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Phytochemical analysis of various solvent extracts of mangrove plant associated *Bacillus* spp.

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ABSTRACT: The aim of the study is to investigate which groups are been produced by mangrove associated bacterial species and to check the effectiveness of the solvents in extracting specific bioactive compound groups. Mangrove associated microbial habitat are proven to produce potential bioactive compounds. In the present study, mangrove plants associated *Bacillus* spp. were bulk grown in the culture broth and the phytochemicals produced were extracted using various solvents viz. butanol, chloroform, ethyl acetate and hexane. The phytochemical testing of these extracts discovered that butanol extracted alkaloid and emodin group of phytochemicals, chloroform extracted phytosterols, anthraquinones and leucoanthrocyanins, hexane extracted phytosterol and emodin groups of bioactive compounds and ethyl acetate extracted phytosterols. The experiment was carried out thrice and every time the results obtained were the same. According to the previous studies we can say that these phytochemicals can be used for new drug discovery and for formulating various pharmaceutical products. Further advanced research on the purification and identification of these compounds can be carried out using techniques like GC-MS and column chromatography. These compounds can further be tested and applied to specific applications.

KEYWORDS: Bioactive compounds, Solvent extraction, Qualitative analysis.

INTRODUCTION

An increasing number of individuals in industrialized and developing nations are turning to the safe and affordable herbal remedies due to the growing abuse of antibiotics and chemotherapeutic agents, which is causing medication resistance. In addition to recognized antibiotics, naturally occurring antimicrobial components in plants can also stop the growth of bacteria by unidentified methods [1]. Mangrove habitats are found throughout the world's tropical and subtropical coastal zones, where freshwater and terrestrial environments converge. Large amounts of organic matter and nutrients are found in these systems. High salinity, high humidity, high sulfide concentrations, and low oxygen contents are the features of mangrove environment. A distinct collection of plant, animal, and microbial species that are suited to survive under harsh environmental circumstances are supported by these ecosystems [2].

As mangroves produce a wide range of naturally occurring materials with both therapeutic and commercial value, they have been the focus of conservation studies. Numerous bacteria, fungi, and algae live in the roots and leaves of mangroves. A sufficient assessment of life forms depending on mangroves does not exist at present. Finding culture-dependent microorganisms that can be harboured from various mangrove hosts is the aim of this investigation. Different microbiomes exist, inside plant tissues (endophytic) and on the surfaces of plants (epiphytic). These bacteria are essential to the plant health and has several advantages. It has been clear how bacteria and plants interact with each other. They eventually show signs of adapting to unfavourable environments and may have genetic changes that enable them to produce certain phytochemicals. The interaction between bacteria and hosts results in the production of many metabolites. Xanthones, steroids, tetralones, alkaloids, terpenoids, quinones, phenolic acids, benzopyranones, and flavonoids are examples of secondary metabolites [3].

When it comes to commercial quantities, bioactive chemicals obtained from microbes are easier to obtain than metabolites originating from plants. It may be possible to cure cancer, inflammation, and bacterial infections with microbial substances. While bioactive compounds from a limited variety of bacteria have been studied, several active chemicals that have potential applications as antibacterial, anticancer, and anti-inflammatory medicines have been identified [4].

Mangrove sediment-associated bacteria's antioxidant capacity of *B. amyloliquefaciens* MBMS5 organic extracts

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was evaluated in vitro, as was their capacity to inhibit the proinflammatory 5-LOX, a key pharmacological target linked to inflammation. The DPPH and ABTS+ radical scavenging experiments showed that the crude extracts have potential antioxidant properties. Interestingly, the antioxidant activities were noticeably higher than those of α-tocopherol, an antioxidant that is sold commercially. Mangrove associated *B. amyloliquefaciens* MBMS5 demonstrated a wide range of inhibition and was effective against pathogenic bacteria, including *S. aureus* that is resistant to multiple drugs. Bacilysocin, a new antibiotic derived from a strain of *Bacillus subtilis*, showed antibacterial efficacy against *S. aureus* and antifungal activity against *Saccharomyces cerevisiae* and *Candida pseudotropicalis*. It was discovered that ethyl acetate extracts of *Streptomyces* genus actinobacterial strains isolated from mangrove sediments in Andhra Pradesh's Krishna district exhibited strong antimicrobial activity against isolates of Gram-negative bacteria like *Pseudomonas aeruginosa, E. coli,* and *Xanthomonas campestris*, as well as Gram-positive bacteria like *Bacillus subtilis*, *Bacillus megaterium,* and *S. aureus*. The chemicals obtained from *Streptomyces cheonanensis* extracts, including tetradecane, 1-tetradecene, octadecane, and cyclotetracosane, were discovered to exhibit possible antibacterial and antifungal properties [4].

