Isolation, Characterization and analysis the biological properties of Endophytic Bacteria isolated from Euphorbia hirta

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***Abstract*—Entophytic bacteria have been considered as an ultimate source for novel bioactive compounds to treat various human diseases, and they are found inside plant tissues. In recent times, plant microbial origin has gained most of the attention due to its application in pharmaceutical and industrial production, plant growth promotion, disease resistance, and bioremediation. The present investigation was determined to isolate and characterize the endophytic bacteria from the stems of *Euphorbia hirta* from the World's biodiversity hotspot region, Western Ghats, India. Three endophytic bacterial species were isolated from surface-sterilized stem samples and characterized based on morphological and biochemical characteristics. Characterization indicated that they were *Bacillus sp*. One bacterial isolate was selected among three bacterial isolates based on their growth and subjected to grow on rice water medium for secondary metabolite production. The bioactive compounds of the bacteria were extracted using acetone and butanol solvent by conventional methods and detected through FTIR and GC-MS analysis. The bacteria were optimized for physicochemical properties such as pH, temperature, NaCl concentration, and incubation time. The optimum pH for growth and secondary metabolite production was pH seven at 36º °C with 5% NaCl concentration and 48 hours of incubation time. The endophytic bacterial secondary metabolites from *Euphorbia hirta* could be a promising source for unique bioactive compounds with medicinal properties to combat diseases.**

***Index Terms*—Endophytic bacteria, *Euphorbia hirta*, NaCl, *Bacillus sp*, FTIR, Rice water.**

I. INTRODUCTION

Bioactive compounds from plant origin have been used in the pharmaceutical field as a lead molecule to synthesize novel drugs to treat various health issues (Mahmoud Rafieian-Kopaei, 2012). Medicinal plants show many properties such as antimicrobial, anticancer, anti-diabetic, immunomodulatory, and hepatoprotective effects. Endophytes are the microorganisms that live inside plants, especially in leaves, stems, roots, and intercellular and intracellular spaces without any damage from biotic and abiotic stress (Rhoden *et al.,* 2015). Endophytes have been regarded as mutualists with potential advantages to the plant to elevate plant growth and nutritional values (Uma Maheshwari & Saranya, 2018). The study of endophytic bacteria has provoked great interest due to important properties like producing auxins, nitrogen fixation, and protecting the host plants against plant pathogens (Vichare Smita & Vora Dipak, 2015). Microbial endophytes are a unique source of various metabolites, namely peptides and alkaloids, to improve their immunity against pathogenic microorganisms.

Euphorbia hirta, also called the asthma plant, belongs to the Euphorbiaceae family and is found worldwide. It has been characterized by white milky latex, which is more or less toxic. The plant produces many phytocompounds such as alkaloids, flavonoids, and terpenes. It is applied in traditional medicines and documented for its antibacterial, antifungal, anthelminthic, antipyretic, antioxidant, anticancer, anti-inflammatory, sedative, antifertility, antiasthmatic, and antimalarial properties (Mark *et al*.,2017). *E. hirta* treats gastrointestinal disorders such as diarrhea, dysentery, intestinal parasitosis, bronchial and respiratory diseases such as asthma, cough, bronchitis, hay fever, and conjunctivitis. The aqueous extract of *E. hirta* decreases the release of prostaglandins I_2, E2, and D_2. The aqueous extract

