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A study on bio-diversity and antiplasmodial activity of rhizosphere soil samples from medicinal plants in Kolli Hills

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Abstract

Background Over the previous two decades, *Plasmodium falciparum* strains have become increasingly resistant to several medications. As a result, there is an urgent need to develop new therapeutic options. Taking this into account, we focused our research on screening microbial extracts from rhizosphere soil samples in specific regions, which increases the likelihood of discovering bacteria capable of producing antiplasmodial activity.

Results In the current study, we aimed to isolate thirty-two different medicinal plant rhizosphere soil samples collected from Kolli Hills (January–December 2016). Isolation was performed on nutrient and starch casein agar medium by serial dilutions, and distinct colonies were chosen from each dilution. A total of two seventy-five bacterial isolates were isolated from the research plants and kept as pure cultures on nutrient agar. In which, maximum count of fourteen Gram-positive spore forming bacilli strains have been identified and further evaluated for morphological, cultural, and biochemical traits and significantly identified as *Bacillus* species. Further, promising anti-plasmodial action was demonstrated by *B. megaterium* bacterial extracts, with IC_{50} values of 24.65 µg/mL at 24 h and 7.82 µg/mL at 48 h. *Bacillus mycoides* showed good antiplasmodial activity with (IC_{50} *P. falciparum* 3D7: 23.52 µg/mL at 24 h and 22.88 µg/mL at 48 h, *Bacillus flexus* showed IC_{50} of 18.36 and 6.24 µg/mL and moderate antiplasmodial activity observed in *Bacillus tequilensis*. Poor antiplasmodial activity was found in *Bacillus subtillis*, *Bacillus macerans, Bacillus pumilus* and *Bacillus larvey*. Interestingly, 16S rRNA sequencing results confirmed that our bacterial species was *Bacillus megaterium* with 99% similarity observed with the accession number KX495303.1. Additionally, GC–MS analysis revealed effective anti-plasmodial bioactive compounds.

Conclusions These findings show the potential of *B. megaterium* from *Achyranthes aspera* as a antiplasmodial agent. However, more research is needed to fully understand the bioactive compound of these strains and further studies are necessary to explore drug formulation and toxicity levels in the future.

Keywords Diversity, Rhizosphere, *Bacillus*, 16S rRNA sequencing

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Background

Malaria remains a serious public health concern, impacting a considerable proportion of the population. It is transmitted in eighty five countries spread across five WHO regions [1]. The great majority of incidents and fatalities have disproportionately affected young children and pregnant women. Thus, prevention and treatment of this condition are more expensive and also it is critical to have effective malaria medications [2]. However, *Plasmodium falciparum* has developed resistance to a variety of antimalarial medications, including artemisinin derivatives. Therefore, development of these resistance phenomena significantly hinders the effectiveness of present global operations to lessen the burden of malaria, and therefore motivates ongoing efforts to discover novel compounds [3].

Considering this, biotechnological research targets microbial diversity to discover novel compounds. The relationship between plants and microbes was widely studied and found to have effective medicinal properties due to their spatial distribution, ecosystems, and climatic changes globally [4]. Among the microbial inhabitants, soil bacteria are most prevalent in the rhizosphere in terms of diversity and community. However, their specificity and complexity are still unknown [5, 6]. Next to bacteria, the rhizosphere may also be overwhelming to numerous microorganisms like fungi, algae, archaea, and protozoa by influencing the plant roots that surround the soil [7, 8]. It facilitates plant growth through nutrient absorption and protects host plants by inducing systemic resistance to deadly pathogens [9]. In recent decades, various bacterial species have been isolated from the rhizosphere soil, namely *Klebsiella, Pseudomonas, Alcaligens*, *Serratia, Bacillus, Azospirillum, Enterobacter*, and *Clostridrium* etc., [10–12]. Between the microbes, rhizosphere soil bacteria, especially *Bacillus* species, were extensively studied as compared to other bacterial communities [13]. In spite of, Gram-negative and Gram-positive bacteria naturally support plant growth and are bio-controllers, and they belong to the genera *Bacillus* [14, 15]. This *Bacillus* bacterium interacts with the rhizosphere of plant roots, produces complex substances that are neutral, beneficial, and promotes novel antibiotics [16, 17].

The study aimed to enumerate, distinguish, and group the diverse bacterial species present in the rhizosphere of soil samples gathered from 32 different medicinal plants located in the Kolli Hills. This discovery is consistent with studies on the evolution of traditional pharmacopoeia to identify novel compounds to reinforce the therapeutic range against malaria. From the perspective of their antimalarial effect, these rhizosphere soil bacteria have not received much research, especially on field isolates. Therefore, the main goal of this research was to assess the diversity and in-vitro antiplasmodial activity.

Methods

Collection of rhizosphere soil sample from various medicinal plants

The rhizosphere soil samples were collected from thirty-two medicinal plants from January 2016 to December 2016 in Kolli Hills, Tamil Nadu and India. After collection, by uprooting the plant, the soil samples from the root were kept in a sterile, labelled, airtight zip-lock cover and refrigerated at 4 °C for further analysis [18]. Plant specimens were identified according to the herbarium guidelines [19].

Isolation of bacteria from rhizosphere soil sample

In order to examine the rhizosphere soil from each root sample, it was collected and transferred into individual, sterile Petri dishes. Furthermore, 10 g of rhizosphere soil from each plant was incorporated into individual conical flasks containing 100 mL of triple sterile water. These flasks were kept under a shaker heated at 80 °C for 10 min in order to eliminate vegetative cells. The supernatant was then diluted in sequence from 10^{-2} to 10^{-6} and transferred 10^{-5}, 0.1 mL of the diluted sample was placed in triplicate using Nutrient Agar and Soybean Casein Digest Medium agar by pour plate method supplemented with 100 g/mL of Terbinafine fungicide and further incubated for 24–48 h at 37 °C ± 2 °C [20, 21]. Finally, bacterial cultures were prepared using a nutrient agar slant, and stored in a glycerol stock medium refrigerated at 4 °C.

Morphological characterization of isolated bacteria

The morphology of each bacterial isolate was analysed under a microscope after incubation for 24–48 h. The colour, appearance, transparency, shape, and diameter of each isolate were evaluated. In this study, Gram's Method was used to identify Gram-positive and Gram-negative bacteria [22].

Biochemical screening of bacteria

The biochemical characterization of bacterial isolates was evaluated based on their chemical nature. We have studied glucose fermentation, lactose fermentation, sucrose fermentation, fructose fermentation, indole production test, methyl red test, Voges–Proskauer test, Simmons citrate agar test, urease utilization test, H_2S production test, starch hydrolysis, oxidase test, catalase test, ornithine decarboxylase test, 6.5% NaCl growth, nitrate reduction, mannitol salt agar test, and amylase production test according to the standard protocols [23].