By using GC-MS analyses, the volatile chemical components of the ethyl acetate extracts of three endophytic bacteria with the most promising biological characteristics - *Bacillus sp.* RAR_GA_16, *Rossellomorea vietnamensis* RAR_WA_32, and *Bacillus spp.* RAR_M1_44 were examined. It's interesting to note that numerous compounds found in the extracts of three endophytic bacteria have been shown in earlier research to possess biological activities (such as antibacterial, antifungal, antivirus, anticancer, anti-inflammatory, antioxidant, nematocidal, anti-quorum sensing, tyrosinase inhibitory, antibiofilm, and antimutagenic properties) [5].

One study adds credence to the mounting evidence that the fungal endophytes *F. oxysporum, Clonostachys sp.*, and *F. solani* produce bioactive compounds. Chemical analysis of the ethyl acetate extract of *Clonostachys sp*. and the *F. solani* strain obtained from *C. decandra* resulted in the identification of efficient secondary metabolites, continuing our laboratory's search for bioactive secondary metabolites from natural sources. The investigation's findings demonstrated the potential of fungal strain CEDBE-1, CEDLE-6, CEDLE-10 crude extract as bioactive substances against cytotoxicity, antimicrobial, and antioxidant tests. As data suggested that the strains of *F. oxysporum*, *Clonostachys sp.*, and *F. solani* are abundant in bioactive substances [6].

MATERIALS AND METHODS

Collection of mangrove plant leaves

The different species of mangrove plant leaves viz., *Excoecaria agallocha Linn.*, *Acanthus ilicifolius Linn.* and *Rhizophora mucronate Poir* were collected from Dahisar, Andheri and Borivali (Mumbai, Maharashtra, India) mangrove areas respectively [7]. Healthy leaves without any signs of infection were collected from the mangrove forest and after the collection the plant leaves were authenticated by a Botanist [8]. After collection, each sample was put into sterile plastic bags. Every sample was shipped to a lab and stored at 4 °C until further processing [9].

Production and extraction of secondary metabolites

Two potential *Bacillus subtilis spp.* (S4WR and S6A) and *Bacillus licheniformis* (Isolate No. 8) bacterial strains were isolated from mangrove plant leaves that were procured from different mangrove regions in Mumbai, Maharashtra, India. For six days, the bacterial strains were cultured in a 100 ml Erlenmeyer flask with 50 ml of nutrient broth and shaken at an orbital speed of 120 rpm at 37˚C. The culture was centrifuged for 15 minutes at 10,000 rpm to separate the cell pellet. The resulting supernatant was filtered through a 0.45 µm membrane filter in order to exclude any bacterial cells. The resultant cell free supernatant (CFS) was then combined with an equal volume of the following solvents: ethyl acetate, butanol, hexane, and chloroform separately. The mixture was then shaken at 120 rpm for 48 hours. Under a rotary vacuum evaporator, the solvent phase was concentrated after separation. The resultant crude extract was kept for later research in a glass vial that had been previously weighed [10].

Phytochemical screening

Standard screening tests of 16 solvent extracts were carried out for various bacterial constituents. The crude bacterial extracts were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins, and anthraquinones using standard procedures.

1. ***Detection of flavonoids & Tannins:*** Lead acetate Test: The extract was mixed with a few drops of lead acetate solution. Appearance of yellow coloured precipitate confirms the presence of flavonoids and tannins.
2. ***Detection of leucoanthocyanins:*** The extract was mixed with isoamyl alcohol. Appearance of red coloured layer indicates that leucoanthocyanins are present in the extract.

3. Detection of alkaloids: The extract was treated with Mayer’s reagent i.e Potassium Mercuric Iodide solution. Formation of a yellow-coloured precipitate detects the presence of alkaloids in the extract.

4. Detection of phenols: Ferric Chloride Test: The extract was treated with a few drops of freshly prepared 1% Ferric Chloride and potassium ferrocyanide solutions respectively. End point of bluish-green colour indicated the test to be positive.

