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Thin Layer Chromatography Bio-autography guided identification of Antibacterial constituents of leaf extract of Stereospermum kunthianum Cham. (Bignoniaceae)

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ABSTRACT

Background: There is an incessant quest for the discovery of novel antibacterial agents. *Stereospermum kunthianum* Cham. (Bignoniaceae) is used in traditional medicine in Nigeria and other parts of Africa in the treatment of venereal diseases e.g., syphilis, wounds, refractory cough, malaria, tooth ache leprosy etc. The present work investigates the antimicrobial activity and phytochemical constituents of the ethanolic leaf extract of the plant.

Methods: Ethanolic leaf extract of *S.Kunthianum* was obtained by macerations method. Agar well diffusion method was used to carry out preliminary antimicrobial susceptibility testing. The test was carried out at extract concentration of 100, 200 and 400 mgmL^{-1}. The agar dilution method was used to determine the Minimum Inhibitory Concentration (MIC) of the extracts. Phytochemical screening was conducted using standard procedures. Thin layer chromatography bioautography analysis was carried out using the agar overlay method. Six micro-organisms including two gram positive- *Bacillus subtilis* and *Staphylococcus aureus*, two gram negative *Salmonella typhi* , and *Pseudomonas aeruginosa*, two fungi, *Aspergillus niger* and *Candida albicans* were used as test organisms.

Results: The ethanol extracts of *S.kunthianum* leaves showed antimicrobial activity against *B.subtilis*, *S.aureus* and *S.typhi* with zones of Inhibition ranging from 13±1.41 to 29.5 ± 1.00 mm and MIC values ranging from 16 -64 mgmL^{-1}. Phytochemical screening revealed the presence of saponins, flavonoids and terpenoid compounds

Conclusion: The study showed that the antibacterial activities of the leaf extract are probably due to polyphenolic and steroidal compounds.

1. Introuction.

Medicinal plants have been used in folkloric medicine to treat infectious diseases. Examples include the leaves of *Mangifera indica* L. (Anarcadiaceae) [1]. *Ocimum gratissimum* L. (Lamiaceae) [2] . *Xylopia aethiopica* (Danal) A. Rich (Annonanaceae), *Alchornea cordifolia* (Schumach & Thonn.) Mull.Arg. (Euphorbiaceae), *Azadiractha indica* A..Juss. (Meliaceae) [3], *Phylantus amarus* Schum. & Thonn. (Euphorbiaceae) [4]. The secondary metabolites present in these plants account for their antimicrobial activities. Combrestatin, Geranium, Bereberine are examples of alkaloids, flavonoids and terpenoids respectively that studies have shown to have antimicrobial activities. [5,6,7]. Antimicrobial resistance remains a global health crisis[8]. New strains of microbes with resistance to current antimicrobial agents are constantly emerging. This is putting at risk the ability to treat common infections in the community and hospital. Globally coordinated steps must be taken to prevent the world from going into a post antibiotic era where minor injuries and common infections can lead to the death of patients [9]. Hence there is an urgent need for the search for novel infection-fighting agents [10].

Natural products have for centuries been used as anti-infective agents. A number of antibiotics in use today were derived from nature. Examples include penicillin from the fungi *Penicillium chrysogenum* discovered in 1928 by Alexander Fleming a Scottish bacteriologist [11], Tetracyclin from *Streptomyces auriofaciens* [12], Vancomycin from *Amycolatopsis orientalis* [13], Erythromycin from *Saccharopolyspora erythraea* [14].

Stereospermum kunthianum is a slender tree about 15 m high, in the wood savanna and less (5-6 m) when in the drier Sahel region. It is present throughout the region and widespread across Africa to the Red sea and Southwards to Malawi, the Congo basin and Angola. In Nigeria the root, along with other roots is a remedy for a disease called rana with symptoms of haematuria. It is used in Senegal internally and externally in the treatment of primary syphilis. Roots and leaves are given by the Fula in Senegal for venereal and respiratory diseases and gastritis [15]. The plant has hypoglycaemic effect, used to treat haemorrhoids, kidney stones and malaria. It is also used as an aphrodisiac. The twigs are chewed to clean teeth and to treat toothache [16]. The plant is also used traditionally to treat wounds, rheumatic arthritis, ulcers, dysentery and leprosy [17]. Anti-inflammatory and antiplasmodial activities of the plant have been reported in literature [17,18] . Compounds with the activities have also been isolated from the plant. Examples include stereospermin and sterekuthal A and B respectively [17]. However, there is no report in literature on the isolation of antibacterial phytoconstituents of the leaf extract. In this study we investigate the antibacterial phytoconstituents of the ethanolic **leaf extract** by TLC guided bioautography as a preliminary step for the isolation of antibacterial compounds from the leaf extract.