inhibits aflatoxin contamination in rice, wheat, maize, and mustard crops. Sunil Kumar *et al*. (2010 reported that the methanol leaf extract of *E. hirta* possesses antifungal and antibacterial activities. Secondary metabolites of plant endophytes are a unique source of various bioactive compounds widely employed in the pharmaceutical field (C. Arunachalam & P. Gayathri, 2010). India comes under the 16 megadiverse countries of the World, having 17,500 plants. Western Ghats of India are considered a biodiversity hotspot region that includes 4050 plants used to treat diseases and infections. Western Ghats Mountain regions run parallel to the western coast of the Indian peninsula, which includes the states of Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra, and Gujarat, and covers an area of around 160,000 km^2 and is considered a UNESCO World Heritage Site. More than 700 medicinal plants are found in the Western Ghats region and are endemic to this region (Webster *et al*.,2020). To explore endophytic strains for novel bioactive metabolites extraction, the present study was carried out to isolate the endophytic bacteria present in the stems of the medicinal plant, *Euphorbia hirta*, of the Western Ghats of India. The bacterium was grown at optimum environmental conditions, secondary metabolite was extracted with organic solvents, and analysed through Fourier Transform - Infrared spectroscopy and Gas chromatography-mass spectrometry (GC-MS).

II. MATERIALS AND METHODS

2.1 Sample collection and surface sterilisation (Abhini & Zuhara, 2018)

Healthy plants of *Euphorbia hirta* were collected from the Velliangiri mountain range of Western Ghats, Coimbatore, Tamil Nadu, at an altitude of 600 m above Mean Sea Level on September 25, 2021. They were carried in a sterile polypropylene bag and transported to the laboratory. The plant samples are processed within 48 hours of collection, and the healthy stems are thoroughly washed under running tap water and double-distilled water to remove dirt. The surface sterilization of the plant explant was carried out by immersing it in 70% ethanol for one minute, then dipping it in 2% sodium hypochlorite solution for 2 - 3 minutes. The sample was again immersed in sterile double-distilled water and then dried.

2.2 Isolation and Quantification of Endophytic Bacteria

After proper drying, the stems were cut into small segments of approximately 1 cm length to remove external tissues using a sterile scalpel and placed onto nutrient agar plates (HiMedia Laboratories, India), further incubated at 37ºC for 24 hours for the growth of endophytes under aseptic conditions, and surface-sterilized uncut stems were considered as control. The unique bacterial colonies around the stem were observed and subcultured to obtain pure culture, and the selected isolates were subcultured in nutrient agar slants. The purified endophytes were stored at 4°C for further analysis. All the isolates were identified through standard procedures described in Bergey's Manual of Systematic Bacteriology. The different morphological traits such as colony type, margin, elevation, colour, and the opacity of colony, Gram's staining, and specific biochemical tests such as carbohydrate fermentation test, indole test, citrate test, catalase test, oxidase, MR-VP, hydrogen sulphide production test, starch and lipid activity, motility test.

2.3 Effect of Physiological characteristics

To study the effect of physiological factors such as pH, temperature, NaCl concentration, and incubation temperature on a selected bacterial strain. The overnight culture of bacterial isolates was inoculated on nutrient broth having the pH in the range of pH 1, 3, 5, 7, and 9, and incubated for 48 hours at 37 °C. The growth of bacteria was measured at 610nm in an ELICO CL 223 Colorimeter, and values were noted. The temperature effect is studied by inoculating the bacterial isolate in nutrient broth and incubating it at varying temperatures, such as 6°C, 16°C, 36°C, and 46°C for 48 hours. The optical density was measured at 610 nm in a Colorimeter. The optimization of incubating time was analysed by inoculating the bacteria on nutrient broth media and incubating at 37°C. The values were taken between 24, 48, 72, and 96 hours, and the absorbance was recorded using a colorimeter at a wavelength of 610 nm. To analyse the effect of concentration of NaCl, the nutrient broth medium was supplemented with various concentrations of NaCl, and the endophytic bacterial isolates were inoculated and incubated at 37°C for 72 hours. Biomass was observed by measuring the optical density at 610 nm.

