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Characterization of Natural Compounds Produced by *Bacillus subtilis* Using GC-MS and Investigation of Its antimicrobial activity

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Abstract: Aims and Objectives: Bacterial metabolites have shown promise in the treatment of a wide range of disorders, and they play an essential role in maintaining human health. The purpose of this research was to find out how effective Bacillus subtilis bioactive chemical compounds are against bacteria and other microbes. Methods: The bioactive chemical components, also called secondary metabolites, were analysed using gas chromatography-mass spectrometry (GC-MS) methods. Then, in vitro evaluation of the antibacterial activity of the Bacillus subtilis methanolic extract was carried out.

Results: The GC-MS analysis of Bacillus subtilis detected the presence of the following: The compounds listed include tert-Butyl 12-aminododecanoate, 1,12-Diaminododecane, Ethylidenehydroxylamine, 5,6-Diamino-2,3-dicyanopyrazine, 1-methylpiperidine-3-carboxylic Acid, 3,3-Dimethyl-2-acetyloxirane, 8-Hexadecanol, 4-methyl-hexadecane, 5,7-Octadecadiynoic acid, 4,6-dimethylpyridin-2-amine. The current study examined the bioactivity of the ethanolic extract of Bacillus subtilis and the standard antibiotics AP-Ampicillin and KN-Kanamycin against five tested pathogens: Staphylococcus saprophyticus (11.67±0.06, 21.07±0.09, and 12.31 ±0.06), Streptococcus pneumonia (12.09±0.07, 22.00±0.08, and 15.86±0.05), Streptococcus pyogenes (14.51±0.64, 23.05±0.73, and 17.74±0.07). Streptococcus agalactiae (10.92±0.11, 18.96±0.11 and 18.01±0.09) and Klebsiella pneumoniae (10.70±0.05, 22.12±0.08, and 19.13±0.09). The metabolites of Bacillus subtilis exhibited significant activity against Streptococcus pyogenes (14.51±0.64).

Keywords: Bacillus subtilis, Secondary metabolites, Antibacterial, GC/MS.

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Introduction

Bacillus species are known to be distributed almost to every part of the natural environment. They are very metabolically versatile and have been studied extensively for producing relatively different chemicals that display biological actions. Further study concerning the pharmacological functions of metabolites, including the impairment of cancer cells, by Bacillus spp. has become a topic of interest in the recent past especially in research studies. Glioblastomas are amongst the most perilous types of brain cancer, they develop rapidly and cannot be treated. Many scientists are focusing on the natural chemicals and their derivatives to provide cure for this dreadful disease [1, 2]. Figuing out whether metabolites from the Bacillus species have the ability to restrict the growth of glioblastoma cancer cells this work examines their effects on the epithelial glioblastoma cancer genes by computational analysis and in vitro analysis. GC-MS is a powerful technology to simply and analyse various chemical entities in microbial extracts and it has given new directions in natural products leads. Thus, the analysis of Bacillus spp.by means of GC-MS. it is possible to find a lot of metabolites in the extract and further study their biological activity. This analytical method creates the framework to envisage the possible pharmacological properties of metabolites being released by Bacillus spp. by giving valuable information on its composition. This research primarily focuses on biological analysis with the use of in vitro apparatus. Especially, we would like to know the following points: In particularly, we want to know that are the identified Bacillus spp metabolites. and characterized on glioblastoma cancer cells. These metabolites have the possibilities of inhibiting glioblastoma cell growth, and inducing apoptosis; their cytotoxicity and anticancer effect will be demonstrated through the in vitro studies [3]. The following experiments must however be carried out in order to offer the following findings: All the experiments should be conducted in adherence to relevant protocols and methodologies. The impacts of the metabolites depending on Bacillus spp. genes that are associated with epithelial glioblastoma cancer are being explored utilizing computational as well as the in-vitro investigation. This strategy will help us uncover the possible impact of these metabolites on the alterations in gene expression and regulation of glioblastoma by using modern sources and equipment in the field of bioinformatics [4]. We would like to find out all that is possible about the possible therapeutic qualities of metabolites from Bacillus spp. for glioblastoma treatment, and integrate the experimental and computational information. This research aimed to looking at specific chemical compounds in bioactivity and then evaluate the degree of their efficacy on bacteria.

