# Screening for the Antibacterial Activity, Antibiotic Sensitivity and Antioxidant properties of Bacterial Endophytes Isolated from *Biophytum sensitivum* (L.) DC.

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**ABSTRACT:** The synergetic activity of the endophytic bacteria within the host creates a consortium that triggers physiological changes within the host plant thereby synthesising various compounds beneficial for plant growth and survival. In the present study five endophytic bacterial strains isolated from *Biophytum sensitivum* (L.) DC. were screened for antibacterial activity, antibiotic sensitivity and antioxidant properties. The results indicated that all the five isolates were inhibitory to the growth of *Proteus vulgaris* and *Salmonella typhi*. Antibiotic sensitivity revealed that all the isolates were sensitive to Ampicillin. Screening for antioxidant properties revealed that the isolate BS2 showed the highest total phenol content and DPPH assay has resulted in significant antioxidant activity of all isolates with BS2 showing optimal action. GC-MS analysis has shown that the isolate BS2 produced many compounds that impart antioxidant property. Thus it can be concluded that the endophytic bacteria isolated from *B. sensitivum* possess antibacterial, antibiotic and antioxidant properties that may contribute to its medicinal value.

**Keywords**: Endophytic bacteria, Antibacterial activity, Antibiotic sensitivity, TPC, DPPH Assay, GC-MS.

# INTRODUCTION:

Plants harbour a myriad of microorganisms both epiphytically and endophytically that play a significant role in plant growth productivity and existence. Endophytic bacteria are those which reside in the internal tissues of the host plant without causing any harm and gaining benefit other than residency. [Kloepper *et al.*,1992; Kado *et al*.,1992]. They were initially defined in various reviews as colonists [Kloepper *et al* 1992], endogenous bacteria [Sturdy *et al* 1974] and as xylem residing bacteria [Gardner *et al* 1982]. The multifaceted interaction between endophytes and host plant produce a particular environmental niche within the internal tissues where it shelters a community of beneficial bacteria that are capable of existing endophytically [Lodewyckx et al., 2002; Schulz et al.,2006; Marasco et al 2012]. The endophytes in turn synthesize various host metabolites that help the host system to improve their fitness with the surrounding environment [Ludwig- Muller, 2015]. The interdependency between the duo causes evolution and genetic modification at cellular and molecular level thus establishing an intimate relationship between the plant and the host [Aravind *et al* 2009; Costa *et al* 2012; Khare *et al*; 2018]. Over the years, an exponential growth of population resulted in a tremendous increase in the incidence of infectious diseases making the pathogens resistant to antibiotics (Bisht *et al*., 2009). This paved the way to the global demand of synthesising new drugs from natural products which led to increased researches in the field of endophytic bacteria to exploit them as an alternate source for bioprospecting of bioactive compounds and as probiotics. The anti-microbial property of most endophytic bacteria confer resistance to the plants from pathogenic attack by producing novel bioactive constituents thus playing a major role in regulating the plant health [Khare *et al* ; 2018].Various reviews substantiate the role of bacterial endophytes in the production of antibiotics and secondary metabolites [Singh *et al*; 2017]. L-Asparaginase and antioxidant activity of endophytic bacteria associated with ethno medicinal plants were reported with *Serratia marcescens*, cenA showing the highest activity (Nongkhlaw *et al* ; 2014). Antimicrobial and antioxidant properties of bacterial endophyte *Methylobacterium radiotolerans* isolated from *Combretum erythruphyllum* seeds was studied recently (Photolo *et al* ; 2019)

The plant selected for the present study was *Biophytum sensitivum* (L). DC that belongs to the family Oxalidaceae of dicots and is a major ingredient of 'Dashapushpam' in Ayurveda. Extensive research has been carried out to find the medicinal benefits of *B. sensitivum* and phytochemical analysis revealed the presence of various phytochemical compounds (Sakthivel *et al*.2012). Five different bacterial endophytes were already isolated from *Biophtyum sensitivum* and were characterized based on biochemical tests, plant growth promotion activity, 16S rDNA sequencing and phylogenetic analysis (George *et al.* 2020). The present investigation was carried out to screen the above isolated strains based on their antibacterial property, antibiotic sensitivity and antioxidant properties.