2.4 Production and extraction of metabolites (Swarnalatha *et al*., 2015)

The selected bacterial isolate was inoculated in 250 ml of nutrient broth in a 1 L Erlenmeyer flask and incubated for 24 hrs at 37°C. 2% of inoculum was added into a 1 L Erlenmeyer flask containing 250 ml of rice water medium and incubated at 36°C in a shaking incubator (180 rpm) for 5 days (Swarnalatha *et al.,* 2015). After 5 days, it was centrifuged at 8000 rpm for 5 minutes. After centrifugation, collect the supernatant with an equal volume of solvents like butanol and acetone. The supernatant and an equal volume of solvents were mixed for 60 to 120 minutes and kept at 1000 rpm. The solution was separated by using a funnel, and the endophytic extract was obtained.

2.5 Characterization of Bioactive compounds

The bioactive compounds of bacterial endophytes were subjected to Fourier Transform Infrared Spectroscopy (FTIR) analysis on Shimadzu 8400S spectrophotometer (Shimadzu Corporation, Japan) in the mid-IR region of 500 to 4000 cm-1. FTIR analysis was carried out by using the solvent extract and performed to identify the structural composition of the bioactive compound to know the different functional groups present in the extracts (K. E. Vivekanandan and Ajeesh, 2018). The solvent extracts were separated, and bioactive compound identification was analyzed using Gas chromatography-mass spectroscopy (GC-MS). This analysis was performed with an Agilent 7820A gas chromatograph equipped with a –DB-5 column (30 m × 0.25 mm; 0.25 µm film thickness), interfaced with an Agilent mass selective detector 5977E inter MSD. Oven temperature program was from 100 to 270°C at 10°C/min; helium was used as carrier gas, and the flow rate was 1.2 ml/min.

III. RESULTS AND DISCUSSION

3.1 Isolation and purification of endophytic bacteria

To identify the endophytic bacteria from *the Euphorbia hirta* plant, the samples were collected from the Western Ghats region, Tamil Nadu, and maintained in the laboratory. The present work claimed successful isolation of 3 bacterial isolates from stems of *Euphorbia hirta* and indicated them as AP1, AP2, and AP3. Surface sterilization was done to remove dirt from plant surfaces and isolate the endophytic origin. Visible colonies were identified on the plant's edges and isolated and processed. The bacterial isolates were subcultured to get the pure culture and stored at 4°C for future research. Various parts of indigenous plants, such as stems, leaves, and fruits, are widely used in pharmaceutical research and medicinal industries to treat diseases due to their therapeutic properties. Different plants of the Western Ghats of India have been reported to have several endophytic microbes with various agricultural, industrial, and medicinal applications (Webster *et al*.,2020).

3.2 Isolation and Quantification of Endophytic Bacteria

The three bacterial colonies were isolated and named AP1, AP2, and AP3, subjected to phenotypic studies such as colony morphology, Gram's staining, and biochemical Characterization studies, and identified as *Bacillus sp*. The results are tabulated in Table 1 and Fig. 1.

Fig 1: a) Purified culture of AP1 b) Gram staining

Table 1: Colony morphology and Gram stain of bacterial isolate

S.No	Characteristics	AP 1	AP2	AP3
1	Color	Pale White	Pale White	White creamy
2	Margin	Irregular	Irregular	Erose
3	Elevation	Raised	Convex	Flat
4	Opacity	Opaque	Opaque	Opaque
5	Size	Large	Small	Large
6	Consistency	Creamy	Rough	Rough, Wrinkled
7	Gram staining	Gram positive	Gram positive	Gram positive

Table 2 Biochemical properties of Endophytic bacteria

S.No	Name of Biochemical test	AP1	AP2	AP3
1	Indole	-	-	+
2	Methyl Red	+	+	+
3	Voges Proskauer	-	-	+
4	Citrate	+	-	+
5	Catalase	-	-	+
6	Oxidase	+	+	-
7	Starch Hydrolysis	+	+	-
8	Lipid Hydrolysis	+	+	-
10	Urease	-	-	-
11	CF-Sucrose	+	+	+
12	CF-Glucose	+	+	+
13	CF- Fructose	+	+	+
14	CF-Starch	+	+	+
15	CF- Maltose	+	+	+
16	CF-Lactose	-	-	-
17	CF- D Sorbitol	-	-	-

* +- Positive , – - Negative,
* CF-Carbohydrate Fermentation.