Motility test

The hanging drop method was used to measure the motility of a bacterial culture. To begin, a consistent layer of Vaseline was placed on the borders of a cover slip. Then, in the centre of the cover slip, a little drop of the bacterial culture was inserted. After that, inverted cavity was placed over the cover slip. The slide was flipped over, and the dangling drop was examined for motility with a high-powered objective. This test determines whether the bacterial isolates are motile or non-motile. Motile bacteria will leave the drop, while non-motile bacteria will remain inside [24].

Extraction of genomic DNA from isolated bacteria

To begin the process, bacterial cells were grown in monolayers of 1–3 colonies suspended in 450 µL of "B Cube" lysis buffer. The cells were then lysed through repeated pipetting. After lysing, neutralisation buffer (4 µL of RNAse A and 250 µL of "B Cube") was added and vortexed. The mixture was incubated for 30 min in a water bath at 65 °C. It was then subjected to centrifugation at 14,000 rpm at 10 °C for 20 min, and the supernatant was collected in a fresh 2 mL microcentrifuge tube. Afterwards, 600 µL of "B Cube" binding buffer was added and incubated for 5 min. The mixture was then centrifuged at 14,000 rpm for 2 min. Next, 500 µL of washing buffer I was added to the spin column, followed by washing buffer II. Finally, 100 µL of elution buffer was added, and the tubes were incubated for 5 min and centrifuged at 6000 rpm for 1 min [25]. The resulting buffer contains the DNA, which was measured for concentration through a 1% agarose gel. The DNA samples were then stored at − 20 °C for future use.

16S rRNA molecular identification by amplification and sequencing

To amplify the 16S rRNA, we used a universal forward primer 5′-AGA GTT TGA TCM TGG CTC AG-3′ and a reverse primer 5′-TAC GGY TAC CTT GTT ACG ACT-T 3′. The amplification process began with denaturing at 94 °C for 3 min. For each cycle, we found that 30 s was the ideal time for denaturation at 30 °C. The annealing temperature was set at 60 °C for 30 s, and the extension temperature was set at 72 °C for 1 min. The final extension was done for 10 min at 72 °C. We purified the PCR product using the Montage PCR Clean-up kit and sequenced it using the same primer 27F/1492R. The reactions were done using the ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The amplified sample ran in an ABI 3730×l sequencer (Applied Biosystems). To align the sequenced 16S rRNA, we used BLAST for similar matches with accessible reference sequences. Further it was aligned using the MUSCLE 3.7 program and Gblocks 0.91b for multiple sequence alignment. Lastly, we carried out phylogenetic analysis using PhyML 3.0 aLRT and HKY85 as substitution models [26–28].

Mass culture of rhizosphere soil bacteria

Nearly fourteen fresh rhizosphere bacterial isolates were inoculated in 500 mL of nutrient broth at 37 °C ± 2 °C for 24 h with constant shaking. Later, 50 mL of culture broth was subjected to freshly prepared nutrient broth. It was then incubated at 37 °C ± 2 °C and kept in a shaker for 48 to 72 h continuously [29].

Extraction of bioactive metabolites

After culturing the mass, the fourteen isolates underwent treatment with 1N HCl and 1N NaOH, with a pH of 5.0. Then they were filtered. A separate funnel was used to combine equal volumes of filtered broth and ethyl acetate in a ratio of 1:1. The mixture was shaken continuously for 30 min. After this, the upper organic layer was collected and concentrated at 40 °C using a rotary vacuum evaporator to obtain the crude extract. This process was repeated three times until all bioactive compounds had been extracted [30–32].

GC–MS analysis

We performed a comprehensive analysis of bacterial extracts to identify a variety of bioactive compounds using GC–MS. To conduct this analysis, we utilised a state-of-the-art Perkin-Elmer GC Clarus 500 system equipped with an AOC-20i auto-sampler and Gas Chromatograph interfaced to a Mass Spectrometer. Our system also featured an Elite-5MS fused capillary column, which measured 30×0.25 µM ID×0.25 µM df. The GC–MS detector utilized an electron ionization system with ionisation energy of 70 eV and carried Helium gas (99.999%) with a constant flow rate of 1 mL/min [33, 34]. Our injection process involved 2 µL of sample with a split ratio of 10:1, and we used a Turbo-Mass Gold-Perkin-Elmer-mass detector. The injector, ion source, and oven were all kept at consistent temperatures of 250 °C, 200 °C, and 110 °C for 2 min, respectively. We then increased the temperature by 10 °C/min to 200 °C, followed by 5 °C/min to 280 °C, and finally ended with 9 min of isothermal at 280 °C. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and fragments from 45 to 450 Da. Our solvent delay was 0 to 2 min, and the total GC–MS

running time was between 3.00 and 45.00 min. We calculated the percentage of relative components by comparing its average peak area to the total areas, utilizing Turbo-Mass ver-5.2 software to handle mass spectra and chromatograms [35].

Cultivation of parasites

The isolated bacterial extracts were analyzed for antiplasmodial activity by *Plasmodium falciparum* (3D7) obtained from the National Institute for Malaria Research (NIMR), New Delhi. Then, it was cultivated in human "O" Rh positive Red blood cells from 1×10^3 to 8×10^4 parasites per µL. To create a blood-medium mixture (BMM), RPMI 1640 liquid medium was mixed with the blood sample in a 1:9 ratio. Specifically, 1 mL of BMM was generated for every 100 µL of the blood sample and the hematocrit was adjusted at 5% for parasite cultures [36, 37].

In vitro antiplasmodial assay

Fourteen crude extracts were obtained and added to 96 well culture plates containing 200 µL of *Plasmodium falciparum* along with fresh red blood cells. Additionally, 2% of parasitized *P. falciparum* diluted into 2% haematocrit, parasitized blood cells culture were treated with Chloroquine drug and maintained with positive and negative controls using various concentrations of 1.56, 3.12, 6.125, 12.5, 25, 50, and 100 µg/mL of extracts. After 24–48 h, parasitemia was evaluated by making a blood smear using Giemsa staining and observing under a microscope by counting the stage-wise growth of *P. falciparum* (3D7) [38, 39]. The average percentage of parasitemia suppression and parasites was calculated using the formula,

$$\text{Average \% suppression of parasitemia} = \frac{\text{Average \% of PC} - \text{Average \% of PT}}{\text{Average \% of parasitemia in control}} \times 100$$

(PC-parasitemia in control; PT-parasitemia in test).