## MATERIALS AND METHODS

Endophytic bacteria were isolated from *Biophytum sensitivum* (L.) DC and identified using 16s rDNA sequencing (George *et al.* 2020). The sequence data were submitted to NCBI GenBank and accession numbers were obtained. The endophytes isolated are *Staphylococcus* sp. strain (MH050396)(BS1); *Bacillus* sp. strain (MH050388)BS2); *Bacillus* cereus strain (MH050384)(BS3); *Bacillus subtilis* strain (MH050389)(BS4) and *Bacillus* sp. strain (MH050399)BS5). Suspension culture of all the bacterial isolates were prepared separately in 500ml nutrient broth and incubated at 25±2ºC for 5 days. From this, culture supernatant was prepared by centrifuging the suspension cultures at 8000 rpm for 10 minutes followed by extraction with double the volume of ethyl acetate. The supernatant thus obtained was further concentrated and used for antibacterial and antioxidant studies.

***Screening for Antibacterial Activity:***

All the five isolated endophytic strains were screened for antibacterial properties using cross-streak method against *Eschericia coli, Klebsiella pneumonia, Proteus vulgaris* and *Salmonella typhi.* The nutrient agar plates were inoculated with bacterial endophytes as a single streak at the centre of the petri plate and incubated for seven days (30°C). The overnight grown cultures were streaked at right angles to the endophyte and observed for growth/ inhibition after 24-48 hr of incubation (30°C) (Kumar *et al* ; 2015).

***Screening for Antibiotic Sensitivity****:*

Bacterial lawn culture was done on previously prepared agar plates for each isolate. Bacterial broth cultures were swabbed using cotton swabs. The antibiotic discs were placed above the inoculum and incubated at 37°C for 24hrs (Kumar *et al* ; 2015). The antibiotic discs used for the assay were Ampicillin,

Chloramphenicol, Streptomycin and Gentamycin. After incubation the plates were observed for the zone of inhibition, the zone diameter was measured and compared with the standard chart to decide the sensitivity towards each antibiotic.

***Determination of Total Phenol Content (TPC):***

Suspension culture of all the bacterial isolates were prepared separately in 500ml nutrient broth and incubated at 25±2ºC for 5 days. Supernatant of cultures were prepared by centrifuging the suspension cultures at 8000 rpm for 10 minutes followed by extraction with double the volume of ethyl acetate and concentrated. The powder thus obtained was then dissolved in methanol and used for further studies. The reaction mixture was prepared by mixing 0.5ml and 1ml of ethyl acetate fraction of the extract, 2.5ml of 10% Folin Ciocalteau reagent and 2.5ml of 7.5% $NaHCO_3$. The samples were incubated for 45minutes at 30ºC. The absorbance was determined using spectrophotometer at $\lambda_{max}$ = 765nm. The samples were prepared in triplicates for each analysis and the mean value of the absorbance was obtained. Gallic Acid was used as standard (Fig1) and the calibration line was constructed. Based on the measured absorbance, the concentration of the total phenolics was expressed as gallic acid equivalents (µg/ml).

***Determination of Antioxidant Activity by DPPH (2,2 Diphenyl Picrylhydrazyl) Assay by Radical Scavenging Method:***

Each isolate was grown in 100mL nutrient broth incubated at 30℃ for 3days. The bacterial culture broth was centrifuged at 1000rpm for 15min and the supernatant was taken. Ethyl acetate was added to the supernatant and was extracted. The ethyl acetate fraction of the extract was filtered and concentrated. Fresh DPPH solution was prepared and kept in the dark. Ethyl acetate fraction of the extract (0.1mL) was added to 3.9mL of a $6 \times 10^{-5}$mol/litre mol/litre ethanol DPPH solution. The reaction mixture was vortexed thoroughly and left in the dark at ambient temperature for 30min. After incubation the variation in the colour was observed and their absorbance was read at 517nm. A solution of ethanol DPPH without the extract was used as control. The percentage radical scavenging activity was calculated by using the formula:

$$\textbf{Scavenging Effect (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample extract or standard. Ascorbic acid was taken as known antioxidant for comparative analysis. Then percentage inhibitions were

plotted against the respective concentrations used and the extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the standard graph. Ascorbic acid was used as standard (mg/mL). The experimental results of biological activity tests were expressed as mean ± standard deviation.