The bacterial isolates colonize in the inner tissues of plant tissues without damaging plants for a better adaptation as a mutualist. The endophytic bacterial isolate of the Western Ghats region of *Euphorbia hirta* could be an excellent source of novel bioactive lead compounds. As reported in Shivaji S, et al., 2006, the isolates were indicated as *Bacillus* through phylogenetic relationship.

3.3 Effect of Physiological characteristics

The pH, temperature, NaCl concentration, and incubation time are distinct parameters. Their effects on three different *Bacillus sp* AP1, AP2, and AP3 were observed by measuring the bacterial growth at 610nm in the ELICO CL 223 Colorimeter. The results are tabulated in Table 2 and Fig. 2. Fig. 2 indicates the effect of physiochemical properties on *Bacillus sp*, the x-axis indicates physical parameter variables, and the y-axis indicates OD values at 610 nm. The effect of pH on *Bacillus* survival *in situ* was examined in the range of 1,3,5, 7, and 9. The pH of the culture medium strongly influenced the growth of bacteria. pH 7 showed the optimum range of bacterial growth, as shown in Fig. 2a. The effect of temperature on bacterial isolate is explained in Fig. 2b, and 36 ºC was found to be the best temperature for bacterial growth, which is the best-suited temperature for bacterial growth and declined at higher and lower temperatures. Results of the effect of NaCl concentration on bacterial growth reveal that 5% NaCl concentration shows significant bacterial growth, and 1%,3% and 10% have no significant growth rate. The bacterial strain showed maximum growth at 48 hours, which is the best-suited incubation time for bacterial growth. Among three bacterial isolates, AP1 shows the maximum ideal growth compared to AP2 and AP3, and was further selected for secondary metabolite production.