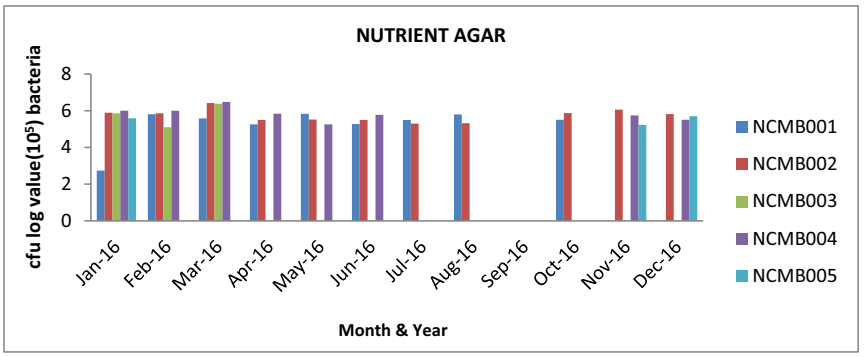
Antiplasmodial activity calculation and statistical analysis

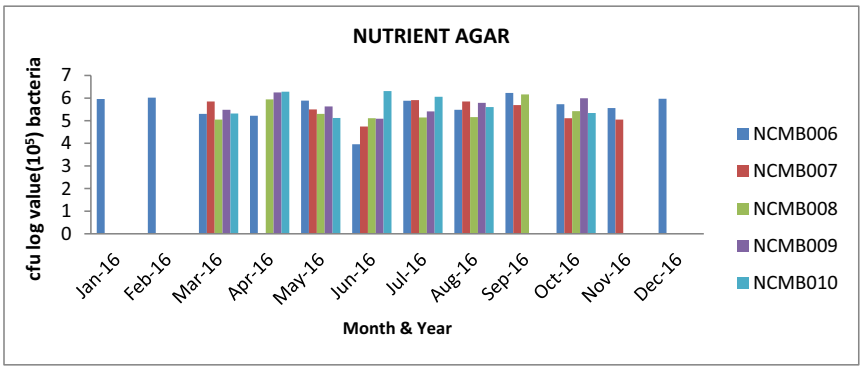
To measure the ability of bacterial extracts to combat malaria, the inhibitory concentration (IC_{50}) of the drug was calculated. This value represents the concentration of the extract required to reduce parasitemia by 50% compared to the control which had 100% parasitemia. The AAT Bioquest online tool (aatbio.com/tools/IC_{50}-calculator) was used to calculate the IC_{50} values, which were plotted on a graph with concentration on the x-axis and inhibition percentage on the *y* axis using a linear regression equation [40, 41].

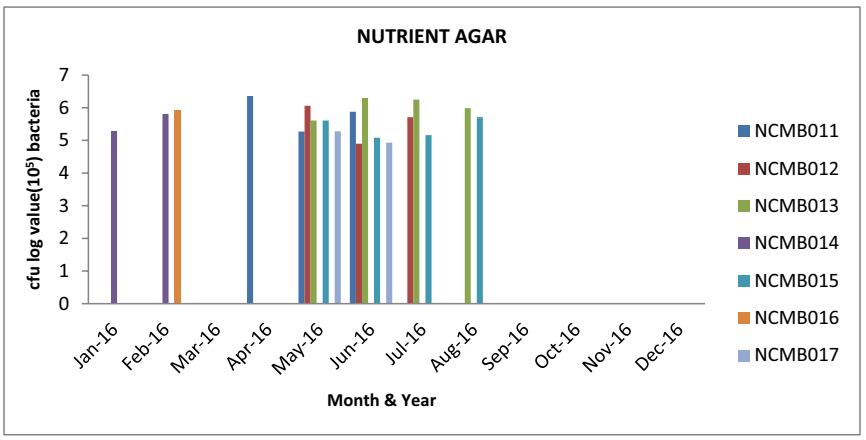
Results

Isolation of bacteria from rhizosphere soil sample

In 2016, a study was conducted in Kolli Hills to isolate bacteria from the soil of 32 different medicinal plants. A total of 275 bacterial strains were identified from plants including *Achyranthes aspera* (NCMB001), *Mimosa pudica* (NCMB002), *Hemidesmus indicus* (NCMB003), *Centella asiatica* (NCMB004), *Acalypha indica* (NCMB005), *Stachytarpheta indica* (NCMB006), *Curcuma aeruginosa* (NCMB007), *Malaxis versicolor* (NCMB008), *Zingiber officinale* (NCMB009), *Leucas aspera* (NCMB010), *Euphorbia hirta* (NCMB011), *Curculigo orchioide* (NCMB012), *Asparagus racemosus* (NCMB013), *Cardiospermum helicacabum* (NCMB014), *Arisaema leschenaultia* (NCMB015), *Sida rhombifolia* (NCMB016), *Asclepias curassavica* (NCMB017), *Lindernia oppositifolia* (NCMB018), *Iphigenia indica* (NCMB019), *Alpinia calcarata* (NCMB020), *Commelinaceae species* (NCMB021), *Solanum nigrum* (NCMB022), *Cheilanthes tenuifolia* (NCMB023), *Hemionitis arifolia* (NCMB024), *Borassus species* (NCMB025), *Alpinia galangal* (NCMB026), *Dioscorea alata* (NCMB027), *Eletteria cardamomum* (NCMB028), *Solanum torvum* (NCMB029), *Scilla hyacinthine* (NCMB030), *Ornithogalum umbellatum* (NCMB031), and *Sansevieria roxburghiana* (NCMB032) during the year (January-December). Throughout the period of January to March 2016, we observed a significant increase in bacterial colony growth at 10^5 dilutions on Nutrient and Starch casein agar, especially in NCMB001, NCMB002, NCMB003, and NCMB004. However, it must be emphasized that by September 2016, there were absolutely no bacterial isolates present. Therefore, bacterial colonies were found in the rhizosphere soil of *Acalypha indica* only in January, November, and December. On the other hand, the bacterial isolates of NCMB006 grew throughout most of the year, with the exception of January, February, and December. The highest number of isolates at 10^5 concentrations was observed in NCMB007-NCMB010. The lowest bacterial colonies were found in the soil sample of NCMB016. Additionally, the rhizosphere soil samples of NCMB011, NCMB012, NCMB013, NCMB014, NCMB015, NCMB016, and NCMB017 did not have any bacterial colonies between March and September to December. Some rhizosphere soil samples, such as (NCMB025–NCMB030), had the most colonies from