5. Detection of phytosterols & reducing sugars:

Salkowski's test: The extract was agitated with chloroform in a test tube. Concentrated Sulfuric acid was carefully added to form a reddish-brown colour which indicated the presence of a phytosterols and reducing sugars.

6. Detection of Anthocyanins: The extract was mixed with 2N HCl and ammonia. Development of pink-red colour indicates the presence of anthocyanins.

7. Detection of Emodins: Ammonium hydroxide solution and Benzene was added to the extract. Formation of reddish or pink colour indicates the presence of emodins in the extracts.

8. Detection of anthraquinones: Borntrager's test: The extract was shaken vigorously with benzene and then treated with 10% ammonia solution. The presence of a pink, red, or violet colour in the ammonia confirms the presence of free anthraquinones.

9. Detection of Fatty acids: The extracts were mixed with ether and were allowed to evaporate on filter paper. The appearance of transparent spots on the filter paper indicates the presence of fatty acids in the extract [11].

RESULTS

According to the observations it can be confirmed that ethyl acetate extracts of *Bacillus subtilis* and *Bacillus licheniformis* were containing phytosterols/reducing sugars (Table 1, Figure 1). Hexane extracts of *Bacillus subtilis* and *Bacillus licheniformis* were tested positive for phytosterols/reducing sugars while hexane extracts of *Bacillus subtilis* were also detected with emodin group (Table 2, Figure 2).

Choloform extracts *Bacillus licheniformis* were detected with leucoanthrocyanin group and phytosterol group, also *Bacillus subtilis* extracts of chloroform were found to contain anthraquinones and phtosterols (Table 3, Figure 3 and 4). Butanol extracts of *Bacillus subtilis* were present with alkaloid and emodin groups (Table 4, Figure 5). Chloroform had the highest efficiency as it could extract maximum number of phytochemical groups followed by hexane and butanol and the least effective solvent was ethyl acetate (Figure 6).

Table 1. Qualitative analysis of bacterial extracts of ethyl acetate.

Bacterial extracts of ethyl acetate / Detection of phytochemical group	S4WR	S6A	8	Control
Flavonoid’s & Tannins	-	-	-	-
Leucoanthrocyanins	-	-	-	-
Alkaloids	-	-	-	-
Phenols	-	-	-	-
Phytosterols/Reducing sugars	Faint red colour (+)	Dark brown colour (+)	Light brown colour (+)	-
Anthocyanins	-	-	-	-
Emodins	-	-	-	-
Anthraquinones	-	-	-	-
Fatty acids	-	-	-	-

-: Indicates negative test; +: Indicates positive test

Table 2. Qualitative analysis of bacterial extracts of hexane.

Bacterial extracts of hexane / Detection of phytochemical group	S4WR	S6A	8	Control
Flavonoids & Tannins	-	-	-	-
Leucoanthrocyanins	-	-	-	-
Alkaloids	-	-	-	-
Phenols	-	-	-	-
Phytosterols/Reducing sugars	Light brown colour (+)	-	Light brown colour (+)	-
Anthocyanins	-	-	-	-
Emodins	-	Pink colour (+)	-	-
Anthraquinones	-	-	-	-
Fatty acids	-	-	-	-

-: Indicates negative test; +: Indicates positive test

Table 3. Qualitative analysis of bacterial extracts of chloroform.

Detection of phytochemical group	**Bacterial extracts of Hexane**			
	S4WR	S6A	8	Control
Flavonoids & Tannins	-	-	-	-
Leucoanthrocyanins	-	-	Orange colour (+)	-
Alkaloids	-	-	-	-
Phenols	-	-	-	-
Phytosterols/Reducing sugars	-	Light brown colour (+)	Light brown colour (+)	-
Anthocyanins	-	-	-	-
Emodins	-	-	-	-
Anthraquinones	-	Pink colour (+)	-	-
Fatty acids	-	-	-	-

-: Indicates negative test; +: Indicates positive test

Table 4. Qualitative analysis of bacterial extracts of butanol.

Detection of phytochemical group	**Bacterial extracts of Butanol**			
	S4WR	S6A	8	Control
Flavonoids & Tannins	-	-	-	-
Leucoanthrocyanins	-	-	-	-
Alkaloids	-	Yellow colour (+)	-	-
Phenols	-	-	-	-
Phytosterols/Reducing sugars	-	-	-	-
Anthocyanins	-	-	-	-
Emodins	Pink colour (+)	-	-	-
Anthraquinones	-	-	-	-
Fatty acids	-	-	-	-

-: Indicates negative test; +: Indicates positive test

Figure 1. Ethyl acetate extracts of Isolate No. 8, S6A, S4WR positive for phytosterol group.