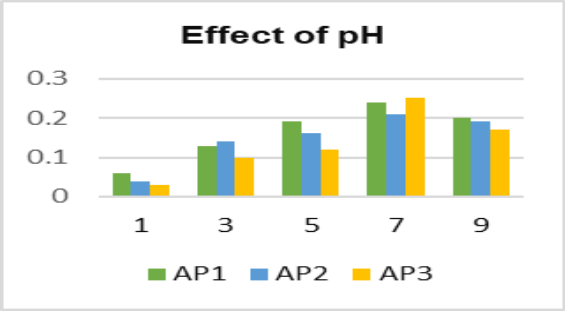

Fig 2a Effect of pH

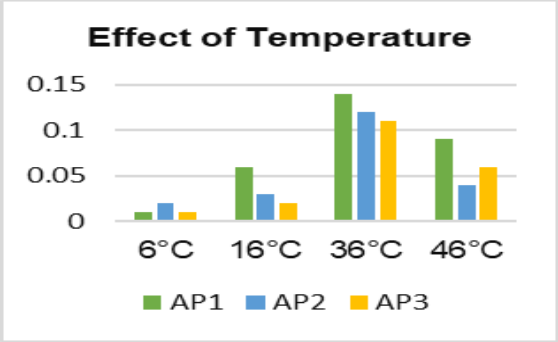

Fig 2b Effect of Temperature

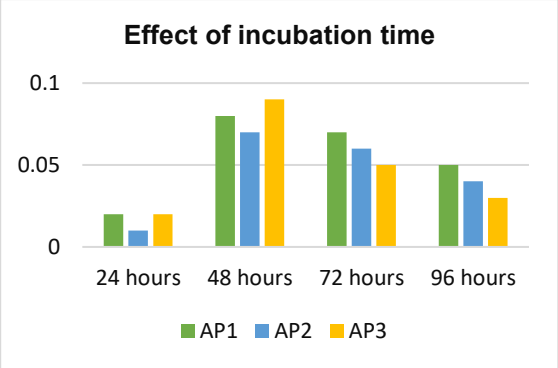

Fig 2c Effect of Incubation time

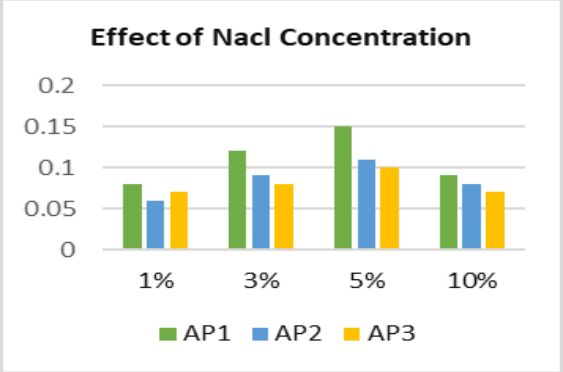

Fig 2d Effect of NaCl concentration

3.4 Production and extraction of secondary metabolites

The secondary metabolite production was performed by inoculating a selected bacterial strain in an Erlenmeyer flask containing 250 ml of nutrient broth and incubating for 24 hours at 37°C. The overnight bacterial culture was inoculated in a 1 L Erlenmeyer flask containing 250 mL of sterilized rice water culture medium and incubated at 36°C in a shaking incubator at 180 rpm speed for 5 days. Bacterial culture was centrifuged at 8000 rpm for 5 minutes to obtain

supernatant. An equal volume of solvents such as butanol and acetone was added and centrifuged at 1000 rpm for 60 to 120 minutes to separate the endophytic extract. The endophytic bacterial extract was screened to detect the presence of secondary metabolites by FTIR and GC–MS analysis. The rice water medium was more efficient in secondary metabolite production from endophytic bacteria.

3.5 Characterization of Bioactive Compounds

3.5.1 FTIR Analysis of Acetone Extract

The secondary metabolites of AP1 bacteria were extracted using solvents such as acetone and butanol. The bioactive compounds of secondary metabolites were analysed through FTIR analysis. The presence of a functional group was determined by FTIR spectrum in the range 300 – 4000 cm^{-1}. The FTIR spectrum of AP1 in acetone extract is shown in Fig. 3, and the results are given in Table 2.