Materials and Methods

Separation and identification of bacterial strains

The sample of soil used in cultures was obtained from botanical garden of the AL-Qasim Green University. LB agar plates were streaked with serial dilutions of the soil samples on top of the plates. Hardening of the plates were done before they were inoculated with successive dilution of the sample. The plates were then incubated at 30 °C for 24 h growth phase at an aerobic shaker incubator. With the help of a sterile loop, the colonies were picked from the bacterial cultures and spread on the other plates for further tests.

Extraction of bacteria

In a 500 mL Erlenmeyer flask, a bacterial isolate was incubated and fermented using a sequential of procedures. Tryptone, sodium chloride, and yeast extract were mixed together and transferred to the flask in order to make the broth. After that, the flask was shaken in an incubator for three or four days. The same flask was used for subsequent incubation at 37°C with shaking at 110 rpm after which the fermentation media containing 5 g of tryptone, 5 g of NaCl, 0.5 g of oatmeal, 0.09 g of phenol red, and 500 mL of distilled water were added.

Extracting chemicals from bacterial byproducts

Once the fermentation process is over, siphon the culture broth to individual funnels; each funnel will contain about 500 mL of soup. Each of them was then presented with the funnels individually in order to determine the effectiveness of extraction solvent, namely ethanol. Layers which had formed in the separating funnel over the night were due to the solvents and the culture broth and this implies that different substances have been successfully extracted from the broth. To ensure that there was no intermixing of the different components that were extracted, this solvent layer was carefully scooped in different beakers. To remove any remaining solvent and condense the extracted chemical the beakers were placed into a water bath at 60° C [5-7]. The rationale for this procedure is to evaporate the solvents so as to keep the concentrated culture broth extracts. They were taken through a process of evaporation and then transferred to test vial's once they were solventless for further tests. To facilitate our further studies and characterization on the extracts, we kept all the extracts in test tubes for neatly and systematically.

Mass Spectrometry-Gas Chromatography

For the raw material analysis we employed the GCMS that bases its operation on size and polarity. The levels of metabolites was quantified using the gas chromatography-mass spectrometry technique. The GC-MS analysis was performed using a PerkinElmer instrument, GC Clarus 500 system for the identification of the compounds. Configuration of GC-MS used in the electron impact mode, the ionisation energy electron ionisation of 70 eV was used. Helium gas of at least 99. 9% purity was used as the carrier gas along with an injection volume of the sample of 2 µL and a flow rate of 1° mL/min. With regards to temperature settings, I had the injector at 250°C through out and ion source at 200°C through out. He began heating the oven at 50 F and increasing it by 1.8 F/min to 350 F, and by 0.9 F/min to 450 F and then a hold at 450 F for 9 minutes. As for the mass spectra, we selected fragments with the molecular weight of ranging from 45 kDa to 450 kDa. A solvent delay of 0-2 minutes and the total GC-MS run time was 36 minutes. Thus, comparing the mean peak areas of each component with the mean total areas allowed us to define the percentage quantitative ratio of each component.

Evaluation of the antimicrobial activity of a secondary metabolite in an ethanolic Bacillus subtilis extract against five different pathogenic bacteria

The agar was prepared by boring five-millimeter-diameter holes with a sterilised cork-borer. Next, the wells were supplemented with 25 µl of the sample solutions that contained metabolites that Bacillus subtilis had generated. Swabs were used to collect the test pathogens, which included *Staphylococcus saprophyticus, Streptococcus pneumonia, Streptococcus pyogenes, Streptococcus agalactiae, and Klebsiella pneumoniae*. The pathogens were then placed onto plates of Muller Hinton agar. Both AP-Ampicillin and KN-Kanamycin were used as reference antibacterial agents [8, 9]. Each experiment was repeated twice .

Data analysis using statistical methods

The data was analysed using a variety of statistical techniques, including statistical tests like analysis of variance (ANOVA) and mean value computation, which were run on the SPSS (Version 11.6) database.

