. The

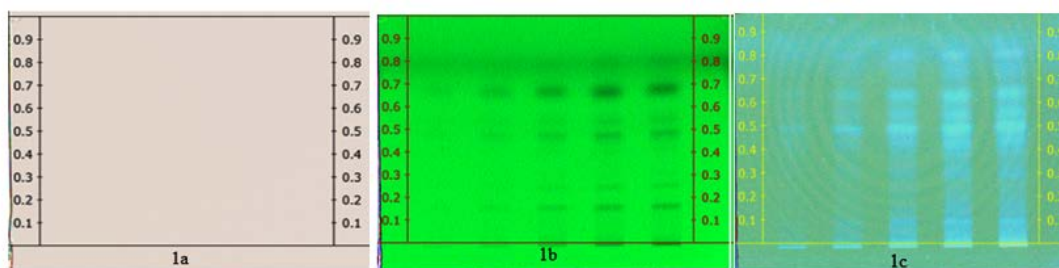


Fig. 1. HPTLC chromatogram 1a) white light 1b) 254 nm 1c) 366 nm for different volumes of extract

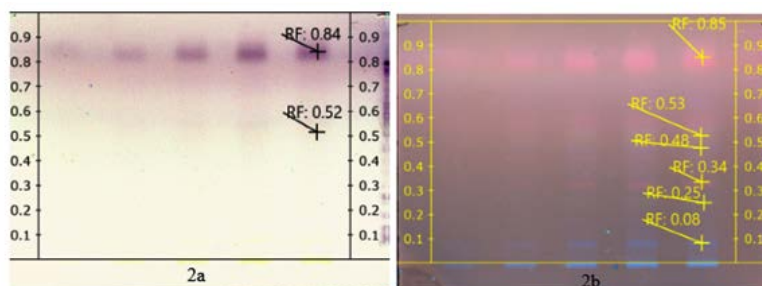


Fig. 2. HPTLC chromatogram after derivatizing with anisaldehyde 2a) white light 2b) 366 nm for different volumes of extract

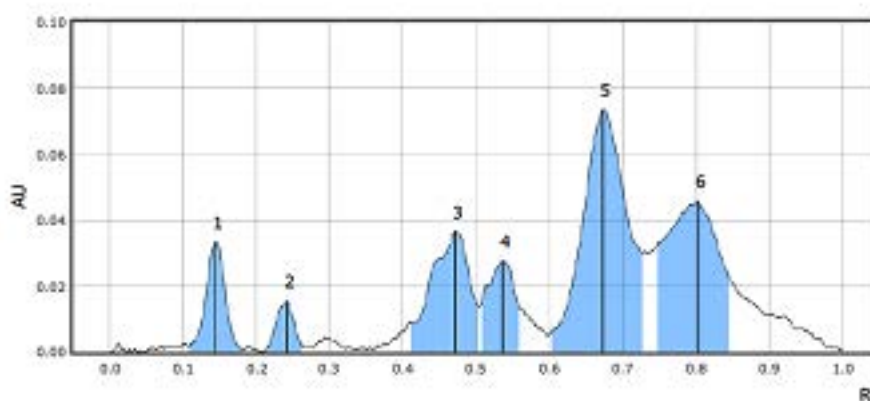


Fig. 3. HPTLC densitometric chromatogram at 254 nm, track 5

annihilation activity of free radicals was calculated in percent inhibition according to the formula  
 Percent Inhibition =  $(A \text{ of control} - A \text{ of Test}) / A \text{ of control} \times 100$ .

#### GCMS analysis

The extracellular extract of CqB14 was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) to predict the compounds present. The Clarus 680 GC used in the analysis, employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m  $\times$  0.25 mm ID  $\times$  250  $\mu$ m df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run.

One  $\mu$ L of extract sample was injected into the instrument, the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C  $\text{min}^{-1}$ , and 300 °C, where it was held for 6 min and total run time 32 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The spectrums of the components were compared with the database of spectrums of known components stored in the GC-MS NIST library.