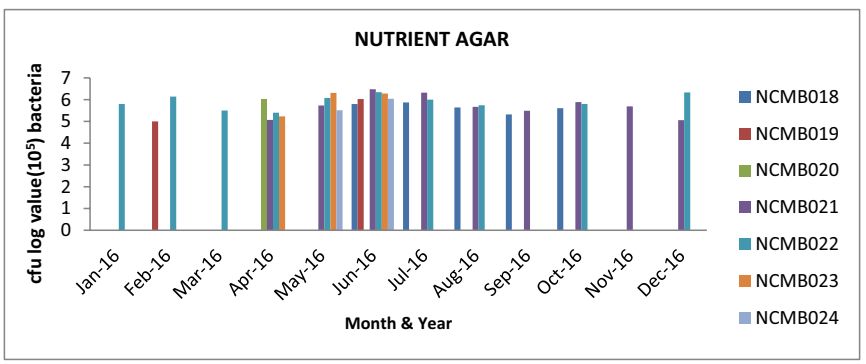
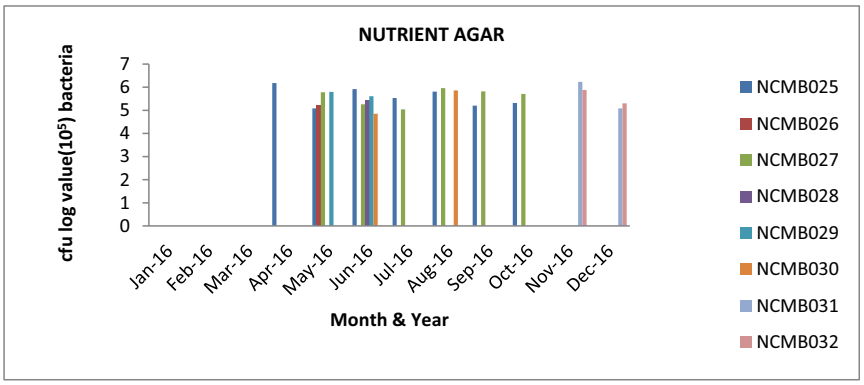
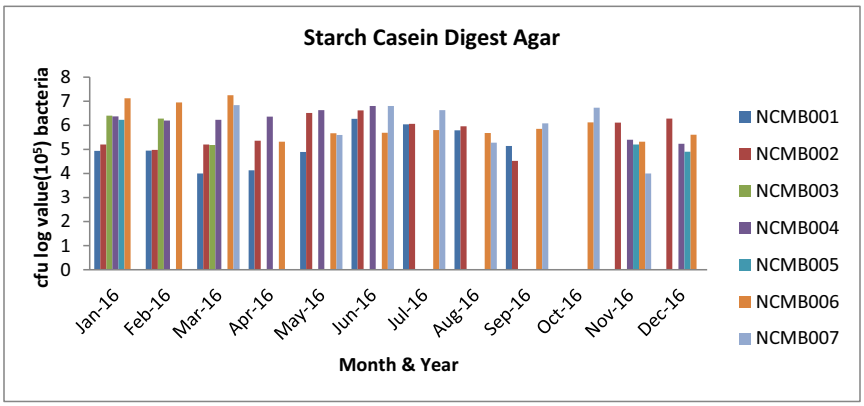
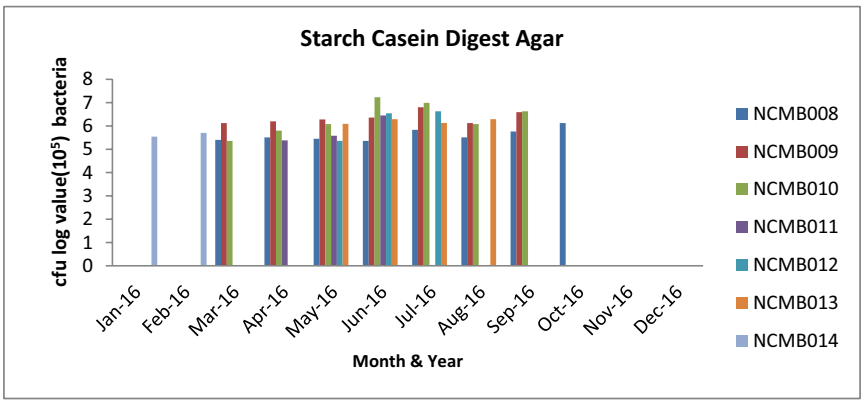


Fig. 1 Bacterial isolates from rhizosphere soil sample on Nutrient and Starch Casein agar







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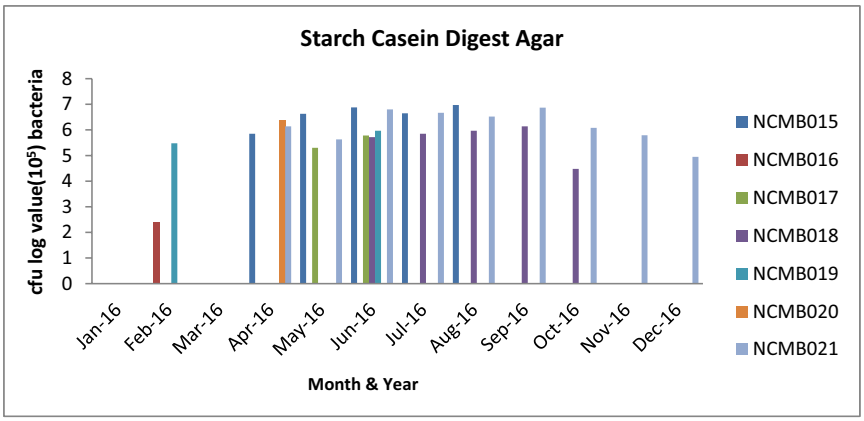
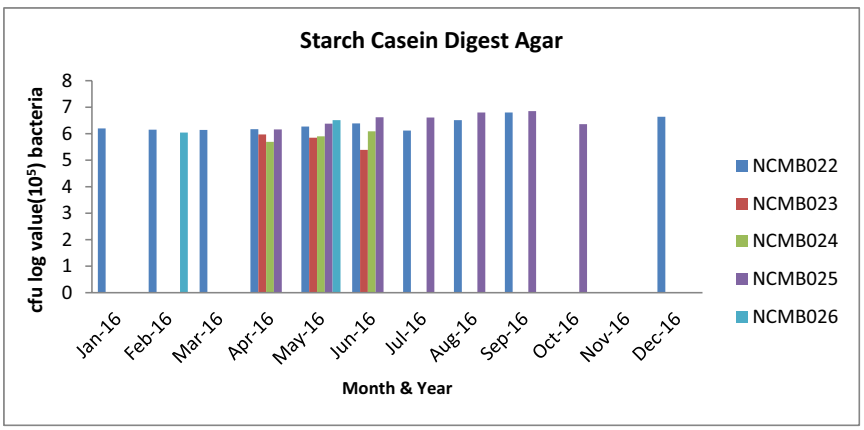