***GC-MS Analysis:***

GC-MS analysis was done to determine the possible compounds responsible for imparting antioxidant property. The tentative identification of chemical compounds present in the isolate was based on GC retention time.

## OBSERVATIONS AND RESULT

**Table 1- Screening for Antibacterial Activity of the endophytic bacteria**

| Isolates | *E.coli* | *Klebsiella pneumonia* | *Proteus vulgaris* | *Salmonella typhi* |
|---|---|---|---|---|
| **BS1** | -ve | -ve | +ve | +ve |
| **BS2** | -ve | -ve | +ve | +ve |
| **BS3** | -ve | -ve | +ve | +ve |
| **BS4** | -ve | -ve | +ve | +ve |
| **BS5** | -ve | -ve | +ve | +ve |

**Table 2- Antibiotic sensitivity of the isolates**

| Antibiotics | BS1 | BS2 | BS3 | BS4 | BS5 |
|---|---|---|---|---|---|
| **Chloramphenicol** | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |
| **Ampicillin** | Resistant | Resistant | Resistant | Resistant | Resistant |
| **Gentamycin** | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |
| **Streptomycin** | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |

**Table 3 – Zone diameter in Millimetre units**

| Antibiotics | BS1 | BS2 | BS3 | BS4 | BS5 |
|---|---|---|---|---|---|
| **Chloramphenicol** | 25mm | 26mm | 23mm | 26mm | 25mm |
| **Ampicillin** | No Zone | No Zone | No Zone | No Zone | No Zone |
| **Gentamycin** | 22mm | 24mm | 18mm | 21mm | 24mm |

| | | | | | |
|---|---|---|---|---|---|
| **Streptomycin** | 24mm | 25mm | 28mm | 23mm | 26mm |