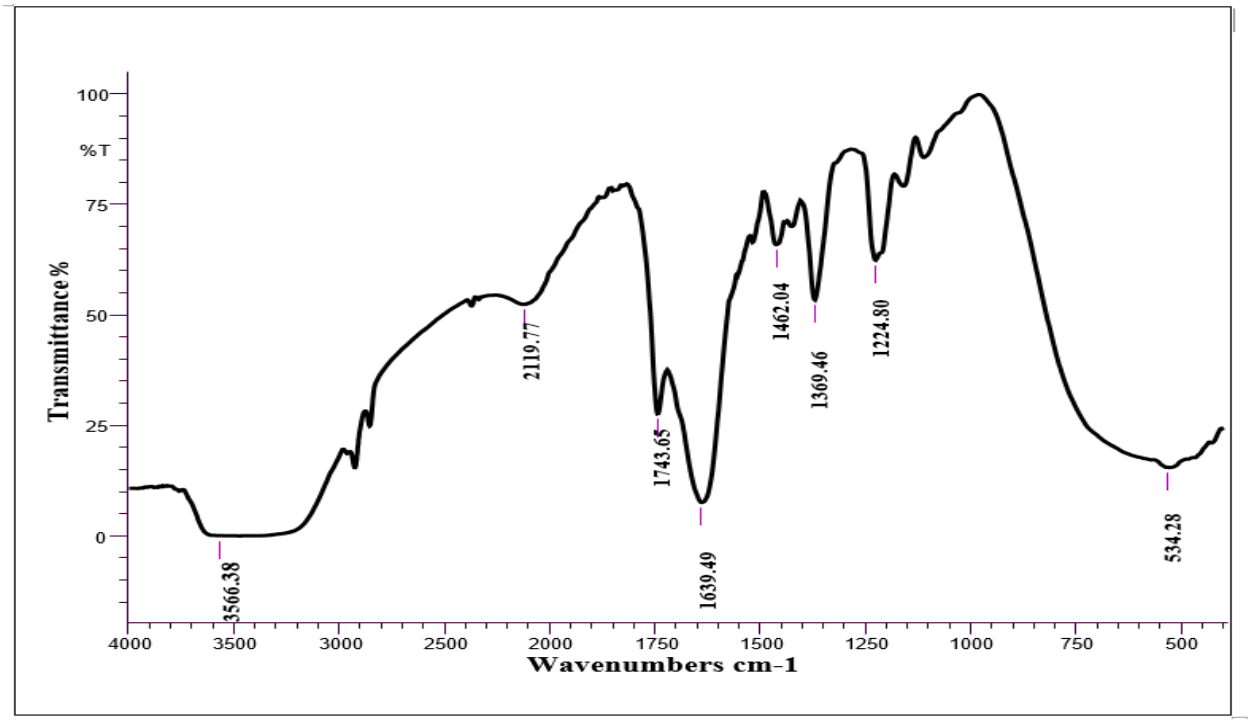

Fig 3a FTIR analysis of AP1 – acetone extract

Table 2: FTIR analysis of the acetone extract of *Bacillus*

S. No	Class of compounds	Absorption, cm^{-1}	Intensity	Type of bond
1.	Halo compounds	534.28	Strong	C-Br stretch
2.	Amine	1224.8	Medium	C-N stretch
3.	Alcohol	1369.46	Medium	O-H bend
4.	Alkane	1462.04	Medium	C-H bend
5.	Alkene	1639.49	Strong	C=C stretch
6.	Ketone	1743.65	Short	C=O stretch
7.	Alkyne	2119.77	Weak	C≡C stretch
8.	Alcohols	3566.38	Medium, broad	O-H stretch

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The IR result of the acetone extracts of the bacterial isolate showed a broad-spectrum range at 3556 cm^{-1}indicating a carboxylic group. The band at 2119 cm^{-1} indicates alkyne, 1743.65 cm^{-1} indicates ketone group, 1639.49 cm-1 indicates alkene group, 1463 cm^{-1} and 1462 cm^{-1} refer to alkane group. The band at 1369.36 cm^{-1} denotes the presence of alcohol (O – H) bend, 1224 cm^{-1} shows amine group, and 534 cm^{-1} indicates halo compounds. The results are similarly reported in (Lal M, 2016).