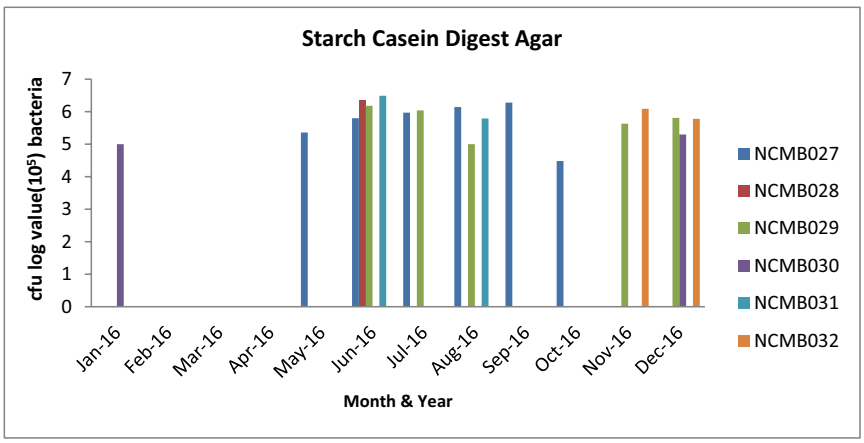


Fig. 1 continued

May to June and no bacterial isolates in January to February (Fig. 1).

Morphological/biochemical identification of bacterial isolates

To determine the full characteristics of the bacterial isolates, we conducted a conventional examination of their colony morphology using the agar plate method and microscopic examination. We identified various shapes of the colony, such as irregular, regular, circular, filamentous, and surface texture with edges and elevation. This allowed for a thorough and accurate analysis of the bacterial specimens. Additionally, Gram staining techniques were utilized to determine the properties of bacterial cell walls. Based on these distinctive features, approximately 14 bacterial strains, designated as BS01-*Bacillus*

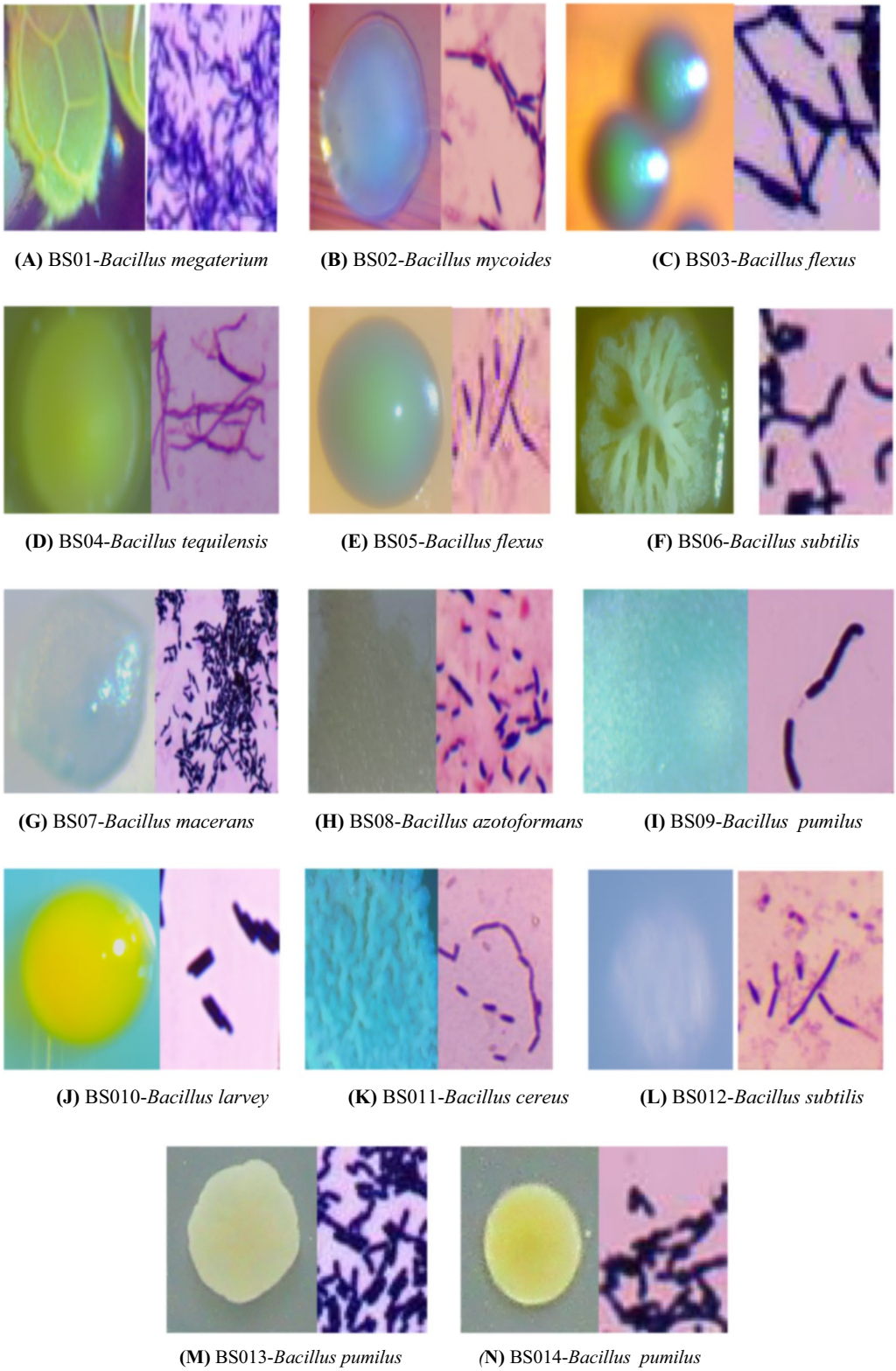


Fig. 2 Morphological characterization of isolated bacteria/Gram staining properties of the selected bacterial isolates

Table 1 Colony morphology of bacterial isolates

Strain No	Name of the isolates from medicinal plants	Shape of colony	Elevation/Margin
BS01	*Achyranthes aspera*	Irregular	Raised/undulate
BS02	*Mimosa pudica*	Irregular	Raised/entire
BS03	*Hemidesmu indicus*	Regular	Convex/entire
BS04	*Stachytarpheta indica*	Circular	Flat/entire
BS05	*Stachytarpheta indica*	Irregular	Raised/entire
BS06	*Curcuma aeruginosa*	Filamentous	Umbonate/undulate
BS07	*Malaxis versicolor*,	Circular (transparent)	Umbonate/entire
BS08	*Asclepias curassavica*	Irregular	Flat/ filliform
BS09	*Lindernia oppositifolia*	Irregular	Raised/undulate
BS010	*Iphigenia indica*	Circular	Convex/entire
BS011	*Alpinia calcarata*	Filamentous	Flat/filliform
BS012	*Cheilanthes tenuifolia*	Circular	umbonate/entire
BS013	*Hemionitis arifolia*	Circular	Convex/Entire
BS014	*Solanacea tarvum*	Circular	Umbonate/entire