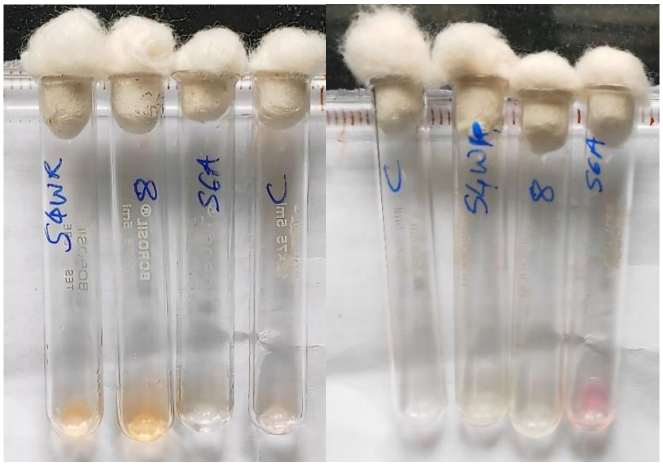

Figure 2. Hexane extracts of Isolate No. 8, and S4WR positive for phytosterol group (Left) and hexane extract of S6A positive for emodins (Right).

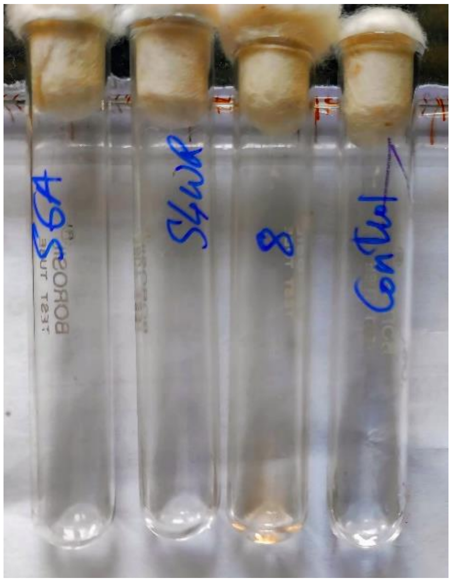

Figure 3. Chloroform extract of Isolate No. 8 positive for leucoanthocyanins.

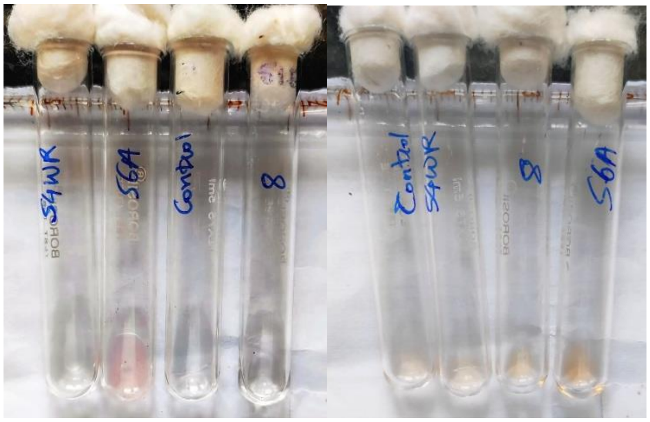

Figure 4. Chloroform extract of S6A positive for anthraquinone group (Left), chloroform extracts of isolate no. 8 and S6A positive for phytosterol group (Right).

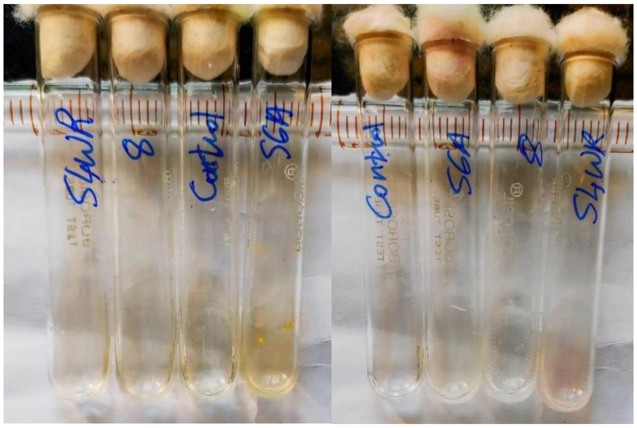

Figure 5. Butanol extract of S6A positive for alkaloids (Left), butanol extract of S4WR positive for emodins (Right).