**Fig1- Standard Graph for Gallic Acid**

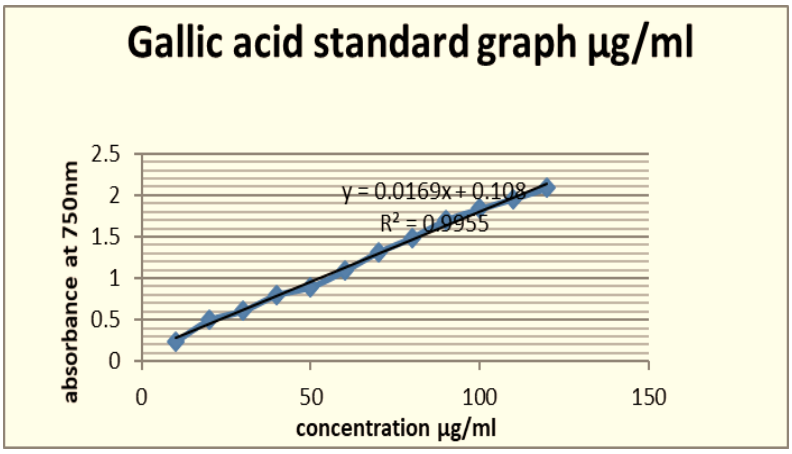



**Fig2- Total Phenol Content of the isolates**

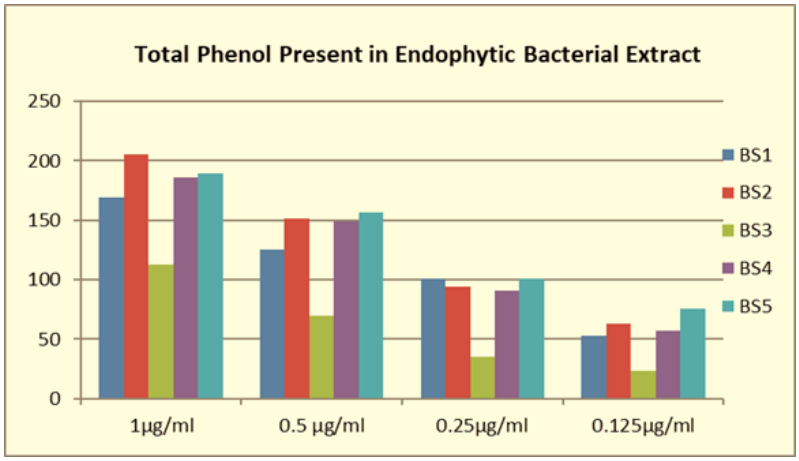
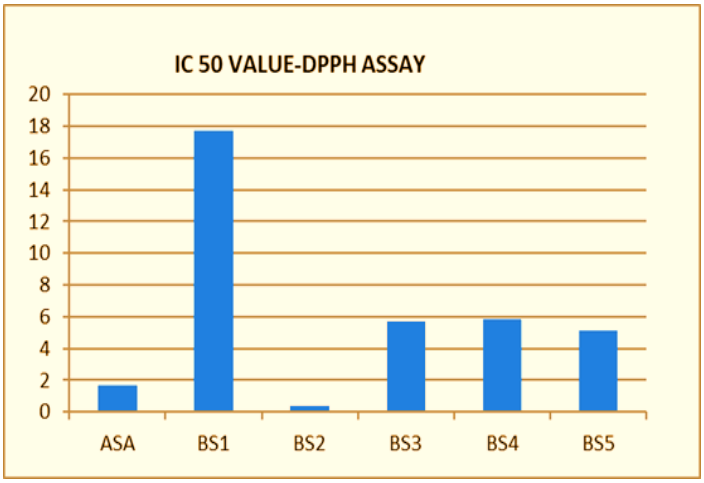
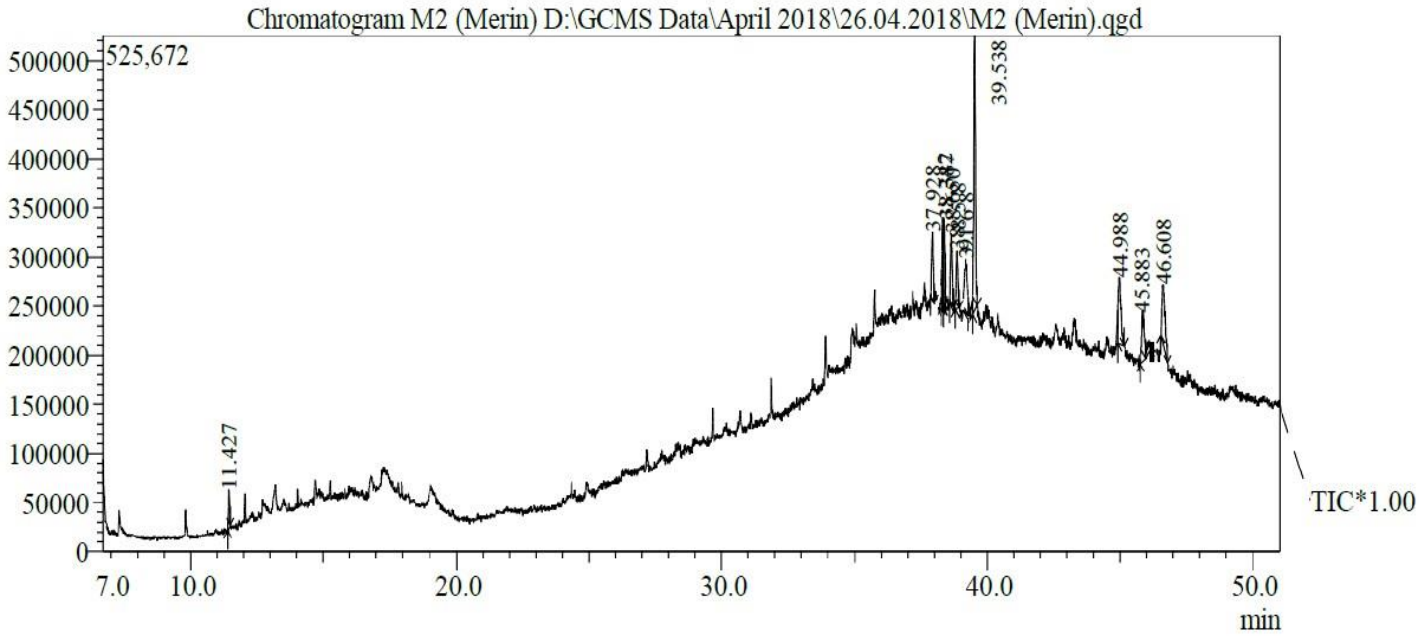


