

## **Original Research Article**

### **GC-MS analysis and antimicrobial activity of the extract and fractions of *Bacillus subtilis* subsp. *subtilis* 168 isolated from a river bank in Nigeria.**

#### **Abstract**

The menace of drug resistant pathogens is increasing and their level of evading conventional antimicrobials is rising. It is therefore important to discover new antimicrobials to counter the current challenges. Our preliminary investigation identified *Bacillus subtilis* subsp. *subtilis* 168 isolated from soil sample sourced from a river bank in Abuja, Nigeria, as the most potent antibiotic-producing bacteria among the other identified producers. The current study screened for the antimicrobial activity of the extract and fractions of the isolate by broth microdilution method. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the ratio of the MBC/MIC were determined. All the tested pathogens were susceptible to the ethyl-acetate extract (MIC between 28.70 mg/ml and 57.40 mg/ml). The extract displayed bactericidal activity against all tested pathogens (MBC/MIC between 1.00 and 2.00) while *Proteus mirabilis* was least susceptible. The extract was purified by vacuum liquid chromatography and the fractions challenged with pathogenic strains. The fraction E was the most potent (MIC between 0.09 mg/ml and 0.75 mg/ml) and also bactericidal against all the tested microbes (MBC/MIC between 2.00 and 2.11). GC-MS analysis of the purified sub fraction obtained from fraction E identified 13 compounds with different Retention time and peak areas. These include: bis(2-ethylhexyl) phthalate, 1,4-epoxynaphthalene-1(2H)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-, D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)-, Ergost-5-ene-3,12-diol, 12-acetate, (3.beta.,12.alpha.)-, 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester, Dibutyl phthalate, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, Androst-5-en-3-ol, 4,4-dimethyl-, (3.beta.)-, 2-((3-Methylbutan-2-yloxy) carbonyl)benzoic acid and Cyclononasiloxane, octadecamethyl-. Our findings suggest that *Bacillus subtilis* subsp. *subtilis* 168 isolated locally could serve as a valuable source of lead compounds for pharmaceutical and biotechnological purposes.

**Keywords:** *Bacillus subtilis* subsp. *subtilis* 168, river bank, antimicrobial activity, minimum inhibitory concentration, minimum bactericidal concentration, chromatography, Gas Chromatography-Mass spectrometry.

#### **1.0 INTRODUCTION**

The discovery of the first antibiotic, penicillin, in 1928 marked the beginning of the “golden era” in the 1960s when the bulk of notable groups of antibiotics was discovered and introduced, therefore saving lives [1]. However, antibiotics are currently failing therapy as pathogens are becoming resistant because of the misuse, overuse, extensive agricultural use and horizontal gene transfer [2,3]. Antimicrobial resistance was captured on the list of top 10 threats to global health in 2019 [4]. The

rising cases of antimicrobial resistance present crucial challenge, as a result prompting the urgent need for replacement of the failing antimicrobial agents.

Microbes with capabilities for antimicrobial (antibiotics) production abound in diverse natural habitats world-wide. Isolating these essential strains from their habitats for pharmaceutical use has become vital considering the global challenges of antimicrobial-resistance. For example, *Staphylococcus saprophyticus* SBPS15, *Streptomyces* spp., *Noardiopsis* spp., *Micromonospora* spp., and *Saccharomonospora* spp. isolated from marine sediment, agricultural soils and rhizosphere of some plants have demonstrated potentials for antimicrobial-production [5–7]. Lotfy et al. [8] isolated Di-(2-ethylhexyl) phthalate (DEHP), an antimicrobial compound from fungus *Aspergillus awamori* cultured from a river sample.

Studies have shown that spore-forming microbes are prolific producers of antimicrobial compounds [9–11]. *Bacillus* species are spore-forming, Gram-positive, rod-shaped, aerobic or facultative-anaerobic known for producing antimicrobial molecules. For example, *Bacillus subtilis* remains a known producer of antimicrobial secondary metabolites including cyclic lipopeptides like surfactins, iturins and fengycins [12]. Other metabolites of *B. subtilis* origin include macrolides like 7-O-succinyl-macrolactin A with cytotoxic activity [13]; including miscellaneous compounds like 3,3'-neo-trehalosdiamine and pyranoids both with antibacterial properties [14,15].

In our previous study we isolated and identified some antibiotic-producing bacteria from natural habitats in Abuja, Nigeria. *Bacillus subtilis* subsp. *subtilis* 168 isolated from a river bank was identified and confirmed as the most potent producer. Therefore, the current study is aimed at investigating the antimicrobial activity of this isolate as well as identify the bioactive compounds by Gas Chromatography-Mass spectrometry (GC-MS) method.