#### Effect of the extract on Saos-2 cell line

Osteosarcoma cell line Saos-2 was procured from National Centre for Cell Science

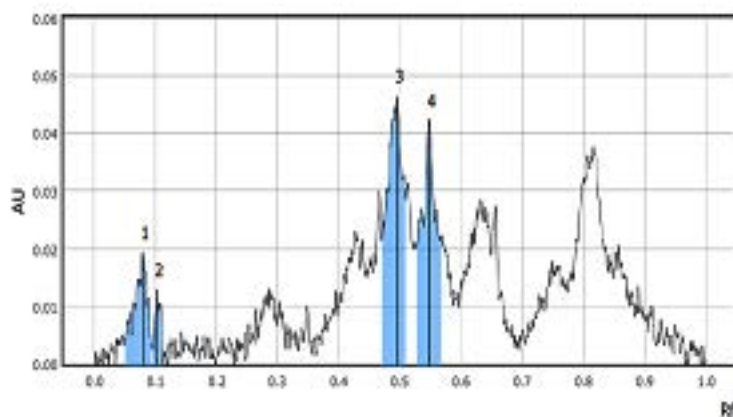


Fig. 4. HPTLC densitometric chromatogram at 366 nm, track 5

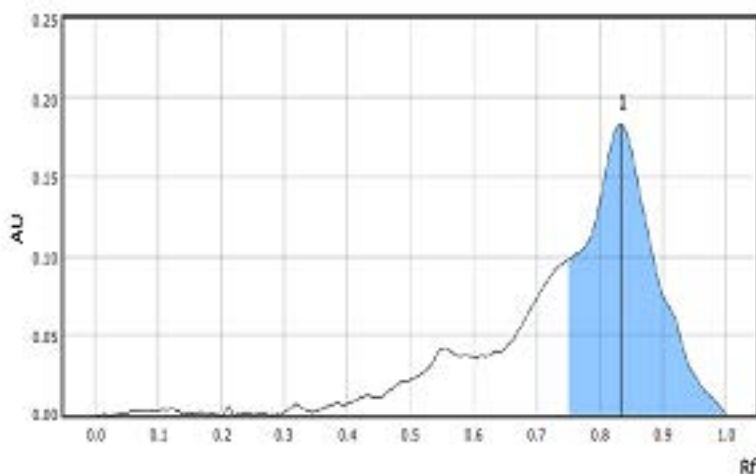


Fig. 5. HPTLC densitometric chromatogram after derivatizing with anisaldehyde at 540 nm, track 5

(NCCS), Pune, India. The cells were maintained in Minimal Essential Media supplemented with 10 % FBS, penicillin in 5 % CO<sub>2</sub> at 37 °C. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to assess the inhibitory effect of the extract at various concentrations<sup>9</sup>. Using the microplate reader, the absorbance at 570 nm was recorded to determine the viable cells. The average absorbance values were deduced from the absorbance values observed three times. Percent inhibition = Control - Test / Control × 100.

#### Identification of endophytic bacteria

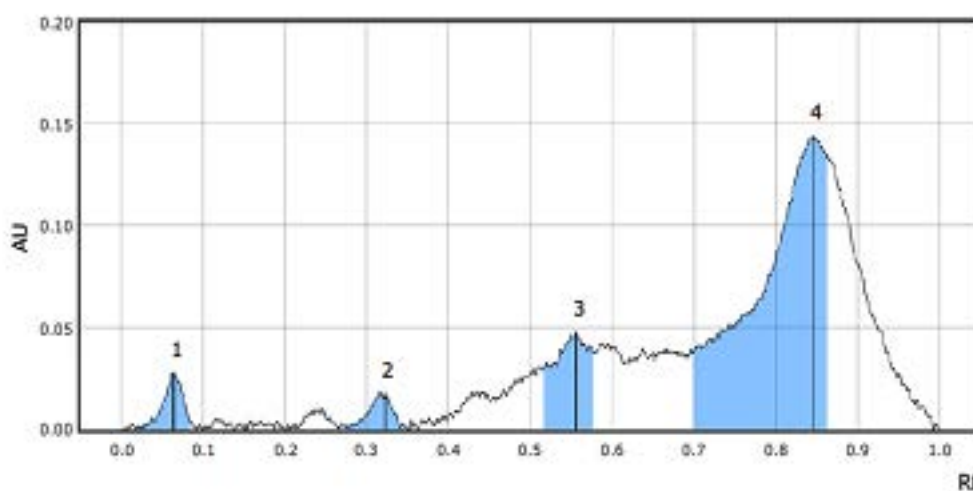
The endophytic bacterium CqB14 was identified by 16S rRNA gene sequence. The genomic DNA of endophytic bacteria was extracted. Bacterial universal primers were

engaged to amplify 16S rDNA in the Polymerase Chain Reaction (PCR) wherein genomic DNA was the template. NCBI BLAST was used to find 16S rRNA genes homologous to the CqB14 sequence. Highly similar sequences were chosen and fed into Mega X. The phylogenetic tree was constituted using the Maximum Likelihood statistical method with bootstrapping as the test of phylogeny. Thousand bootstrap replications were performed to assess nodal support in the tree<sup>10</sup>.