Results and Discussion

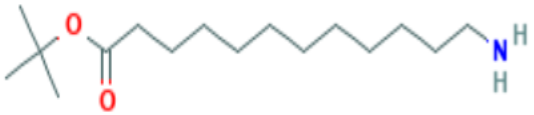
The compounds were identified by the presence of forty-five peaks in the GC-MS chromatogram. Ethylidenehydroxylamine, 5,6-Diamino-2,3-dicyanopyrazine, 1,12-Diaminododecane, tert-Butyl 12-aminododecanoate, and 1-methylpiperidine-3-carboxylic acid are the chemicals that have been mentioned. These compounds are known as 3,3-Dimethyl-2-acetyloxirane, 4-methyl-hexadecane, 5,7-octadecadiynoic acid, and 4,6-dimethylpyridin-2-amine. There is evidence that certain species of Bacillus can create secondary metabolites with antimicrobial properties. Based on the species, culture medium, and profiling technique, Bacillus sp. can create a wide variety of chemicals (Khan et al. 2019). Bacillus sp. lipopeptide

and polyketide compounds are well-known for their antibacterial, anti-inflammatory, antiviral, antiplatelet, antitumor, and anticancer characteristics, among many others. Since they are not volatile, targeted Liquid Chromatography-Mass Spectroscopy (LC-MS) is typically able to detect these lipopetide and polyketide substances. Researchers looked examined the effectiveness of Bacillus subtilis secondary metabolites as antibacterial agents against three different types of bacteria: In this investigation, five different pathogens were investigated to determine the bioactivity of AP-Ampicillin, KN-Kanamycin, and an ethanolic extract of Bacillus subtilis. Staphylococcus saprophyticus (11.67±0.06, 21.07±0.09, and 12.31±0.06), Streptococcus pneumonia (12.09±0.07, 22.00±0.08, and 15.86±0.05), Streptococcus pyogenes (14.51±0.64, 23.05±0.73, and 17.74±0.07). Streptococcus agalactiae (10.92±0.11, 18.96±0.11 and 18.01±0.09) and Klebsiella pneumoniae (10.70±0.05, 22.12±0.08, and 19.13±0.09). The metabolites of *Bacillus subtilis* exhibited significant activity against *Streptococcus pyogenes* (14.51±0.64).

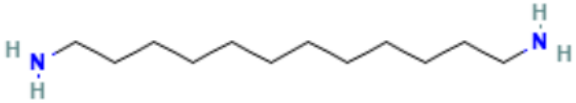
Table 1. Bioactive chemical compounds of ethanolic extract of *Bacillus subtilis*.

Compounds	Molecular Formula	Molecular Weight
tert-Butyl 12-aminododecanoate	$C_{16}H_{33}NO_2$	271.44 g/mol
1,12-Diaminododecane	$C_{12}H_{28}N_2$	200.36 g/mol
Ethylidenehydroxylamine	C_2H_5NO	59.07 g/mol
5,6-Diamino-2,3-dicyanopyrazine	$C_6H_4N_6$	160.14 g/mol
1-methylpiperidine-3-carboxylic Acid	$C_7H_{13}NO_2$	143.18 g/mol
3,3-Dimethyl-2-acetyloxirane	$C_6H_{10}O_2$	114.14 g/mol
8-Hexadecanol	$C_{16}H_{34}O$	242.44 g/mol
4-methyl-hexadecane	$C_{17}H_{36}$	240.5 g/mol
5,7-Octadecadiynoic acid	$C_{18}H_{28}O_2$	276.4 g/mol
4,6-dimethylpyridin-2-amine	$C_7H_{10}N_2$	122.17 g/mol