## 2.0 METHODOLOGY

### 2.1 Isolation of antibiotic-producing strain.

In our previous study, 5 potential antibiotic-producing bacteria were isolated from some natural habitats [16]. An isolate from soil sample collected from a river bank emerged as the most efficient producer; it was confirmed morphologically, biochemically and molecularly as *Bacillus subtilis* subsp. *subtilis* 168 (Figure 1).



Figure 1: Morphological characteristics of *Bacillus subtilis* subsp. *subtilis* 168 in nutrient agar medium on 24-hour incubation.

## **2.2 Preparation of crude antimicrobial compounds.**

### **2.2.1 Fermentation of the candidate antibiotic-producing isolate.**

The identified *Bacillus subtilis* subsp. *subtilis* 168 was fermented in order to obtain its crude antimicrobial compounds as described by Saravanan et al. [17]. Briefly, 10% of the bacteria suspension (adjusted to 0.5 McFarland standard) was aseptically transferred into 3000 ml Erlenmeyer flask containing 2000 ml of Tryptone-soy broth (TSB) (ThermoFisher, UK) and incubated at  $30\pm 2^{\circ}\text{C}$

for 7 days on a rotary shaker (120 rpm). The pH of the medium was adjusted to 7.0 using 1M NaOH and 1M HCl. The fermented culture broth was centrifuged at 6000 rpm for 10 minutes.

### 2.2.2 Preparation of Bacterial extracts

The supernatant was subjected to solvent extraction method as described by Almalki [18] with slight modifications. For this purpose, ethyl-acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase containing the bioactive compounds was separated from the aqueous phase and transferred into the porcelain basin. It was monitored on water bath at 40°C for complete evaporation to obtain the crude ethyl-acetate extract. To increase the quantity of the extract for downstream applications, the biomass (cells) was suspended in ethyl-acetate in the ratio of 1:1 (w/v) and the mixture was shaken vigorously overnight. The extract was ultimately allowed to evaporate to dryness using a rotary evaporator at 40°C. The crude extracts obtained were finally pooled and subsequently used for antimicrobial activity and also subjected to bioassay-guided chromatography for identification of the bioactive compounds.

**Comment [U1]:** Recast ; Concentrated in vacuo

**Comment [U2]:** Pooled, weighed

**Comment [U3]:** Recast; chromatographic characterization of the bioactive compounds.

### 2.3 Antimicrobial activity of the extract

The extract was challenged with microorganisms supplied by the Department of Microbiology and Biotechnology, NIPRD. The organisms were *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), including clinical isolates like *Bacillus subtilis*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Candida albicans*.

#### 2.3.1 Standardization of the test organisms

This was performed following the colony suspension methods described in the Clinical and Laboratory Standard Institute guidelines [19,20]. Briefly, the microbial suspensions were prepared from 24-hour culture plates by suspending the test organisms in 2 ml of sterilized Mueller-Hilton broth (MHB) (ThermoScientific) and incubated at 37°C for 2 h. Thereafter, the suspension was adjusted to 0.5 McFarland turbidity standard (equivalent to optical density 0.08-0.13 at 600 nm). That corresponded to  $1 \times 10^8$  CFU/ml for the bacteria suspension which was diluted 1:200 in MHB to

obtain final concentration of  $5 \times 10^5$  CFU/ml. The inoculum size for yeast (*C. albicans*) was  $1 \times 10^6$  CFU/ml equivalent to 0.5 McFarland standard. The fungal suspension was diluted 1:400 using MHB to achieve an absolute concentration of  $2.5 \times 10^3$  spore forming unit/ml.

### 2.3.2 *In vitro* antimicrobial assay by Broth Microdilution technique

The crude extract was screened for antimicrobial activity and its minimum inhibitory concentration (MIC) determined by broth microdilution technique [19] with slight modification. The cell-free stock solution of 114.8 mg/ml concentration was prepared by dissolving 1.148 g of the extract in 10 ml of dimethylsulphoxide (DMSO), and filtered using 0.45  $\mu$ m disposable membrane filter unit (Millipore brand). From the start concentration of 57.40 mg/ml, the two-fold serial dilutions of concentration ranges of 28.70-0.22 mg/ml were made using MHB in 96-well microplates (Corning®).