## RESULTS AND DISCUSSION

#### Preliminary phytochemical screening

The preliminary phytochemical analysis of extracellular ethyl acetate extract of the endophyte labelled as CqB14 revealed the



**Fig. 6.** HPTLC densitometric chromatogram after derivatizing with anisaldehyde at 366 nm, track 5

**Table 1.** Rf values and area % of HPTLC chromatogram of extracellular ethyl acetate extract of CqB14, track 5

Peak No.	254 nm		366 nm		540 nm after derivitization with anisaldehyde		366 nm after derivitization with anisaldehyde	
	Rf max	Area%	Rfmax	Area%	Rf max	Area%	Rf max	Area %
1	0.144	7.49	0.079	14.79	0.833	100.00	0.064	4.46
2	0.242	3.16	0.103	4.37			0.323	3.49
3	0.471	16.12	0.495	44.44			0.556	14.11
4	0.536	8.50	0.548	36.40			0.846	77.95
5	0.673	37.59						
6	0.803	27.13						

presence of carbohydrates, phenols, flavonoids and alkaloids. Similarly, the extract of aerial parts of *Cissus quadrangularis* showed the presence of carbohydrates, alkaloids, flavonoids, glycosides, saponins, steroids, tannins, triterpenoids<sup>11</sup>. The presence of these metabolites indicates that the extract may possess bioactivities.

#### HPTLC analysis

HPTLC profiling has been attempted by researchers to substantiate the presence of bioactive compounds<sup>12</sup>. One of the several advantages of

HPTLC is the ability to analyze crude samples containing multicomponent. HPTLC application has been proven in analysis of fermentation broth<sup>13,14</sup>. The HPTLC analysis of crude extract of CqB14 showed the presence of peaks before and after derivatization with anisaldehyde. The number of peaks and Rf values differ as per the qualitative variations of the components. The various HPTLC chromatograms are shown in figures 1-10. The chromatograms show separation of constituents without any tailing. Brownish band

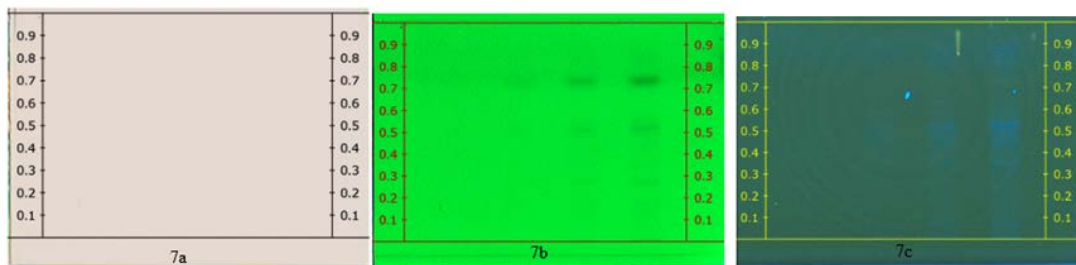


Fig. 7. HPTLC alkaloid chromatogram 7a) white light 7b) 254 nm 7c) 366 nm for different volumes of extract

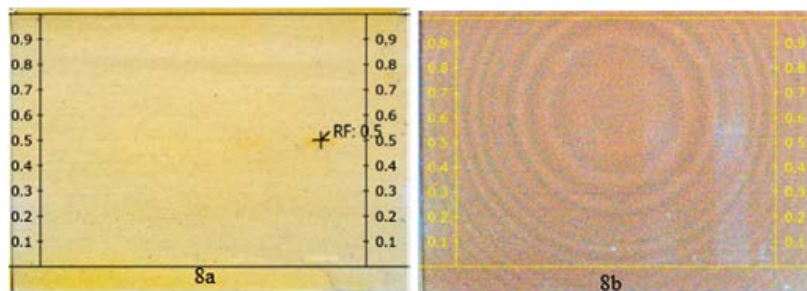


Fig. 8. HPTLC alkaloid chromatogram after derivatizing with dragendorff 8a) white light 8b) 366 nm for different volumes of extract