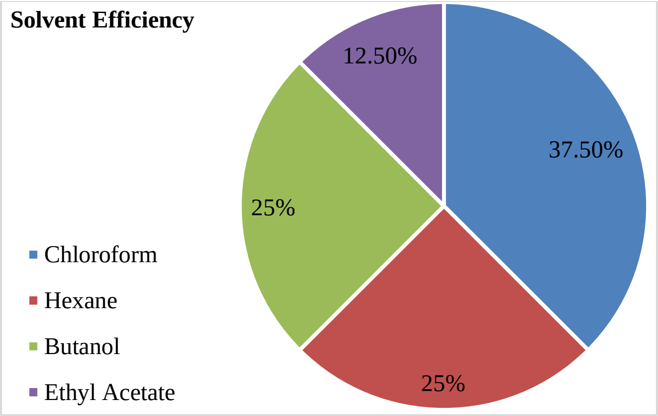

Figure 6. Solvent efficiency of extracting different compounds of various phytochemical groups.

DISCUSSION

The basic qualitative analysis has provided us with the broad groups of phytochemicals that have been extracted in the solvents and have been qualitatively detected. Statistical analysis shows that the test performed have given accurate results as every test was carried out thrice and have ended up with similar results. The basic analysis cannot confirm the names of the bioactive compounds present in the extracts. Further the extracts can be sent for advanced analysis and the potential compound names can be found out. In the future, these compounds can be formulated into novel drugs. A lot of previous study has been focused on the findings of phytochemicals of various mangrove leaves extracts and bacterial isolate extracts from mangrove soil, roots and water.

In previous research, a team had isolated the fungus *Pseudofusicoccum sp.* J003 from the mangrove species *Sonneratia apetala* Buch.-Ham. Acorenone C, two alkaloids, four phenolic compounds and four steroid derivatives have been extracted as a result of the chemical analysis of the methanol extract of the strain's culture broth. Acorenone C showed very little acetylcholinesterase activity, one of the steroid derivatives showed inhibition of nitric oxide production in mouse macrophages and it also affected the growth of two human tumors [12].

The objective of one of the studies was to separate fungal endophytes from eleven mangrove plants, evaluate their enzyme synthesis and look into their antifungal effectiveness against phytopathogenic fungi. The antioxidant and anti-mutagenic properties of a few endophytic fungal ethyl acetate crude extracts were assessed and the active ingredients in the extracts were identified. Thirty of the endophytic fungal isolates were obtained from leaves and seven from stems. Five plant infections were used to examine each endophyte's anti-pathogenic activity. Fungal strains

AmL-02 and CtL-06 were selected for phytochemical analysis and further investigation of their anti-mutagenic properties, after the results showed that they had the maximum growth inhibition of *Curvularia sp.* and *Fusarium sp*., respectively [13].

The *Glutamicibacter* group of microorganisms is well-known for producing enzymes and antibiotics. They produce enzymes and antibiotics that are crucial for the prevention, management, and treatment of long-term human illnesses. In this investigation, mangrove soil in the Mangalore region of India yielded the *Glutamicibacter mysorens* strain MW647910.1. This study demonstrated that tiny molecular weight bioactive chemicals derived from microorganisms have two functions: they function as anticancer peptides (ACPs) and antimicrobial peptides (AMPs) [14].

Mangrove environments have a remarkably diverse and abundant microbial diversity that holds great promise for biotechnology. In a thorough analysis covering the years 2021–present, they have looked at the identification of 165 new secondary metabolites from 41 different microbial strains. This study has identified 82 polyketides, 44 nitrogen-containing chemicals, 16 terpenoids, and 23 halogenated compounds in the last four years. Nearly half of these secondary metabolites have one or more biological actions, indicating their potential as a starting point and a guide for the development of new therapeutic approaches [15].

According to a prior study, one of the key factors influencing the conversion of a natural product into a medication is species abundance as measured by the Global Biodiversity Information Facility (GBIF) dataset. Understanding the particular needs for drug development based on natural products was their main goal. For studies on alkaloids in conjunction with plant systematics and taxonomy, Web of Science was consulted. Between 2014 and 2020, the average number of GBIF occurrences increased by 8.66 for all species that contain alkaloids. Alkaloids found in medicinal species are more abundant than those found in non-medicinal species, and they are frequently associated with cultivation as well. Compared to "rare" alkaloids, alkaloids with high biodiversity are more likely to be included in early-stage medication research because they are frequently simple alkaloids that are present in several species with the existence of driver species [16].