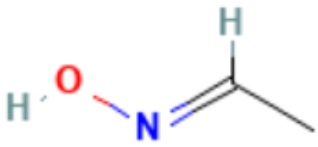
tert-Butyl 12-aminododecanoate



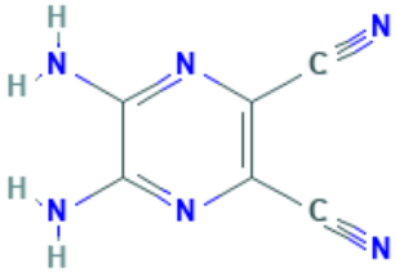
1,12-Diaminododecane



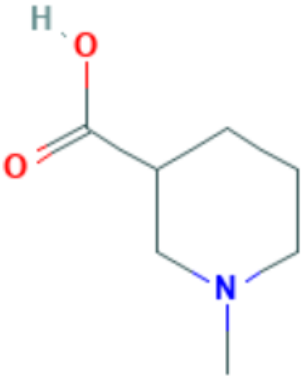
Ethylidenehydroxylamine



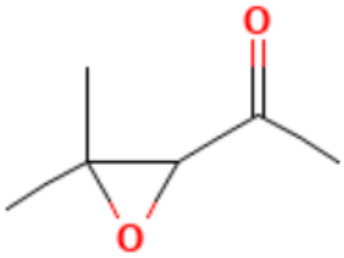
5,6-Diamino-2,3-dicyanopyrazine



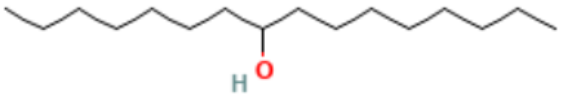
1-methylpiperidine-3-carboxylic Acid



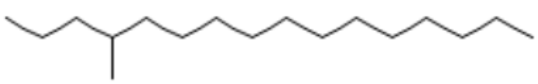
3,3-Dimethyl-2-acetyloxirane



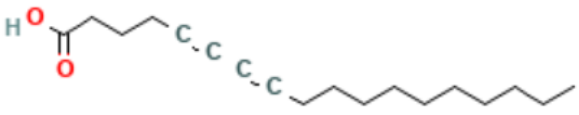
8-Hexadecanol



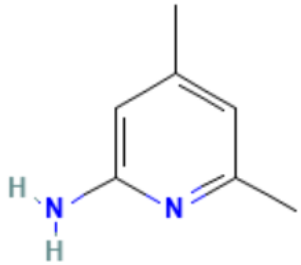
4-methyl-hexadecane



5,7-Octadecadiynoic acid



4,6-dimethylpyridin-2-amine



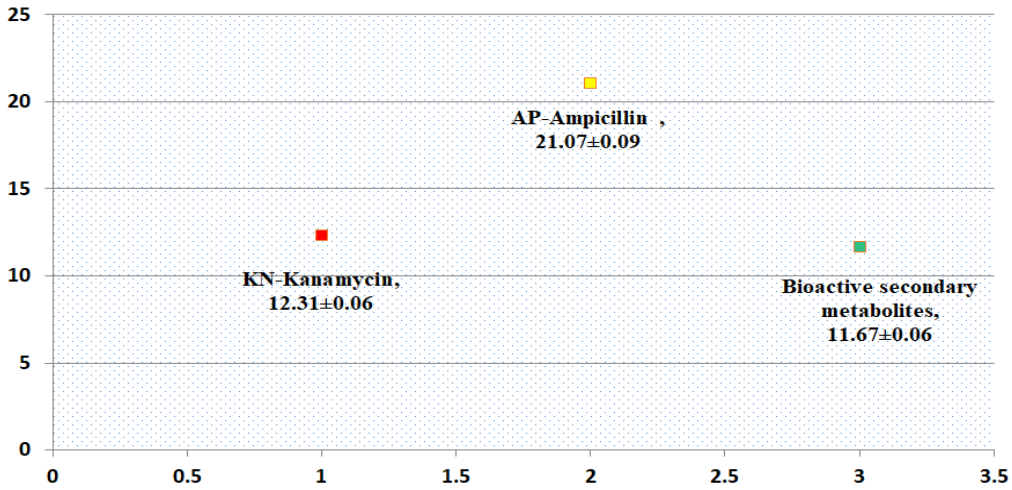


Figure 1. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Bacillus subtilis* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Staphylococcus saprophyticus*

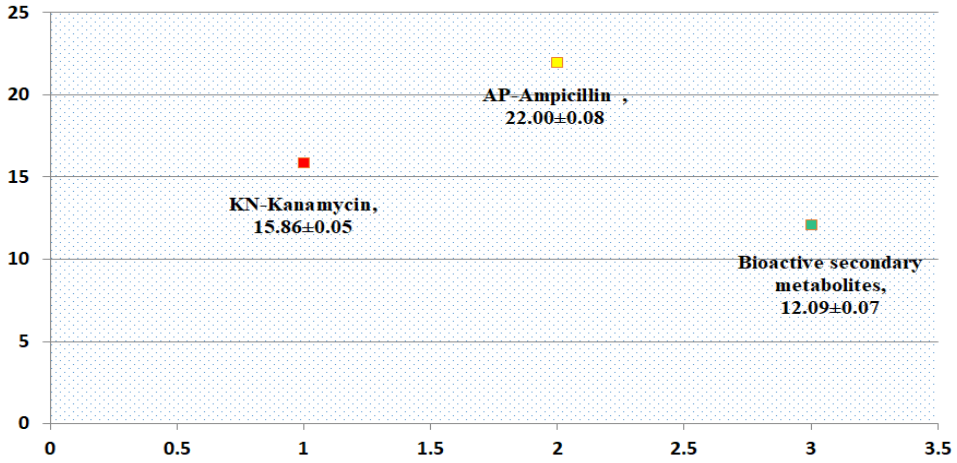


Figure 2. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Bacillus subtilis* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Streptococcus pneumonia*