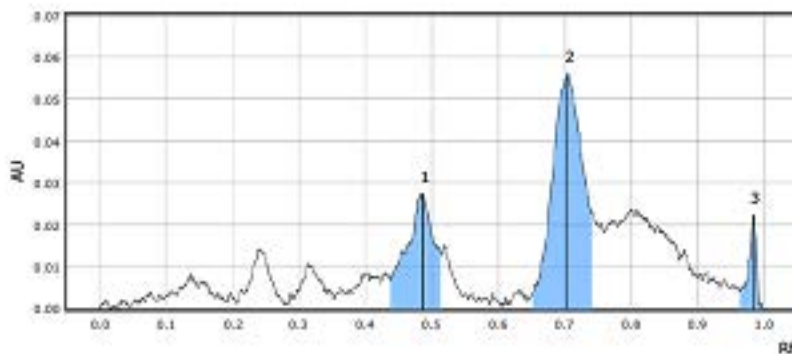


Fig. 9. HPTLC alkaloid densitometric chromatogram at 254 nm, track 4

(Rf 0.5) after derivatization with dragendroff's reagent confirms the presence of alkaloids<sup>15</sup>. The Rf values and peak area % are tabulated in table 1 and 2. It is observed in the above HPTLC studies that the extracellular ethyl acetate extract of CqB14 contain a range of chemical constituents with different Rf values including alkaloids. Alkaloids

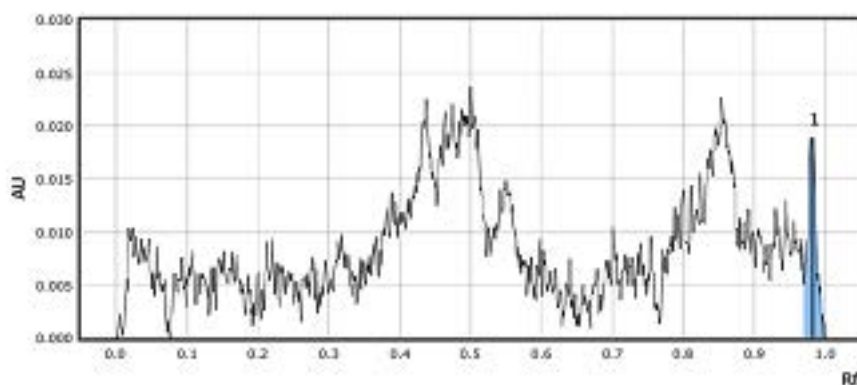
**Table 2.** Rf values and area % of HPTLC alkaloid profile chromatogram of extracellular ethyl acetate extract of CqB14, track 4

Peak No.	254 nm		366 nm	
	Rf max	Area%	Rf max	Area%
1	0.487	28.54	0.982	100.00
2	0.705	64.60		
3	0.985	6.86		

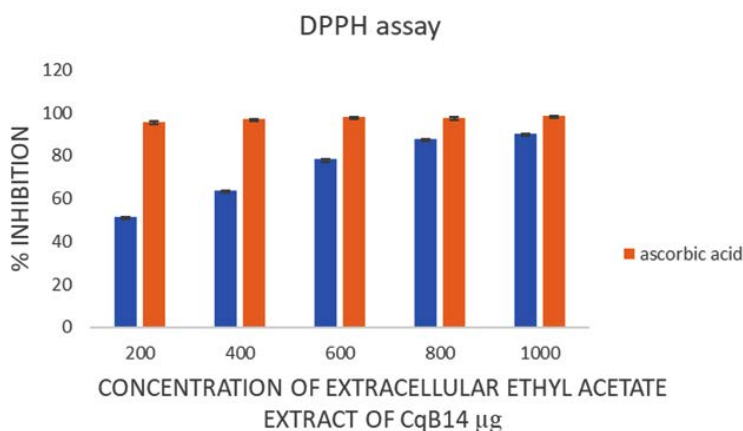
are significant class of chemical compounds that provide a rich reservoir for drug discovery. The antioxidant activities of polyphenols and alkaloids have been demonstrated in several studies. Numerous alkaloids isolated from natural herbs display antiproliferation and antimetastatic effects on various types of cancers both in vitro and in vivo<sup>16</sup>. The developed fingerprint analysis will help to isolate and identify new compounds, which will offer a possibility to discover lead molecules for drug development.