**Fig3- Screening for Antioxidant property through DPPH Assay**




| Sample ID | IC 50 |
|---|---|
| ASA | 1.654*** |
| BS1 | 17.699*** |
| BS2 | 0.35*** |
| BS3 | 5.701*** |
| BS4 | 5.87*** |
| BS5 | 5.13*** |

**Fig6- GC-MS Analysis of BS2 isolate exhibiting various constituents**




Peak Report TIC

| Peak# | R.Time | Area | Area% | Height | Height% | Name | Base m/z |
|---|---|---|---|---|---|---|---|
| 1 | 11.427 | 112639 | 2.55 | 40125 | 4.26 | Undecanal | 57.05 |
| 2 | 37.928 | 273447 | 6.20 | 69976 | 7.43 | 22,23-Dibromostigmasterol acetate | 55.10 |
| 3 | 38.342 | 332569 | 7.54 | 91693 | 9.74 | METHYL COMMATE C | 218.20 |
| 4 | 38.387 | 338565 | 7.67 | 92010 | 9.77 | Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)- | 81.10 |
| 5 | 38.650 | 287125 | 6.51 | 73699 | 7.83 | Glaucyl alcohol | 190.15 |
| 6 | 38.858 | 246717 | 5.59 | 59120 | 6.28 | .gamma.-Sitosterol | 55.05 |
| 7 | 39.168 | 382162 | 8.66 | 55407 | 5.88 | METHYL COMMATE E | 218.20 |
| 8 | 39.538 | 1190086 | 26.97 | 279827 | 29.71 | CHOLEST-5-EN-3-YL CHLORIDOCARBONATE # | 147.15 |
| 9 | 44.988 | 457332 | 10.36 | 67990 | 7.22 | .beta.-Sitosterol | 55.05 |
| 10 | 45.883 | 322871 | 7.32 | 50877 | 5.40 | 2(1H)-Naphthalenone, 7-ethynyl-4a,5,6,7,8,8a-hexahydro-1,4a-dimethyl-, (1.alpha.,4a.beta.,7.beta.,8a.alpha.)- | 173.15 |
| 11 | 46.608 | 469084 | 10.63 | 61106 | 6.49 | 2-CYCLOHEXEN-1-OL, 1,2,4,4-TETRAMETHYL-3-(3-METHYL-1,3-BUTADIENYL)-, (E)-(.+-.)- | 190.20 |
| | | 4412597 | 100.00 | 941830 | 100.00 | | |

## RESULTS AND DISCUSSIONS:

***Anti-bacterial Activity:***

In the present study, the antibacterial assay conducted against the four-test human pathogenic strains reveal that all the five isolates were found to prevent the growth of *Proteus vulgaris* and *Salmonella typhi* whereas *E. coli* and *Klebsiella pneumonia* were resistant to the antibacterial property of the bacterial isolates (Table1). Various reviews substantiate the antibacterial activity shown by endophytic bacteria against plant and human pathogenic microbes. Recent reports by Uche-Okereafor *et al.* (2019) revealed the antibacterial activity of crude secondary metabolite extracts of *Pantoea* species from the stem of *Solanum mauritianum*. Their study displayed that the crude secondary metabolites of the endophytes had antibacterial properties when tested against human pathogenic microbes such as *Escherichia coli, Staphylococcus aureus,*

*Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. 90 bacterial isolates from 30 medicinal plants when subjected to antibacterial assay against *S. aureus, E. coli, B. subtilis, P. aeruginosa, Shigella sp, K.pneumoniae, Proteus mirabilis* and *Listeria monocytogenes* revealed that few isolates showed antibacterial activity whereas contrasting results were shown by five of the isolates(Sushma *et al* ; 2018). Indrawati *et al* 2018 demonstrated the successful isolation of nine bacterial endophytes from Kupa plant (*Syzygium polycephalum*) of which four endophytic bacteria were identified; *Bacillus* sp. (1), *Bacillus* sp. (2), *Bacillus pumilus* and *Bacillus amyloliquefaciens*. Among the isolates, *Bacillus* sp (2) derived from the leaves of the plant showed maximal antibacterial activity against pathogenic bacteria Methicillin-resistant *Staphylococcus aureus* and to *Bacillus cereus*, respectively. Whereas *Bacillus* sp (1) isolated from stem inhibited *Klebsiella pneumoniae* and *B. cereus*. Bacterial endophytes isolated from *Curcuma longa* were also reported with antibacterial activity by Kumar et al 2016. A total of six strains *Bacillus cereus* (ECL1), *Bacillus thuringiensis* (ECL2), *Bacillus* sp. (ECL3), *Bacillus pumilis* (ECL4), *Pseudomonas putida* (ECL5) and *Clavibacter michiganensis* (ECL6) were isolated, of which all the strain exhibited antibacterial activity against *E. coli*, whereas *B. cereus* ECL1, *Bacillus* sp. (ECL3), B. pumilus (ECL4), *C. michiganensis* (ECL6) exhibited antibacterial activity against *Klebsiella pneumonia*. On the other hand, none of the strains inhibited the growth of *Pseudomonas aeruginosa*. Efficient antibacterial activity was shown by the endophytic bacteria *Bacillus cereus* isolated from *Ocimum sanctum* against *E. coli* by nasal swab culture (Nayak *et al* ; 2019). Thus, it is clear that the bacterial endophytes of *Biophytum sensitivum* has the ability to resist the attack of other bacteria, adding to its resistance against diseases.

***Antibiotic Sensitivity:***

The antibiotic sensitivity profile in the present study indicated that all the isolates were susceptible to Chloramphenicol, Gentamycin and Streptomycin as indicated by the zone of inhibition however all the isolates were resistant to Ampicillin (Table 2,3). Our findings exhibit that all the isolates showed antibiotic property against the test antibiotics except Ampicillin. It also discloses that since these strains show antibiotic sensitivity, they do not cause much harm to the human body. The antibiotic properties of endophytic bacteria can enhance the host plant's resistance to pathogens thus promoting their growth and survival (Christina *et al ;* 2012). Arunachalam *et al ;* 2010 assessed the antibiotic susceptibility of all the 20 isolated bacterial endophytes using six antibiotics. It was found that most of the isolates were sensitive to

Ampicillin, Ciprofloxacin, Erythromycin, Chloramphenicol, Amikacin and Gentamycin whereas some were resistant to all antibiotics. Similar results were reported by Gayatri *et al*.(2010) during the isolation of 104 bacterial endophytes from the leaf samples of mangrove plants of Pichavaram, Tamil Nadu. Their study revealed that more than 20 endophytic bacteria were sensitive to antibiotics like Streptomycin and Trimethoprim whereas 31 isolates (86.1%) were resistant to Vancomycin and Bacitracin. The antibiotic sensitivity profile of endophytic bacteria CER5(*Klebsiella oxytoca*), CER6(*Klebsiella* sp) and CER11(*Agrobacterium* sp) isolated from chilli root was determined by disc diffusion method against the antibiotics Norfloxacin, Cefotaxime, Ceftriaxone, Ciprofloxacine and Ofloxacin. Results exhibited showed that the isolate CER5 was sensitive to the test antibiotics (Syed *et al;* 2017). All these reports indicate the potentiality of harnessing bacterial endophytes as novel source of antibiotics.

***Total Phenolic Content (TPC)***

Phenolic compounds eradicate free radicals and peroxide radicals by contributing hydrogen atoms which makes them an effective antioxidant compound (Kinsella *et al*; 1993). The TPC of the isolates were calculated using the linear regression equation obtained from the standard plot of gallic acid: 'y' = 0.0169x + 0.108. R2 = 0.9955, where 'y' is absorbance and 'x' is the amount of gallic acid in µg (Fig1). Among the various isolates BS2 yielded a maximum of 0.205mg/ml total phenolics (Fig 2). Recent research on antioxidant property of the endophytic bacteria from *Carica papaya L*. was reported by Sarjono *et al*. (2019).The assay was carried out to determine the correlation between antioxidant activity and total phenol content of the isolate *Bacillus* (EC3). Their results reveal that the extracts of secondary metabolites of the isolate show satisfactory phenolic contents that are proportional to the antioxidant capacity of the isolates. Similar studies on endophytic bacterial extracts with total phenolic content were reported by Swarnalatha *et al.* (2015) from *Adhathoda beddomei.* Nongkhlaw *et al*. (2015) assessed the total phenolic content of the bacterial endopytic isolates from 11 ethnomedicinal plants and the results were compared with the total phenolic contents of the epiphytic plants isolated from the same plants. Phenols are secondary plant metabolites that are unanimously present in plants thereby playing a significant role in enhancing antioxidant (Rice-Evans *et al;*1996; Velioglu *et al;* 1998) and antagonistic activities of endophytic bacteria (Sousa *et al*; 2006; Pereira *et al.;* 2007) which can be harnessed for the production of bioactive compounds.