Briefly, 50  $\mu$ l of sterile MHB were dispensed into sterile 96-well microplates from wells 2 to 12 (column A2H2- A12H12). One hundred microliters (100  $\mu$ l) of the cell-free extract with concentration of 57.4 mg/ml were dispensed into the first wells (column A1H1). Fifty microliters (50  $\mu$ l) of the extract from the first wells (column A1H1) was transferred into the second wells (column A2H2). It was mixed thoroughly by pipetting up and down for four times to obtain two-fold serial dilution (1:2 dilution) which continued down to the tenth wells (column A10H10). Fifty microliters were discarded from the 10<sup>th</sup> wells. This resulted in concentrations of 28.40 mg/ml, 14.20 mg/ml, 7.10 mg/ml, 3.55 mg/ml, 1.78 mg/ml, 0.89 mg/ml, 0.44 mg/ml and 0.22 mg/ml (A2:H2-A10:H10) respectively. The wells were inoculated with 50  $\mu$ l of standardized testing organisms from columns 1-9 and 12 (exempt columns 10 and 11). The 10<sup>th</sup> (column A10:H10), 11<sup>th</sup> (column A11:H11) and 12<sup>th</sup> wells (column A12:H12) served as the Drug Sterility Control, Media Sterility Control and Organism Viability Control respectively. Amoxicillin (GlaxoSmithKline) and ketoconazole (Hovid) each at starting concentration of 100  $\mu$ g/ml were used as positive controls for the bacteria and the yeast while the DMSO served as negative control. The plates were incubated at  $37 \pm 2^\circ\text{C}$  for 24 h for bacteria and 48 h for yeast.

Antibacterial activity of the extract was confirmed by adding 40  $\mu$ l of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) as microbial growth indicator to all the wells and incubated further at  $37^\circ\text{C}$  for 30 minutes as described by Eloff [21]. Growth was confirmed in the wells that changed to red

color. The lowest concentrations where no visible growth occurred were taken as the MIC endpoint (the first clear well) and recorded as the MIC value. All assays were carried out in triplicate.

The determination of MBC was performed as described by El-Azizi [22]. The MBC was recorded as a lowest concentration of the extract that killed 99.9% of the test bacteria after 24 h incubation at 37°C. Ten microliters (10µl) were taken from the MIC well and two wells above the MIC and spread on MHA plates. The number of colony was counted after incubation at 37°C for 24 h. The concentration of the sample that produces < 10 colonies was recorded as MBC value.

## 2.4 Bioassay-guided fractionations of the isolated extract

The fractionations of the crude ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 were performed according to Oladosu et al. [23] methodology with slight modification.

The extract (24.70 g) was chromatographed on a silica gel column (particle size 90% < 55 µm) loaded on a 1000 ml vacuum flask connected to B100 DEC pressure pumps device (Charles Austen). The mobile phase was methanol, ethyl-acetate, n-hexane and water. The column was gradually eluted with solvent mixtures to extract both the polar and non-polar components by stepwise increase in polarity. This includes n-hexane: ethyl-acetate (5:0, 4:1, 3:2, 2:3, 1:4, 0:5 v/v), ethyl-acetate: methanol (5:0, 4:1, 3:2, 2:3, 1:4, 0:5 v/v), and methanol: water (5:0, 4:1, 3:2, 2:3, 1:4, 0:5 v/v) gradients. Eighteen (18) fractions were finally obtained and were combined to form 6 fractions.

### 2.4.1 *In vitro* antimicrobial assay of the fractions.

Only 5 out of the 6 fractions produced enough quantity for testing and were also screened for antimicrobial activity by broth microdilution technique [19]. Two-fold serial dilutions of start concentration not more than 1.6 mg/ml of each fraction according to Koeth et al. [24] was prepared to challenge standardized pathogenic microbes in the 96-well microplates. Amoxicillin and ketoconazole (each at starting concentration of 100 µg/ml) were also used as positive controls for the bacteria and the yeast while the negative control was DMSO. The plates were incubated at 37±2°C for 24 h for bacteria and 48 h for yeast. TTC served as microbial growth indicator and the MICs and MBCs were determined.

#### 2.4.2 Fractionations of the fraction E extract by Column Chromatography

The fraction E extract which showed the strongest antimicrobial activity was further purified by the column chromatography technique. The glass pipette column was uniformly packed using 6.0 g VLC-graded silica gel powder as the stationary phase and glass blocked at the exit end using glass wool plug.

The fraction E extract (4.53 g) was homogenized with 10 ml methanol. Vacuum Liquid Chromatography-graded silica gel powder (6.0 g) (particle size 90% < 55  $\mu\text{m}$ ) was added to the solution and thoroughly mixed to slurry form. The slurry-form mixture was kept in well-ventilated space at 28°C for 24 hrs for complete evaporation of the solvents. The dried extract mixture was loaded on packed-column and the stepwise gradient of the mobile phase was added at interval to effect the elution. The mobile phase was ethyl-acetate, methanol and water. The prepared column was eluted with gradients of solvent mixtures which were ethyl-acetate/methanol (5:0, 2:3, 1:4, 0:5 v/v), and methanol/water (4:1, 3:2 v/v) respectively. The sixth procedure (methanol/water, 3:2), the column appeared exhausted as the silica gel appeared colorless. The sub fractions obtained were allowed to dry at room temperature in well-ventilated space in preparation for the Thin Layer Chromatography (TLC).