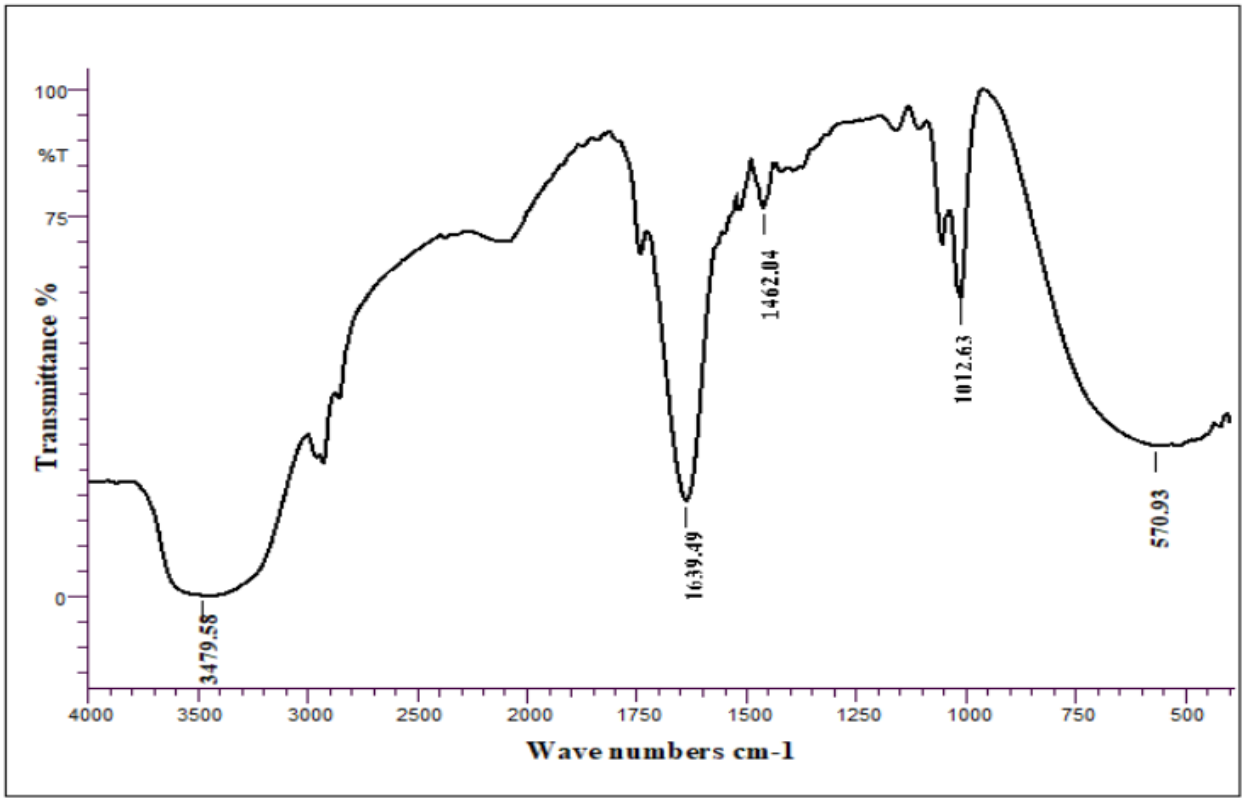
3.5.2 Butanol extract

Fig 3: FTIR analysis of AP1 – Butanol extract

The presence of a functional group was determined by FTIR spectrum in the range 300 – 4000 cm^{-1}. The FTIR spectrum of AP1 in butanol extract is shown in Figure 3. The spectrum results are given in Table 3.

Table 3: FTIR analysis of the butanol extract of *Bacillus*

S. No	Class of compounds	Absorption, cm^{-1}	Intensity	Type of bond
1.	Halo compounds	570.93	Strong	C-Br stretch
2.	Fluro compounds	1012.63	Strong	C-F stretch
3.	Alkanes	1462.04	Medium	C-H bend
4.	Alkene	1639.49	Strong	C=C stretch
5.	Alcohol	3479.58	Strong	O-H stretch



The IR result of the butanol extracts of the bacterial isolate AP1 showed that a broad-spectrum range stretching at 3479.58 cm^{-1} indicates a carboxylic group. The band at 1639 cm^{-1} indicates an alkene group, and 1462 cm^{-1} refers to an alkane group. The band at 1012.63 cm^{-1} denotes fluoro compounds, and the band at 570.93 cm^{-1}indicates the presence of halo compounds.

3.5.b GC – MS analysis of Bacterial analysis.