***Invitro Antioxidant Activity by DPPH Assay:***

The antioxidant activity of the isolates was assessed by the radical scavenging method DPPH. The scavenging effect of the extracts on the DPPH was expressed as % inhibition and they were compared with standard antioxidant, ascorbic acid. During the DPPH free radical reaction, the degree of discoloration (decrease in absorbance) of the DPPH solution indicates the scavenging potentials of the sample antioxidant. The $IC_{50}$ values of the isolates were calculated. Among the extracts, the optimum DPPH activity was reported in BS2 (35µg/ml) as opposed to that of ascorbic acid ($IC_{50}$ 165.4µg/ml) standard. Statistical analyses were performed by using one-way analysis of variance (ANNOVA). Tukey's test was conducted (***p < 0.001-highly significant, **p < 0.05-significant, *p > 0.05-not significant) which indicated highly significant antioxidant property of all the five isolates. In our present study it was found that the endophytic isolates were able to reduce the radicle DPPH with BS2 showing optimal $IC_{50}$ value. Sulistiyani *et al.* in 2016 isolated eight endophytic bacteria from *Curcuma longa* rhizome of which the antioxidant activity during DPPH assay was exhibited by K3(72.3 %), K2 (51.3 %) and M1b (64.6 %) with K3 showing the highest antioxidant activity. Nongkhlaw *et al* in 2015 assessed the antioxidant property of various bacterial endophytes isolated from ethnomedicinal plants through free radical scavenging activity. They demonstrated the ability of the isolates to reduce DPPH, among which Bacillus sp. F21 showed maximum radical scavenging activity. Endophytic bacteria isolated from *Centella asiatica* exhibiteda a satisfactory antioxidant profile with the highest free radical scavenging. obtained with *Pantoea agglomerans* followed by *Providencia vermicola.* Ethyl acetate was often used as an extraction solvent to determine the highest phenolic content which is in turn associated with the antioxidant property (Conde *et al* ; 2008). The presence of phenolic contents could be a contributing factor that determine the antioxidant property of the endophytic ethyl acetate extract.

***Identification of various compounds by GC-MS Analysis:***

GC-MS identification confirmed that the ethyl acetate fraction of the extract BS2 have 11 detectable compounds. The GC-MS retention time (RT) and percentage peak of the individual compounds were demonstrated in table, figure. The major phytoconstituents present in the ethanolic extract of BS2 are Cholest-5-en-3-yl- chloridocarbonate with a peak height of 29.71%, Methyl Commate C with a peak height of 9.74 and Stigmasta-5, 22-Dien-3-ol- acetate (3beta) with a peak height of 9.77. Of the three, Cholest-5-en-

3-yl- chloridocarbonate was found to be the major constituent. Cholesterol based compounds are found to be a contributing factor for antioxidant property (Albuquerque *et al* 2018). Previous reports by Chandrawat *et al*. (2015) substantiated the occurrence of Methyl Commate C and Stigmasterol with antioxidant property. From this it can be concluded that these compounds could be a contributing factor in determining the antioxidant property of the endophytic bacteria.

***Conclusion:***

In the present investigation, antibacterial, antibiotic and antioxidant properties of the five endophytic bacterial strains isolated from *B. sensitivum* were carried out. The antibacterial assay exhibited that these strains show satisfactory inhibition to the growth of human pathogenic bacteria, thereby playing a significant role in disease prevention. Antibiotic sensitivity profile depicts these strains do not cause much harm to the human body; they can be easily destroyed by the antibiotics. The present study reveals the presence of many phytoconstituents from endophytic bacteria, which could be sources of raw materials for new natural medicines and bioactive products in near future (Strobel, 2003). The multifaceted interaction between the host and the endophytic bacteria resulted in good biological activities such as antibacterial, antibiotic, antioxidant etc making them more advantageous through the production of various pharmacologically beneficial phytoconstituents which can be sources of novel natural products that can be exploited for drug discovery for medical and pharmaceutical applications.

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