#### 2.4.3 Purification of the active compounds by TLC

The isolated sub fractions were tested for their complexity by TLC. The 6 sub fractions obtained from fraction E extract after chromatography were spotted at 1.0 cm from the base of a 9.5 x 10.5 cm silica gel plate (200  $\mu\text{m}$ ). Approximately 10  $\mu\text{l}$  from each sub fraction were spotted separately on the TLC plate and left to dry for 15 minutes. The spots were samples from elutes produced as a result of the following solvents as the mobile phase: spot #1 was from ethyl-acetate alone elute; spot #2 was from ethyl-acetate: methanol (2:3); #3 was from ethyl-acetate: methanol (1:4); #4 was from methanol alone; #5 and 6 were from methanol and water (4:1). The plate was developed using a 3: 2 ethyl-acetate: methanol mobile phase. Afterwards, the side of the plate close to the samples spots was suspended in the solvent system in the developing chamber (the spotting line was about 0.4 cm away from the mobile phase). The solvent travelled upward by capillary system by passing through the

spotted line. The migration was aborted as the mobile phase approached the solvent front (7.4 cm). The chromatogram was removed from the chamber and air-dried for 10 minutes. The separated compounds were visualized under the UV at a wavelength of 365 nm. The Retention Factor (RF) values for the separated bands on the chromatogram were determined (the ratio of the distant travelled by the solute to the distant travelled by the solvent).

### 2.5 GC-MS analysis of the most active fraction.

The bioactive compounds present in the pure sub fraction (from the most active fraction E) obtained from the ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 were determined using GC-MS according to the method described by Ugbabe et al. [25]. The analysis was performed using GC-MS QP 2010 SE (Shimadzu Corporation, Japan). The QP-2010 Mass Selective Detector (MSD) operated in the electron ionization mode (electron energy) of 70 eV, scan range of 45-700 amu, scan rate of 3.99 scans/sec, and Shimadzu GC-MS solution data system. The Gas Chromatography column was Optima-5 ms fused silica capillary with 5% phenyl-methyl polysiloxane stationary phase, with length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25  $\mu$ m. Helium gas was used as the carrier gas with a flow rate of 1.61 ml/min. The program used for Gas chromatography oven temperature was 60°C for 1 min, and then increased from 60°C to 240°C at a rate of 15°C/min. That was sustained at 240°C for 2 min, followed by an increase from 240°C to 300°C at a rate of 12°C/min, then again held at 300°C for 5 min. The injection port temperature was 250°C while detector temperature was 300°C. The diluted sample (1:100 in hexane, v/v) of 1.0  $\mu$ l was injected using auto sampler in the split mode with a ratio of 20:80.

The qualitative identification of all the detected chemical components was determined by comparing their mass spectra with annotated compounds in the National Institute of Standards and Technology (NIST) Mass Spectra library (NIST 11). The relative amount or quantity of each compound was calculated as the percentage of the peak area relative to the total peak area.

## 3.0 RESULTS AND DISCUSSION

Screening for lead compounds for the replacement for the currently failing conventional antimicrobial agents is crucial to the global solution to the challenges of antimicrobial resistant microorganisms. Microbes have been excellent sources of bioactive molecules, as

majority of antimicrobials currently in use are of microbial origin [26]. Antimicrobial-producing microorganisms have been isolated from natural habitats like marine, river, endophytes, soil etc [27,28]. In the present study, ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 isolated from a river bank was challenged with some pathogenic strains; the extract was chromatographed and the most potent fraction was analyzed by GC-MS.

### 3.1 Extraction of crude bioactive metabolites from fermented broth

The ethyl-acetate extract of the fermented antibiotic-producing isolate produced a final yield of 24.7g crude extract.

#### 3.1.1 Antimicrobial activity of the ethyl-acetate extract

Table 1 summarizes the antimicrobial activity of ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 as determined by the broth microdilution method.

The ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 displayed inhibitory activities against 2 Gram-positive bacteria including *Staphylococcus aureus* (ATCC 25923) (MIC: 57.40 mg/ml) and *Bacillus subtilis* (MIC: 57.40 mg/ml) (Table 1). The extract inhibited 5 Gram-negative bacteria including *Escherichia coli* (ATCC 25922) (MIC: 57.40 mg/ml), *Pseudomonas aeruginosa* (ATCC 27853) (MIC: 57.40 mg/ml), *Klebsiella pneumoniae* (MIC: 28.70 mg/ml), *Salmonella typhi* (MIC: 57.40 mg/ml) and *Proteus mirabilis* (MIC: 57.40 mg/ml) including a yeast, *Candida albicans* (MIC 28.70 mg/ml). Susceptibility of *P. mirabilis* to the extract is contrary to the previous report where the organism showed resistance [16]. This could be due to the high concentration of the antibacterial metabolites in the extract than the filtrate. The MIC values of the ethyl-acetate extract are notably higher (between 28.70 and 57.40 mg/ml) than the MIC breakpoint of the control antimicrobials (amoxicillin between 1.56 and 100 µg/ml; ketoconazole at 100 µg/ml). The extract was bactericidal against Gram-positive *S. aureus* (ATCC 25923) and *B. subtilis*, each with a MIC of 57.40 mg/ml and MBC also of 57.40 mg/ml (MBC/MIC=1.00). The extract was also bactericidal against the Gram-negative *E. coli* (ATCC 25922) (MBC/MIC=1), *P. aeruginosa* (ATCC 27853) (MBC/MIC=1.00), *K. pneumonia* (MBC/MIC=2.00), and *S. typhi* (MBC/MIC=1.00). Likewise, the extract showed