Table 2 Identification of bacterial isolates using various biochemical tests

Biochemical tests	BS01	BS02	BS03	BS04	BS05	BS06	BS07	BS08	BS09	BS010	BS011	BS012	BS013	BS014
Oxidase	+	+	+	+	+	+	−	+	+	+	+	+	−	−
Catalase	−	−	−	+	−	+	+	−	−	−	+	+	−	−
Indole	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Methyl red	+	+	+	+	+	−	+	−	−	−	−	−	−	−
Voges Proskauer	−	−	+	−	−	+	−	−	+	+	+	+	+	+
Simmon Citrate agar	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Starch hydrolysis	+	−	+	+	+	+	+	−	−	−	+	+	−	−
Urease utilization	+	+	+	−	+	−	−	−	−	+	−	−	−	−
Glucose fermentation	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Lactose fermentation	−	−	+	−	+	−	−	−	−	−	−	−	−	−
Sucrose fermentation	−	−	+	+	+	+	−	−	+	+	+	+	+	+
Fructose fermentation	−	−	+	+	+	+	+	+	+	+	+	+	+	+
H_2S Production	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6.5% NaCl growth	−	−	+	+	−	+	−	−	−	−	−	+	−	−
Nitrate reduction	−	+	−	+	−	−	−	−	−	−	−	−	−	−
Mannitol salt agar	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Amylase production	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Motility test	MR	NR	MR	MR	MR	MR	MR	MR	MR	MR	MR	MR	MR	MR

+ indicating positive; − indicating negative, *MR* motile rods, *NR* non-motile rods

megaterium, were identified in a rhizosphere soil sample taken from the *Achyranthes aspera* plant, BS02-*Bacillus mycoides* from *Mimosa pudica*, BS03-*Bacillus flexus* (*Hemidesmu indicus)*, BS04-*Bacillus tequilensis* (*Stachytarpheta indica),* BS05- *Bacillus flexus* (*Stachytarpheta indica),* BS06-*Bacillus subtilis* (*Curcuma aeruginosa),* BS07-*Bacillus macerans* (*Malaxis versicolor)*, BS08-*Bacillus pumilus* (*Asclepias curassavica),*BS09-*Bacillus pumilus* (*Lindernia oppositifolia)*, BS010-*Bacillus larvey* (*Iphigenia indica),* BS011- *Bacillus cereus* (*Alpinia calcarata),* BS012-*Bacillus subtilis* (*Cheilanthes tenuifolia),* BS013- *Bacillus pumilus* (*Hemionitis arifolia*) and BS014- *Bacillus pumilus* from *Solanacea tarvum* respectively (Fig. 2). According to the findings presented in Table 1, it has been discovered that all fourteen bacterial isolates can be classified as Gram-positive spore forming bacilli. In order to confirm their identity, these isolated strains have been subjected to a variety

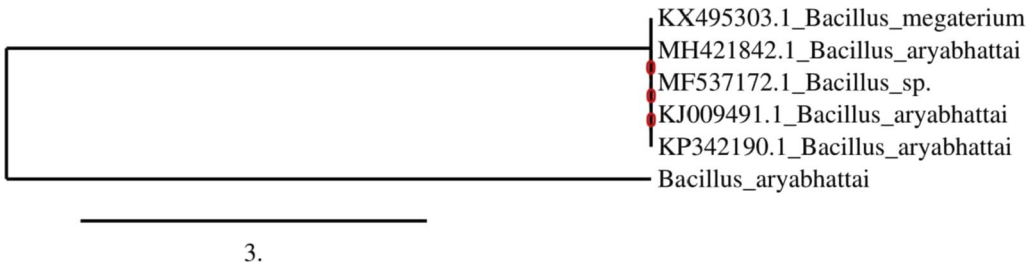


Fig. 3 Phylogenetic tree of 16S rRNA sequence of *Bacillus megaterium* and the scale bar representing the evolutionary distance

of biochemical parameters, except *Bacillus mycoides* all other 13 bacterial species found to be motile as outlined in Table 2. The results of this identification process have conclusively determined that all 14 strains belong to the *Bacillus* species.

Molecular identification of *Bacillus megaterium*

Upon conducting a comprehensive analysis of the bacterial isolates of the 16S rRNA sequence BS01, utilizing the BLAST tool available on the National Centre for Biotechnology Information (NCBI), it has become apparent that these isolates have been accurately identified as *Bacillus megaterium*. The findings of this investigation have revealed an impressive 99% similarity to the Gene bank sequence database with the accession number KX495303.1. In order to ensure that these sequences can be accessed by all individuals, we have taken the initiative to deposit the corresponding sequences in GenBank, which have been assigned the accession numbers MT937315.1 (https://www.ncbi.nlm.nih.gov/nuccore/1897301275?log$=activity). To further analyze the details of the sequence, we have employed the MEGA7 software and the neighbor joining method to generate a phylogenetic tree, which has been presented in Fig. 3. This process has enabled us to gain a more comprehensive understanding of the evolutionary relationships among the different organisms, based on the sequence data obtained.

GC–MS analysis

Through the utilization of GC–MS analysis, we have successfully determined the presence of bioactive compounds in *B. megaterium* extracts. These compounds were identified through the meticulous analysis of their retention time, molecular weight, and formula, utilizing