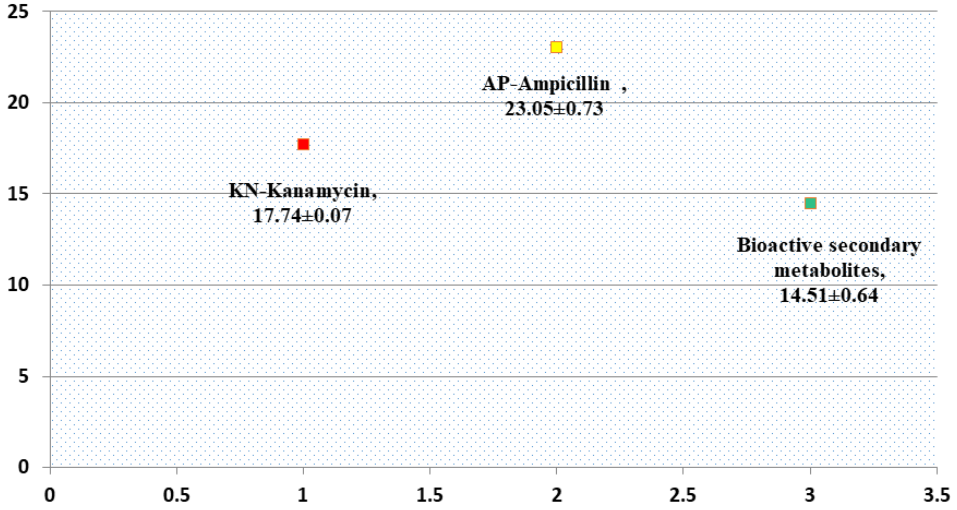


Figure 3. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Bacillus subtilis* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Streptococcus pyogenes*

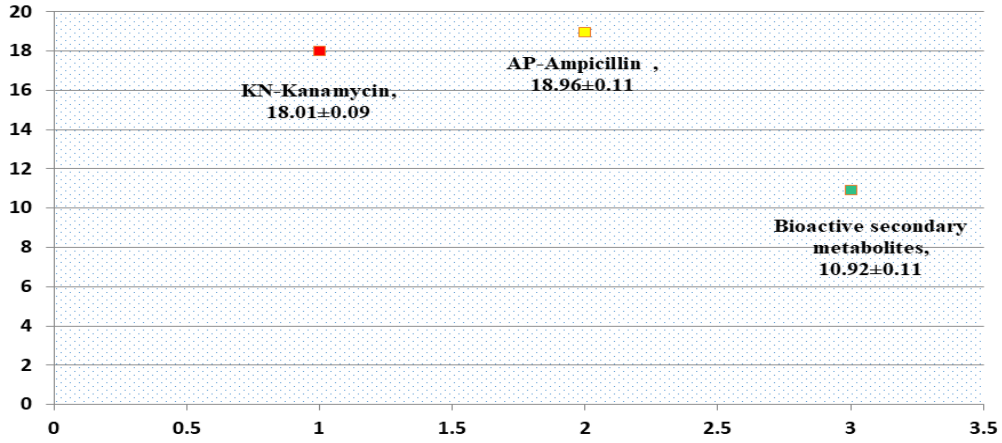


Figure 4. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Bacillus subtilis* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Streptococcus agalactiae*