bactericidal activity against the yeast *C. albicans* with a MIC of 28.70 mg/ml and MBC of 57.40 mg/ml (MBC/MIC=2.00). The MBC value of the extract against all the tested isolates was 57.40 mg/ml, however there was no MBC against *Proteus mirabilis*. Therefore, *Proteus mirabilis* was the least susceptible of all the isolates tested.

3.2 Chromatographic fractionations of the extract

Fractionations of the extract obtained from the candidate isolate performed by adsorption chromatography (vacuum liquid chromatography) produced a total of eighteen fractions which was combined to form 6 fractions. However, only 5 (fractions A, B, C, D and E) which produced enough quantity were screened for antimicrobial activity.

3.2.1 Antimicrobial activity of the fractions by Broth Microdilution Method

Of the 5 fractions tested, the fraction E displayed the highest MIC values (Table 1). It exhibited activity against all the tested pathogens with the MIC range between 0.09 mg/ml and 0.75 mg/ml. It inhibited 2 Gram-positive bacteria vis *Staphylococcus aureus* (ATCC 25923) (MIC: 0.09 mg/ml) and *Bacillus subtilis* (MIC: 0.75 mg/ml). Likewise, the fraction displayed activity against four (4) Gram-negatives bacteria like *Escherichia coli* (ATCC 25922) (MIC: 0.09 mg/ml), *Pseudomonas aeruginosa* (ATCC 27853) (MIC: 0.37 mg/ml), *Klebsiella pneumoniae* (0.37 mg/ml) and *Salmonella typhi* (MIC:0.75 mg/ml) including a yeast, *Candida albicans* (MIC: 0.19 mg/ml). Meanwhile, the MIC values of the fraction E are considerably higher (between 0.09 and 0.75 mg /ml) than the breakpoint of the control antimicrobials (1.56 and 100 µg/ml). Fraction E was bactericidal against Gram-positive *S. aureus* (ATCC 25923) (MBC/MIC=2.11) and *B. subtilis* (MBC/MIC=2.00). The fraction was also bactericidal against the Gram-negative pathogens including *E. coli* (ATCC 25922) (MBC/MIC=2.11), *P. aeruginosa* (ATCC 27853) (MBC/MIC=2.03), *K.pneumonia* (MBC/MIC=2.03), and *S. typhi* (MBC/MIC=2.00). Likewise, the extract also showed bactericidal activity against the yeast *C. albicans* (MBC/MIC= 2.00).

Table 1: Summary of antimicrobial activity of the extract and fractions obtained from *Bacillus subtilis* subsp. *subtilis* 168 by broth microdilution method.

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	Minimum Inhibitory Concentration												
	EA	MBC <sup>EA</sup>	MBC <sup>EA</sup> /MIC <sup>EA</sup>	(mg/ml)						MBC <sup>FE</sup>	MBC <sup>FE</sup> /MIC <sup>FE</sup>	(μg/ml)	
				FA	FB	FC	FD	FE	A			K	
<i>S. aureus</i> (ATCC 25923)	57.40	57.40	1.00	+	0.34	0.31	1.38	0.09	0.19	2.11	1.56±0	NT	
<i>B. subtilis</i>	57.40	57.40	1.00	+	+	0.31	0.69	0.75	1.50	2.00	50.0±0	NT	
<i>E. coli</i> (ATCC 25922)	57.40	57.40	1.00	+	+	0.61	0.69	0.09	0.19	2.11	1.56±0	NT	
<i>P. aeruginosa</i> (ATCC 27853)	57.40	57.40	1.00	+	0.17	0.38	1.38	0.37	0.75	2.03	100±0	NT	
<i>K. pneumonia</i>	28.70	57.40	2.00	+	0.34	0.61	0.34	0.37	0.75	2.03	50.0±0	NT	
<i>P. mirabilis</i>	57.40	-	-	NT	NT	NT	NT	NT	NT	-	100±0	NT	
<i>S. typhi</i>	57.40	57.40	1.00	+	0.68	0.61	0.69	0.75	1.50	2.00	3.13±0	NT	
<i>C. albicans</i>	28.70	57.40	2.00	+	+	0.31	0.69	0.19	0.38	2.00	NT	100±0	