#### Antioxidant Activity

In present study, extracellular ethyl acetate extract of endophytic bacteria CqB14 was investigated for antioxidant potential by using DPPH method. An increase in antioxidant activity was noted as the concentration of the



**Fig. 10.** HPTLC alkaloid densitometric chromatogram at 366 nm, track 4



**Fig. 11.** Antioxidant activity by DPPH assay



extracellular extract was increased. The extract showed antioxidant activity of 90.23% at 1000 $\mu$ g/ml [figure 11]. This antioxidant potential may be due to the various metabolites detected by

HPTLC. It is reported that 100 ppm concentration of ethyl acetate extracts of *Cissus quadrangularis* fraction exhibited 61.6% in the 1,1-diphenyl-2-picrylhydrazyl system. This fraction showed the

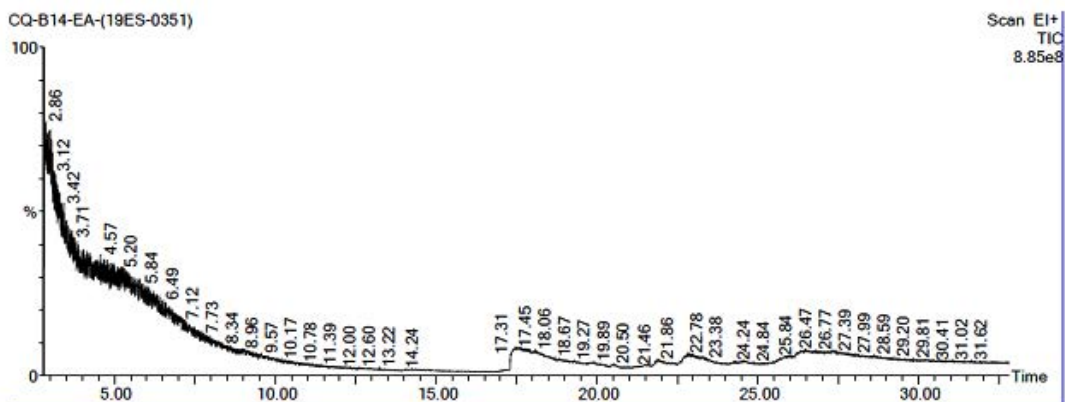


Fig. 12. GC-MS spectrum of extracellular extracts of CqB14

Table 3. GC-MS analysis of extracellular extracts of CqB14

S. No	Compound	Molecular Formula	Molecular Weight	RT	Area %
1.	3-Nonanol, 1,2;6,7-Diepoxy-3,7-Dimethyl-, Acetate	$C_{13}H_{22}O_4$	242	27.298	4.780
2	2-Myristinoyl-Glycinamide	$C_{16}H_{28}O_2N_2$	280	27.163	2.264
3	Unknown	No hits found	-	26.833	6.090
4	Dl-leucine, n-dl-leucyl-	$C_{12}H_{24}O_3N_2$	244	26.468	10.525
5	Benzaldehyde, 4-methoxy	$C_8H_8O_2$	136	17.464	69.500
6	Acetophenone, 4'-methoxy	$C_9H_{10}O_2$	150	18.795	4.505
7	Cyclopropane, 1,2-dibutyl	$C_{11}H_{22}$	154	21.891	2.337