Emodin, a significant bioactive anthraquinone derivative that is isolated from rhubarb, has several health benefits and can be used to treat a variety of illnesses, including metabolic syndrome, bacterial or viral infections, tumor progression, and immune-inflammatory abnormalities. The multi-targeting therapeutic mechanisms that underling emodin's effective therapeutic potential including its anti-inflammatory, immunomodulatory, anti-fibrosis, anti-tumor, antiviral, antibacterial, and anti-diabetic qualities have been better understood by the emerging data. Reviewers have looked at current initiatives to use structural alteration and innovative material-based targeted delivery to enhance the pharmacokinetic characteristics and biological activities of emodin. To sum up, emodin still has a lot of potential to develop into a promising treatment for endocrine disorders, organ fibrosis, common cancers, pathogenic bacterial or viral infections, and immunological and inflammatory abnormalities [17].

A member of the tetracyclic triterpene class, stigmasterol which is an unsaturated phytosterol. Stigmasterol's diverse biological effects on numerous metabolic disorders was been investigated using molecular docking and in vitro/in vivo experiments. Strong pharmacological effects, including anti-inflammatory, anti-diabetic, immunomodulatory, antiparasitic, antifungal, antibacterial, antioxidant, and neuroprotective qualities, are shown by the results. Plant and algae-derived stigmasterol is a promising chemical for the creation of cancer treatment medications since it activates intracellular signalling pathways in a variety of malignancies. In gastric and ovarian tumors, it affects the JAK/STAT and Akt/mTOR pathways. Furthermore, through down-regulating vascular endothelial growth factor receptor-2 signalling and tumor necrosis factor-α, stigmasterol significantly hampered angiogenesis in human cholangiocarcinoma. In breast cancer, the combination of stigmasterol and sorafenib increased caspase-3 activity and decreased levels of the anti-apoptotic protein Bcl-2. The chemoprotective effects of stigmasterol in skin cancer are attributed to antioxidant activities that guarantee lipid peroxidation and DNA damage reduction. Along with dopamine depletion and acetylcholinesterase inhibition, stigmasterol's neuroprotective actions are also influenced by the regulation of reactive oxygen species. Inducible nitric oxide synthase and cyclooxygenase-2 are inhibited, inflammatory mediator release is reduced, and anti-inflammatory cytokines are produced as a result of phytosterols anti-inflammatory qualities. By lowering oral glucose tolerance, blood insulin levels, and fasting glucose, it can be concluded that stigmasterol has anti-diabetic actions [18].

CONCLUSION

The mangrove plants are abundant in bioactive compounds and they are proved to have therapeutic potential according to the past research material. Mangrove plant leaves isolates were grown in culture broth and was subjected to extraction with various solvents. Solvents used were ethyl acetate, butanol, chloroform and hexane. The extracts were evaluated for bioactive compounds. Extracts of ethyl acetate of two different strains of *Bacillus subtilis* and one strain of *Bacillus licheniformis* were found to be positive for phytosterol group (Table 1, Figure 1). Chloroform extracts of *Bacillus subtilis* were found positive for phytosterol and anthraquinone group, Chloroform extracts of *Bacillus licheniformis* were found positive for phytosterol and leucoanthrocyanin group (Table 3, Figure 3 and 4). Hexane extracts of *Bacillus subtilis* were found to contain emodins and phytosterols, while hexane extracts of *Bacillus licheniformis* were found to contain phytosterols (Table 2, Figure 2). Butanol extracts of two different strains of *Bacillus subtilis* were positive for alkaloids and emodins (Table 4, Figure 5). Chloroform was found to be the most efficient in extracting different phytochemical groups as compared to the other solvents (Figure 6). Further these compounds can be identified, purified using various techniques and each molecule can be analysed for different aspect like antiviral activity, antimicrobial activity, anti-inflammatory activity, etc, as they have a history of having various potential applications. Further advanced level research can be carried out in future.

DECLARATIONS

Authorship contributions

All the guidance and concept has been contributed by Dr. Rajesh C. Patil and the practical work, data processing and writing work has been carried out by Ruchi Malik.

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Competing interests

The authors declared that there is no conflict of interest.

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