+: No activity (Growth); E

A: Ethyl-acetate extract; FA: Fraction A extract; FB: Fraction B extract; FC: Fraction C extract; FD: Fraction D extract; FE: Fraction E extract; A: Amoxicillin (antibiotic positive control); K: Ketoconazole (antifungal positive control); NT: Not tested; MIC<sup>EA</sup>: Minimum Inhibitory Concentration of EA; MBC<sup>EA</sup>: Minimum Bactericidal Concentration of EA; MBC<sup>EA</sup>/MIC<sup>EA</sup>: ratio of Minimum Bactericidal Concentration of EA and Minimum Inhibitory Concentration of EA; MIC<sup>FE</sup>: Minimum Inhibitory Concentration of FE; MBC<sup>FE</sup>: Minimum Bactericidal Concentration of FE; MBC<sup>FE</sup>/MIC<sup>FE</sup>: ratio of Minimum Bactericidal Concentration of FE and Minimum Inhibitory Concentration of FE.

### 3.3 Chromatographic fractionation of the fraction E extract

The fractionation of the most potent fraction E resulted in six (6) sub fractions.

### 3.3.1 Purification of active metabolites by Thin-Layer Chromatography (TLC)

Figure 2 shows the chromatogram from TLC of the sub fraction from fraction E visualized under the UV at a wavelength of 365nm.

The separation of compounds was observed by the display of spots or smudge on the plate. The RF values for the separated compounds were evaluated. The sub fraction spotted on lane 1 displayed a bold violet pale blue complex spot of distance 5.8 cm and an RF value of 0.8. However, the sub fraction spotted on lane 2 showed a pale-blue spot also of distance 5.8 cm and an RF value of 0.8. The sub fraction spotted on lane 3 displayed a smudge including a band that covered a distance of 4.7 cm and an RF value of 0.6. The sub fractions spotted on lanes 4,5 and 6 showed slight smudges with no visible migration. The sub fraction spotted on lane 2 with a visibly distinct spot signifies the presence of pure compounds; it was therefore, considered for compound identification by the GC-MS analysis.

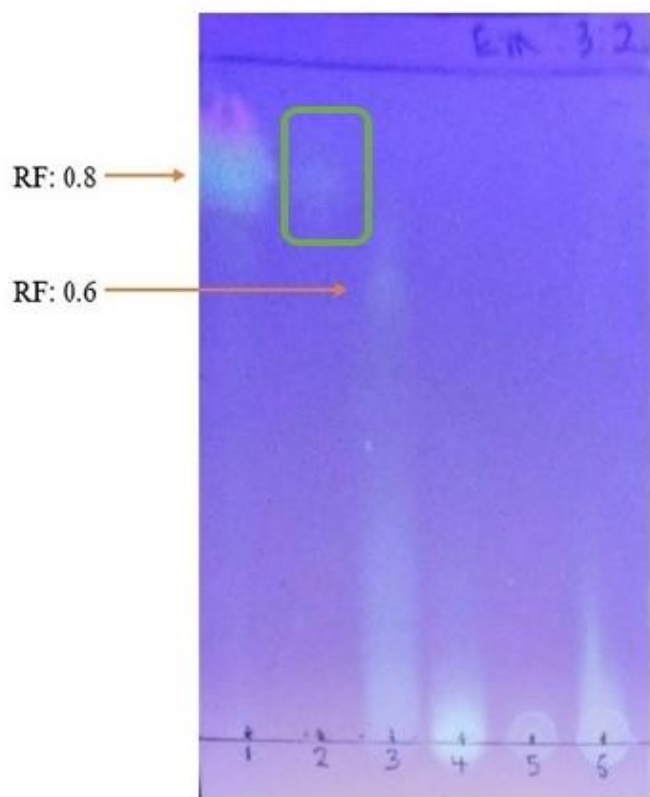


Figure 2: The chromatogram of the 6 sub fractions obtained from fraction E viewed under the UV light at 365nm. Green circle on lane 2 encloses a distinct pale-blue band; E: Ethyl-acetate; M: Methanol; Solvent Ratio of 3:2; RF: Retention factor

### 3.4 GC-MS analysis of the most active fraction

GC-MS analysis of the pure sub fraction 2 (from the most active fraction E) obtained from the ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168 identified thirteen (13) chemical compounds (Table 2 and Figure 3). Given the peak area of the molecular mass, three major compounds including others were identified.

Among the confirmed three (3) major compounds is bis(2-ethylhexyl) phthalate, a phthalate ester at Retention time (Rt) of 18.29 and peak area (%) of 33.8. Masrukhin et al. [29] similarly reported bis(2-ethylhexyl) phthalate as a major compound from the GC-MS profile of active compounds in the ethyl-acetate extract of a *Bacillus* spp. (*Bacillus siamensis*). Studies have confirmed the antibacterial, antifungal and anticancer activities of bis(2-ethylhexyl) phthalate [29,30].