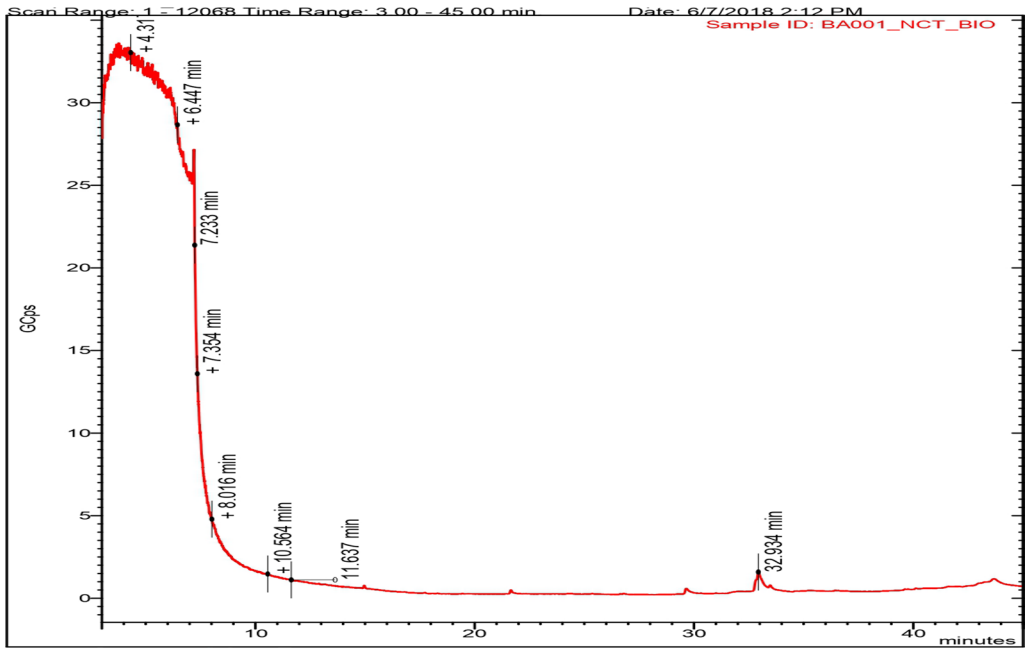


Fig. 4 GC–MS analysis of bacterial extract *B. megaterium* extract

RT chromatogram peaks and NIST libraries. It is important to note that nearly 8 compounds have been identified Cyclopentaneundecanoic acid, methyl ester at RT 4.31, Hexadecanoic acid, 15-methyl-, methyl ester at RT 6.447 and 2-Methyl-Z, Z-3,13-octadecadienol at RT 7.233, Dimethylsulfoxonium formylmethylide at RT 8.016, Dimethylfluoroamine at RT 10.564, Ethane, 1-chloro-2-nitro- at RT 11.637, Propane, 2-chloro- at RT 32.934, and Heptadecane, 2,6-dimethyl at RT 7.354, as depicted in Fig. 4.

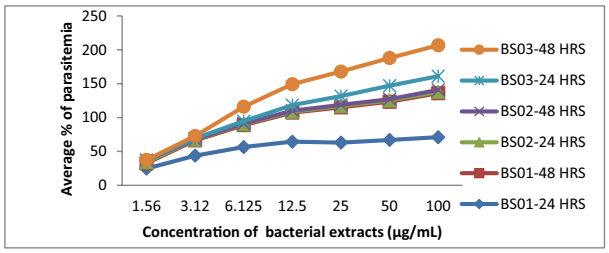
In vitro antiplasmodial assay

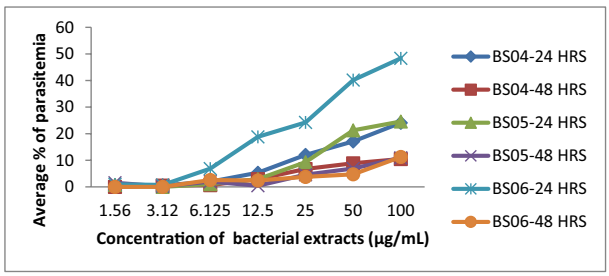
Out of the fourteen bacterial extracts tested for antiplasmodial activity against *P. falciparum*, the extracts of *Bacillus mycoides* displayed good antiplasmodial activity. The IC_{50} values for *P. falciparum* $3D_7$ were 23.52 µg/mL at 24 h and 22.88 µg/mL at 48 h. *Bacillus flexus* also showed moderate antiplasmodial activity with IC_{50} values of 18.36 and 6.24 µg/mL at respective hours. However, *Bacillus tequilensis* showed only moderate antiplasmodial activity with IC_{50} values of 39.48 and 20.06 µg/mL. Similarly, IC_{50} values of 30.15 and 48.36 µg/mL were observed in *Bacillus flexus*.

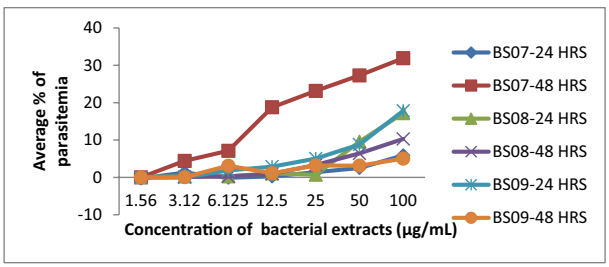
In contrast, *Bacillus subtillis*, *Bacillus macerans*, *Bacillus pumilus*, and *Bacillus larvey* displayed minimal activity against *Plasmodium falciparum* (IC_{50} *P. falciparum* 3D7: >100 µg/mL). Meanwhile, only *B.megaterium* exhibited significant antiplasmodial activity at lower concentrations of 24.65 µg/mL and 71.08 µg/mL within 24 h of treatment, with an IC_{50} of 0.49 µg/mL. At 48 h, the treated groups showed 7.82 µg/mL at lower concentrations and 64.86 µg/mL at higher concentrations of extracts, with an IC_{50} of 0.58 µg/mL, which correlate with the standard Chloroquine drug treated groups, as shown in Fig. 5. As a result of this study, it was revealed that parasitemia percentage was significantly higher when *B.megaterium* extracts were compared to other bacterial extracts in terms of activity. A microscopic examination of the antiplasmodial activity of bacterial extracts indicated inhibition of the trophozoites at the early and middle stages of *Plasmodium falciparum* parasites are lysed (Fig. 6).

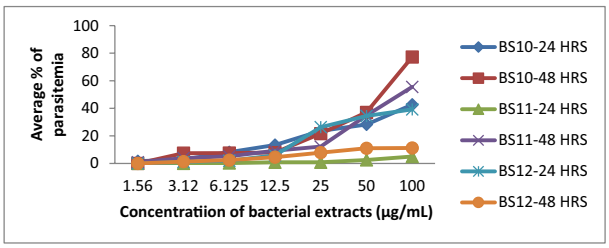
Discussion

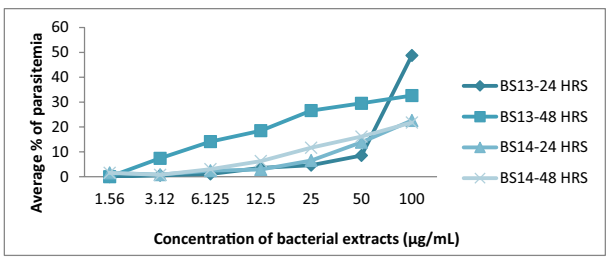
In recent years, most researchers have utilised rhizosphere soil samples for isolating new chemicals and discovered that they are among the best natural sources [42]. A diverse community of bacteria has been discovered in soil habitats. It is thought to produce distinct antibiotics from soil samples. In the present study, we have isolated 275 rhizosphere soil samples from thirty-two medicinal plants. The scientists identified nearly 210