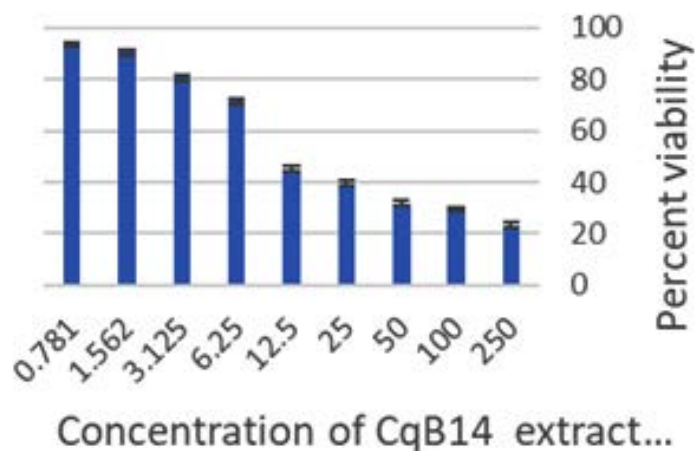


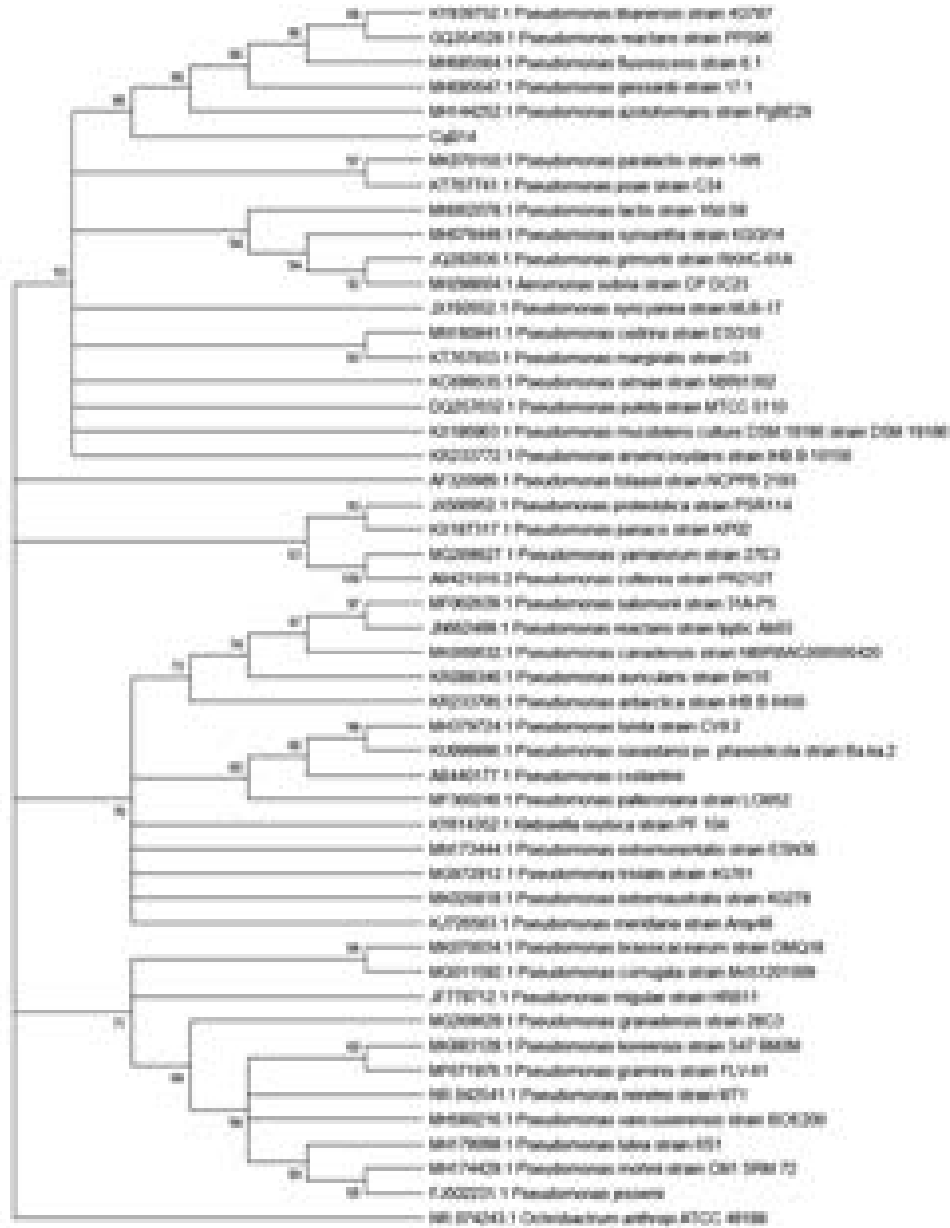
Fig. 13. Cell viability of Saos-2 cells treated with extracellular ethyl acetate extract of CqB14



presence of sterols, vitamin C, and tannins as phytoconstituents<sup>17</sup>. Antioxidant activity of *Cissus quadrangularis* extract is also reported by Badami *et al*<sup>18</sup>. The endophytic bacteria may contribute towards the antioxidant activity of the *Cissus quadrangularis*.