The next major compound identified is 1,4-epoxynaphthalene-1(2H)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro- at Rt of 21.35 and peak area (%) of 26.80. This compound has also been identified in the extracts of actinomycetes, *Nannochloropsis* sp. (a microalgae), and *Aspergillus terreus* [31-33]. Except for its insecticidal properties [34], reports on other biological activities of 1,4-epoxynaphthalene-1(2H)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro- are scarce.

The third major compound identified is D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)- at Rt of 20.14 and peak area (%) of 18.30. Until now, D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)- have been identified in the leave extracts of some medicinal plants [35,36]. Currently, the biological activities of the compound are yet to be studied.

Another identified compound is Ergost-5-ene-3,12-diol, 12-acetate, (3.beta.,12.alpha.)- at Rt of 20.76 (3.98%). The biological activity of the compound is yet to be reported.

Also identified is 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester at Rt of 13.11 (3.68%). Studies have reported the antibacterial and antifungal activities of this compound [37,38]. The compound 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester have been identified from the extracts of *Lysinibacillus sphaericus* KJ872548 (a bacterium) and *Ulva fasciata* (marine macroalgae) with significant biocontrol activities [37,38].

Among the identified compounds is Dibutyl phthalate, a phthalate compound, at Rt of 13.44 and 13.60 (2.34%; 2.26%) respectively. Studies have reported the broad-spectrum antibacterial, antifungal, including mosquito larvicidal activities of the compound [39–41]. Dibutyl phthalate has also been identified in the extracts of *Brevibacterium mcbrellneri*, *Streptomyces* strain KX852460 including a fungus *Fusarium geni* (an endophytic strain from *Rumex madaio*) [39–41].

Another identified compound is 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester at Rt of 12.79, 13.26 and 13.80 (2.02%; 1.95%; 1.22%). Studies have reported the antimicrobial and anticancer activities of this compound [42,43].

Also identified is Androst-5-en-3-ol, 4,4-dimethyl-, (3.β.)- at Rt of 20.47 (1.35%). Currently, there is no report on the biological activity of this compound.

Another compound identified is 2-((3-Methylbutan-2-yloxy) carbonyl)benzoic acid at Rt of 13.50 (1.17%). The biological activities of this compound is yet to be reported.

Cyclononasiloxane, octadecamethyl- at Rt of 24.45 (1.14%) was also among the identified compounds. Ahsan et al. [39] reported the antifungal activity of this compound in synergy with other compounds with promising capability as biocontrol agent.

This is the first study to report these 13 compounds from the secondary metabolites of antibiotic-producing *Bacillus subtilis* subsp. *subtilis* 168 sourced from a river bank soil. All the compounds identified are contrary to the compounds reported by Matloub et al. [44] who characterized the bioactive compounds of hexane extract of endophytic *Bacillus subtilis* NCIB 3610 (both intracellular and extracellular) by GCMS. This could be due to the different solvents employed during the extraction process. Swamy et al. [45] have reported that extraction with different solvents can influence the outcome of chemical composition of biological extracts.

The antimicrobial activities of the extract and the fractions of *Bacillus subtilis* subsp. *subtilis* 168 could be due to the independent and or synergistic activities of compounds like bis(2-ethylhexyl)

phthalate, 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester, Dibutyl phthalate, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, and Cyclononasiloxane, octadecamethyl-.

Interestingly, the biological activities of 4 among the identified compounds are yet to be reported which need to be investigated. The compounds include (i) D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.β.)-, (ii) Ergost-5-ene-3,12-diol, 12-acetate, (3.β.,12.α.)-, (iii) Androst-5-en-3-ol, 4,4-dimethyl-, (3.β.)- (iv) 2-((3-Methylbutan-2-yloxy)carbonyl)benzoic acid.