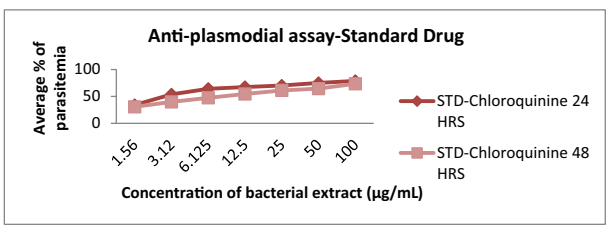
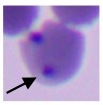
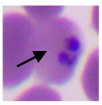
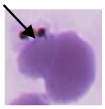
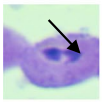
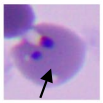
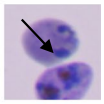
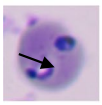
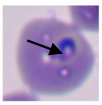
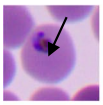
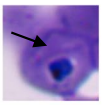
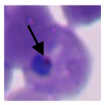
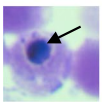
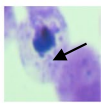
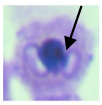
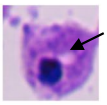
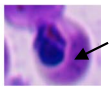
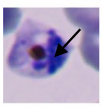
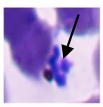
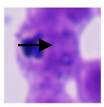
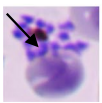
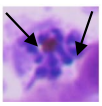
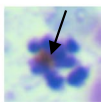


Fig. 5 Inhibitory effect of antiplasmodial activity after exposure of different bacterial extracts at 24 and 48 h

S.No	Intra-erythrocytic Stages of *P.falciparum*	Extract of *Bacillus megaterium*		
		(0 hours)	(24 hours)	(48 hours)
1.	Ring stage Early			
2.	Ring stage Mid			
3.	Ring stage Late			
4.	Trophozoite stage Early			
5.	Trophozoite stage Mid			
6.	Trophozoite stage Late			
7.	Schizont stage Early			
8.	Schizont stage Late			

[Arrows indicating presence of malarial parasites in the erythrocytes, purple stain representing erythrocytes, dark blue stain denoting parasites]

Fig. 6 Morphological changes of parasites after treatment of *Bacillus megaterium* extract

bacterial isolates from rhizosphere soil samples, which correlate with our present study [43]. Likewise, another group reported that 356 isolates were isolated from various regions in Turkey [44, 45]. A similar study was identified in which they selected 3 bacterial strains out of 263 isolates from soil samples [46]. Also, the biodiversity of microbial bacteria was studied in the different places of Mizoram, in which they obtained 248 bacterial colonies, especially in January compared to May [47, 48]. This study was exactly correlated with our present findings,

in which the maximum number of bacterial isolates was observed only in the winter as compared to the summer, respectively. Similar work suggested that a greater number of isolates were observed in the rainy and winter seasons compared to the summer seasons, respectively [49].

The isolation of bacteria was done using the serial dilution method 10^{-2} to 10^{-6} on nutrient and starch casein agar by the pour plate method [50]. As a result, we used the same method for detecting rhizosphere soil samples and isolating bacteria. The bacterial isolates were identified using colony morphological examination and the Gram staining method, which is widely used and considered to be one of the finest traditional methods [51]. Gram staining results revealed that 14 bacterial isolates were naturally Gram-positive bacilli. Previous research indicated that the majority of rhizosphere soil samples were Gram-positive, which matched with our current findings [52, 53]. Several biochemical assays were carried out in order to identify different bacterial strains [43]. For the identification of bacterial isolates, 16S rRNA gene sequencing was performed on various samples [54]. Out of fourteen bacterial isolates, BS01 isolate 16S rRNA was submitted to NCBI BLAST and the sequence was exactly matched with *Bacillus megaterium* respectively. Previously, it was reported that most of the *Bacillus* species were frequently seen in the rhizosphere soil samples [55]. Most of the Gram-positive bacteria were found to be producing novel antibiotics compared to Gram-negative bacilli [56, 57].

Through GC–MS analysis, it was clearly discovered that the presence of cyclopentaneundecanoic acid, methyl ester functions as a natural antioxidant, antiplasmodial substance, which has already been described through many investigations [2]. Similar results were found for hexadecanoic acid and octadecanoic acid in terms of their antibacterial, antimalarial, and anticancer properties. These substances were identified from B. megaterium extract and exhibit significant medicinal activity. These secondary metabolites generate a brand-new antibacterial and antiplasmodial substance that aids in the treatment of a variety of difficult disorders [58–60].

Over a variety of bacterial extracts from rhizosphere soil, possible antiplasmodial activity was only seen in *B. megaterium* extract, although *B. flexus, B. subtilis, B. macerans, B. pumilus*, and *B. cereus* displayed moderate antiplasmodial activity with an IC_{50} value between (1 and 50 µg/mL). Methanolic extracts of *C. planchonii*, which had IC_{50} values of 15 and 50 µg/mL equally, have been used in similar investigations [61]. These plasmodial activities, however, differ from one bacterial extract to the next extracted from the various soil samples.

The worldwide demand for antibiotics is constantly increasing due to the emerging antibiotic resistance among bacteria [62, 59]. For this particular study, our objective was to isolate and characterize soil bacteria over several months. Among the 275 bacterial isolates, we identified fourteen isolates, including the *Bacillus megaterium* from the rhizosphere soil sample of *Achyranthes aspera*, which was previously proven to be effective compound. Previous reports have also indicated that *Bacillus megaterium* exhibits various activities such as antibacterial and antiplasmodial effects, as well as antibiotic production. We anticipate that this discovery will contribute to the development of innovative antibiotic medications.

Conclusion

The rhizosphere soil surrounding various medicinal plants contains a diverse range of bacterial microorganisms. Among these, a majority of the bacterial populations found in the rhizosphere soil belong to the species of *Bacillus*. This type of research is valuable for researchers as it enables them to identify a wide variety of microbial species. This study specifically revealed that approximately 14 bacterial strains exhibited significant biochemical properties. Through an assessment of their antiplasmodial activity, we identified *Bacillus megaterium* as a particularly promising bacterium within the rhizosphere soil sample of *Achyranthes aspera*. This finding suggests that these rhizosphere soil bacterial extracts contain secondary metabolites that have potential in treating malarial parasites. However, further studies are necessary to explore drug formulation and toxicity levels in the future.

Abbreviations

ICMR	Indian Council of Medical Research
CFU	Colony-forming unit cell
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic Acid
NCBI	National Centre for Biotechnology Information
NCMB	National Centre for Marine Biodiversity

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Author contributions

Dr. MG perceived the knowledge and delineated the content. VR contributed to its writing, data collection, editing and submission. All authors reviewed and permitted the final manuscript.

Studies involving plants

We followed Guidelines on the Conservation of Medicinal Plants 1993 (The World Health Organization (WHO) IUCN-The World Conservation Union WWF-World Wide Fund for Nature).

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Availability of data and materials
The datasets analysed during the current study are available in the GenBank sequence database repository NCBI with accession number MT937315.1 https://www.ncbi.nlm.nih.gov/nuccore/1897301275?log$=activity.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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