**GC-MS analysis**

The GC-MS of extracellular crude extract of CqB14 predicted 7 major compounds [Fig 12 and Table 3]. GC-MS analysis indicated that the bacterium CqB14 produced an amino compound 2-Myristinoyl-Glycinamide having antimicrobial



**Fig. 14.** Phylogeny tree constructed using Mega X

activity<sup>19</sup>. Benzaldehyde, 4-methoxy was found to be present at a highest percent (peak area 69.5%) among the seven predicted compounds which is reported to be an insect repellent and also used as flavouring ingredient. Benzaldehyde, 4-methoxy also possesses anticancer and antifungal activities<sup>20</sup>. Acetophenone, 4'-methoxy an aromatic phenol ketone is reported to be a flavouring ingredient and is used as a component of perfumes and as chemical intermediate in the manufacture of pharmaceuticals, resins, flavouring agents<sup>21</sup>. Thus, the results obtained confirm the therapeutic potency of the endophyte CqB14 and forms a good basis for the selection of the isolate for further phytochemical and pharmacological investigation.

#### **MTT assay using Saos-2 osteosarcoma cell lines**

The use of *Cissus quadrangularis* in the management of bone and joint disorders such as osteoporosis, osteoarthritis, and rheumatoid arthritis has been documented in native medicine. The anti-proliferative properties of the *Cissus quadrangularis* methanol extract from aerial parts against MG63 osteosarcoma cells were shown by Suresh et al using cytotoxicity assay<sup>22</sup>. In this study the anticancer potential of the extracellular crude extract of the endophyte CqB14 was tested using MTT assay against Saos-2 osteosarcoma cell line. MTT assay is a colorimetric assay in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) enters the cells. In the mitochondria the yellow colored MTT is reduced to an insoluble purple colored formazan product in presence of mitochondrial succinate dehydrogenase. The cells are then solubilized with an organic solvent (e.g. DMSO) and the released, solubilized formazan reagent is measured using spectrophotometer. Since reduction of MTT can only occur in living cells, the level of activity indicates viability of the cells. The generation of reducing equivalents in metabolically active cells forms the basis of MTT assay which helps quantify the viable cells. Greater the number of viable cells higher is the absorbance value<sup>23</sup>. Through MTT assay it was observed that as the concentration of the extracellular crude extract of CqB14 was increased the viability of Saos-2 osteosarcoma cells decreased [Figure 13]. The extract may serve as a source of therapy against bone tumours. However detailed study to determine the primary mode of antiosteosarcoma effect needs to be carried out.

#### **Identification of potential endophytic bacteria**

The endophytic bacteria CqB14 was characterized by 16S rRNA sequencing. BLAST analysis revealed 100% similarity with *Pseudomonas azotoformans*. A phylogenetic tree was constructed using MEGA X software and bootstrap algorithm [Fig. 14]. The internal node connecting the query sequence CqB14 with *Pseudomonas azotoformans*, *Pseudomonas libianensis*, *Pseudomonas reactans*, *Pseudomonas fluorescens* and *Pseudomonas gessardii* with a bootstrap value of 66% was obtained. The sequence was deposited at NCBI genbank and accession number MN559400 was obtained. Mostly, endophytic bacteria isolated from medicinal plants belongs to *Pseudomonas* and *Bacillus* species<sup>24</sup>. To the best of our knowledge this is the foremost study which reports the isolation of endophytic bacteria similar to *Pseudomonas* species from *Cissus quadrangularis*.

#### **CONCLUSION**

Bacterial endophytes have several capabilities that it can be utilized in pharmaceutical and drug discovery. Endophytes related with plants having medicinal significance serve as a potential source of natural products and for developing new agents exhibiting a wide range of bioactivities. A single endophyte may yield a variety of bioactive metabolites. The utilization of highly automated HPTLC, can be considered as a useful tool in the analysis of complex mixtures of natural products. In the present investigation the endophytic bacteria CqB14 isolated from ethnomedicinal plant *Cissus quadrangularis* seems to be an alternative source of natural antioxidant and anticancer compounds. CqB14 identified to be similar to *Pseudomonas* species is capable of producing various secondary metabolites admissible as therapeutics against human ailments and clinically effective bioactive compounds. So, it could be proposed as an organism of pharmaceutical interest. Further studies will need to be undertaken by isolating individual secondary metabolite and determining the biological activities of individual constituents which may hopefully yield good results.

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