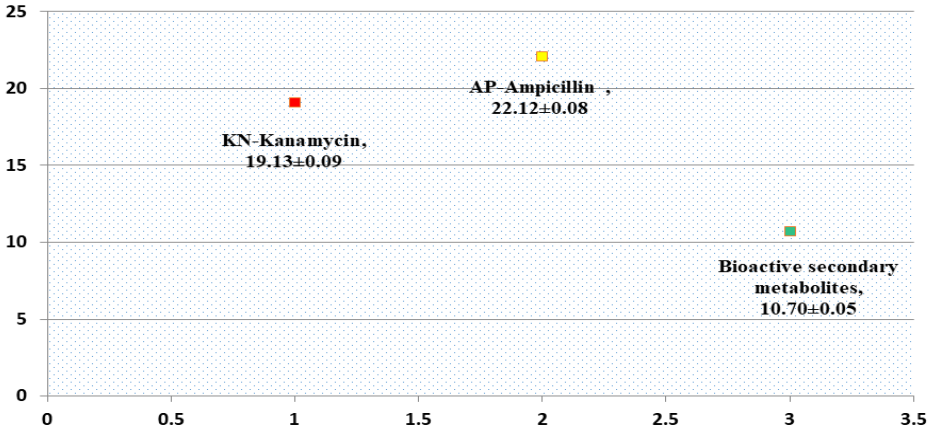


Figure 5. Bioactivity of the ethanolic extract of bioactive secondary metabolites of *Bacillus subtilis* and standard antibiotics GN-Gentamicin and VC-Vancomycin against *Klebsiella pneumoniae*

The extraordinarily active metabolism of the Bacillus species has made it famous for the production of several well-known natural compounds. Iturin, surfactin, fengycin, and cyclic lipopeptide antibiotics are only a few of the important metabolites that Bacillus species create. Some examples of these all-natural cancer treatments are surfactin, streptavidin, and bacillomycin D. They are members of the Bacillus family, which is known to produce many secondary metabolites which have anti-cancer effects. These include terpenes, siderophores, polyketides (PKs), and peptides which are either ribosomally or nonribosomally synthesised. To learn about the possible biological roles of other elements, though, more research is required. Research into the Bacillus genus often leads to the identification of novel active chemicals; this genus is among the most fascinating subsets of natural product manufacturers. New antibiotics have been shown to be abundantly produced by the Bacillus genus. Metabolites produced by Bacillus are abundant and exhibit a wide range of bioactivities. Our understanding of Bacillus's metabolite repertory remains limited [10, 11], even in light of recent studies. Although several bacillus metabolites have been demonstrated to inhibit bacteria and reduce cancer, additional research is needed to confirm their promise as a source of novel medications .

As the leading cause of treatment failure in infectious diseases, antibiotic resistance is an enormous concern with far-reaching consequences. There is an increasing mortality rate due to treatment failure since many bacteria have become resistant to current medications. Although methicillin-resistant Staphylococcus aureus is well-known, other bacteria like Escherichia coli are reportedly resistant to β-lactam medicines and third-generation cephalosporin as well. In addition, the issue of mycobacteria that are resistant to many drugs is becoming worse. Scientists are actively seeking new medications to combat the growing problem of

antibiotic resistance. Antibacterial properties can be derived from a variety of natural sources [12, 13], including microbes and plant secondary metabolites. In instance, fermentation or culture of Gram-positive or Gram-negative bacilli can yield secondary metabolites, which can be used in the search for microbiological sources of natural products. Bacillus sp. is one of the Gram-positive bacilli that produces secondary metabolites that are antimicrobial. Bacillus sp. 4040 produces pumilacidins, Bacillus velezensis iturins and fengycins, and Bacillus subtilis bacillomycins and locillomycins are all examples of Bacillus species that can create antibacterial agents. Bacillus sp. is ubiquitous, appearing in soil environments as well as the deepest parts of the ocean, on land, and in living things. Since several volatile chemicals were discovered as a result of secondary metabolite extraction from Bacillus sp., a metabolomics method can be used by profiling these metabolites using Gas Chromatography Mass Spectrometry (GC-MS) [14-16]. Furthermore, symbiotic Bacillus sp. bacteria found in the Savu Sea sponges had the ability to suppress the growth of E. coli and Klebsiella pneumonia by producing antibiotic compounds. Bacillus sp. was isolated from the digestive tract and stomach contents of sand sea cucumbers (Holothuria scabra) during this study. So yet, this Bacillus sp. has not been investigated for its potential antimicrobial properties. The different species of Bacillus sp. discovered and the uniqueness of their secondary metabolites can be influenced by variances in the location and symbiotic organisms involved in their discovery. A disc-diffusion screening test revealed that one strain of Bacillus subtilis subsp. subtilis HSFI-9 may suppress the growth of Staphylococcus aureus and Escherichia coli.

Conclusion

Recent studies have shown that there are a number of readily available, common household treatments that may be used either in conjunction with conventional therapy or as a substitute for it. Developing nations, who often lack sufficient dental care facilities and financial resources, can greatly benefit from this understanding. *Streptococcus pyogenes* (14.51±0.64) was significantly inhibited by the metabolites of *Bacillus subtilis*.

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