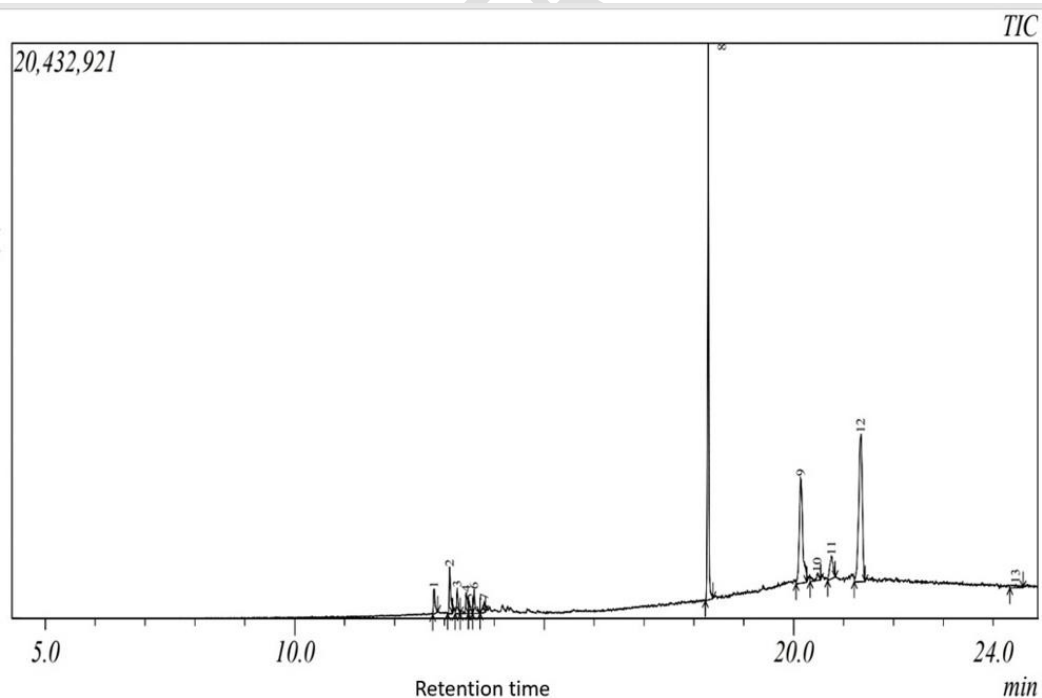


Figure 3: Chromatogram showing identified compounds from the most active purified fraction from ethyl-acetate extract of *Bacillus subtilis* subsp. *subtilis* 168. TIC: Total Ion Chromatogram. 1: 12.793 min; 2: 13.106 min; 3: 13.256 min; 4: 13.441 min; 5: 13.497 min; 6: 13.595 min; 7: 13.797 min; 8: 18.287 min; 9: 20.141 min; 10: 20.472 min; 11: 20.760 min; 12: 21.345 min; 13: 24.447 min.

Table 2: Summary of GC-MS analysis of most active fraction E

S/No	RT (min)	Identified Compounds	Peak area (%)	Formula	Molecular weight
1	12.793	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	2.02	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278
2	13.106	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	3.68	C <sub>20</sub> H <sub>30</sub> O <sub>4</sub>	334
3	13.256	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1.95	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278
4	13.441	Dibutyl phthalate	2.34	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278
5	13.497	2-((3-Methylbutan-2-yloxy)carbonyl)benzoic acid	1.17	C <sub>13</sub> H <sub>16</sub> O <sub>4</sub>	236
6	13.595	Dibutyl phthalate	2.26	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278
7	13.797	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1.22	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278
8	18.287	Bis(2-ethylhexyl) phthalate	33.80	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	390
9	20.141	D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)-	18.30	C <sub>30</sub> H <sub>50</sub> O	426
10	20.472	Androst-5-en-3-ol, 4,4-dimethyl-, (3.beta.)-	1.35	C <sub>21</sub> H <sub>34</sub> O	302
11	20.760	Ergost-5-ene-3,12-diol, 12-acetate, (3.beta.,12.alpha.)-	3.98	C <sub>30</sub> H <sub>50</sub> O <sub>3</sub>	458
12	21.345	1,4-Epoxy naphthalene-1(2H)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-	26.80	C <sub>23</sub> H <sub>36</sub> O <sub>2</sub>	344
13	24.447	Cyclononasiloxane, octadecamethyl-	1.14	C <sub>18</sub> H <sub>54</sub> O <sub>9</sub> Si <sub>9</sub>	666

RT: Retention time

#### 4.0 CONCLUSION

The present study indicates that ethyl-acetate extract and fractions of *Bacillus subtilis* subsp. *subtilis* 168 isolated from soil sample from a river bank exhibited promising antimicrobial activity against the tested pathogenic strains. Fraction E showed the strongest activity among all the fractions and both the extract and fraction E displayed broad-spectrum and bactericidal activities. GC-MS analysis of fraction E identified 13 chemical compounds at different Retention time. The peak area of the molecular mass showed 3 major compounds which includes bis(2-ethylhexyl) phthalate (33.8%), 1,4-epoxynaphthalene-1(2H)-methanol, 4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro- (26.8%) and D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)- (18.3%). The biological activities of nine compounds have been reported while remaining four need to be investigated as this could also be resourceful for biotechnology, pharmaceutical and or biocontrol purposes. The 4 compounds include: (i) D:B-Friedo-B':A'-neogammacer-5-en-3-ol, (3.beta.)-, (ii) Ergost-5-ene-3,12-diol, 12-acetate, (3.beta.,12.alpha.)-, (iii) Androst-5-en-3-ol, 4,4-dimethyl-, (3.beta.)- (iv) 2-((3-Methylbutan-2-yloxy)carbonyl)benzoic acid.

#### COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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