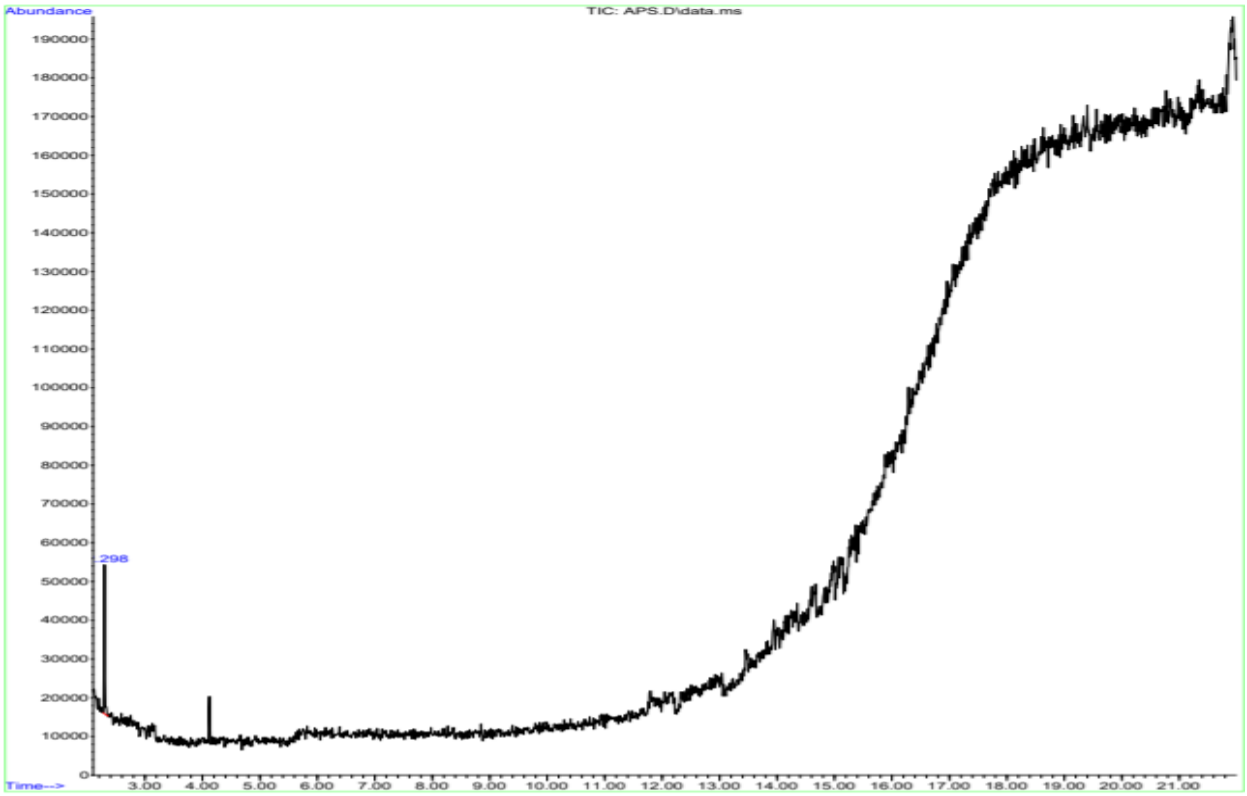


Fig 4: GC – MS analysis of AP1 – acetone extract

The acetone extract of *Bacillus sp* was analysed using gas chromatography-mass spectrometry analysis. The compound was identified based on peak area and mass by charged ions. The primary secondary metabolites in the acetone extract of AP1 were 2, 2-Bis (butoxy) propionic acid and butyl ester. The retention time of the metabolite was 2.295 min, and the % area showed 100%.

IV. CONCLUSION

Plant microbial origin has gained tremendous attention due to its potent, unexplored bioactive compounds having pharmaceutical applications. The current work was intended to isolate the endophytic bacteria from stems of *Euphorbia hirta.* The isolation and identification of bacterial endophytes were determined by morphological and biochemical characterization, and the bacteria were identified as *Bacillus sp.* The effect of various physiochemical characters, namely pH, temperature, NaCl concentration, and incubation time, on the growth of *Bacillus sp* was studied. The secondary metabolites were produced in a rice water medium and extracted using acetone and butanol. The bioactive compounds were identified by FTIR analysis and GC-MS analysis; however, further work is required to explore other pharmaceutical applications for future medical applications. The results of the current work suggest that *the Euphorbia hirta* endophyte could be a novel potential lead for industrial needs and the medicinal field.

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