2. Materials and methods

Muller Hinton and Saboraund dextrose agar (Himedia Laboratories, India.), Clotrimazole (Drugfield Pharmaceuticals, Nigeria), Levofloxacin (USP), TLC plate (Merck, Germany), Chloroform (Supelco, Merck, Germany), Methanol (Sigma Aldrich, Germany), Ethanol, Sulphuric acid, Ferric chloride, Ammonia solution, Sodium Chloride and Vanillin (BDH Laboratory, England), Methyl thiazolyl tetrazolium salt (Merck, Germany).,

2.1 Collection and Preparation of Plant.

Stereospermum Kunthianum leaves collected from Ogun State in South-Western Nigeria in the month of August 2014. Identity of the plant was confirmed at the herbarium of the Department of Botany, Faculty of Science, University of Lagos with voucher number LUH 6287 . Leaves were dried in an oven at 40°C and ground using mechanical grinder then the powder was used for extraction.

2.3 Extraction of Plant material

Stereospermum Kunthianum

The powdered plant material (350 g) was macerated with absolute ethanol with changing of the solvent every 72 h for 9 days. The combined extract was concentrated using a rotary evaporator at 50°C. Further drying was done in a desiccator.

2.4 Preliminary Phytochemical Screening

2.4.1 Screening for Polyphenols

About 0.5 g of plant material was dissolved in 10 mL of water. It was boiled for 15 min and was filtered using a funnel plugged with cotton wool. About 3 drops of 60% ferric chloride was added to 2 mL of the filtrate. Formation of a deep coloured precipitate indicates the presence of polyphenolic compounds [19].

2.4.2 Screening for Alkaloids

About 0.5 g of the plant extract was dissolved in about 10 mL of 2% Sulphuric acid. A little quantity of the resulting solution was tested with Dragendorff's reagent. A brick red precipitate suggests the presence of alkaloids. The acidic extract was basified with ammonia. This was confirmed by a change in colour of litmus paper from red to blue. The resulting solution was extracted with 2 successive 5 mL portions of chloroform. Further purification was done by extracting the chloroform extract with two successive 5 mL portions of 2% Sulphuric acid. 2 drops of Dragendorff's reagent was added to the acidic extract. A brick red precipitate confirms the presence of alkaloids [19].

2.4.3 Screening for Saponins

About 0.5 g of the plant extract was shaken with water in a test tube, frothing, which persists on warming was taken as preliminary evidence for the presence of saponins.

About 0.1g of plant extract was dissolved in water. 2 mL of this was dissolved in 0.18% solution of Sodium Chloride. This dilution gives 0.9% solution of sodium chloride. About 5 drops of blood was added to the test tube. This was achieved by using a sterile syringe to prick the tip of the finger. A negative control was also done using 2mL of distilled water instead of the extract. *Vernonia amygdalina* filtrate was used as positive control. Observation for

haemolysis was done after 48 h. A change in the intense red colour of the filtrate with sedimentation at the bottom of the test tube indicates haemolysis of red blood cells [19].

2.4.4 Screening for anthraquinones Bontrager's test

About 0.5 g of the extract was taken into dry test tube, 5 mL chloroform was added and shaken for 5 min. The extract was filtered, and the filtrate shaken with an equal volume of dilute ammonia solution. A pink violet or red colour in the ammoniacal layer (lower layer) indicates the presence of free anthraquinones [19].

2.5 Subculturing of organisms

The test organisms were clinical isolates obtained from the pharmaceutical microbiology Laboratory of the Faculty of Pharmacy University of Lagos. The clinical strains include *Bacillus subtilis Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhosa, Candida albicans* and *Aspergillus niger*

2.6 Antimicrobial Susceptibility Test.

The agar well diffusion method was adopted to test for the antimicrobial activity of the extract on the test organisms. Sterile media plates of nutrient agar were prepared for the bacteria and fungi using Muller Hinton agar and Saboraund dextrose agar respectively. These plates were then separately flooded with diluted standardized overnight cultures and then drained off to remove excess. Wells of 6mm diameter were made in triplicate in each plate with a central well for the control using 6mm sterile cork borer. The wells were filled with 0.1mL of diluted concentrations of extract with the aid of sterile pipettes per well. 400, 200, and 100 $mgmL^{-1}$ of extract were used, and $1mgmL^{-1}$ of the standard antibiotics (levofloxacin 500 mg and clotrimazole 50 mg) was used as positive controls. Sterile distilled water was used as negative control on a separate plate. Diameters of zones inhibition were measured after incubating the plates at 37∘C for 18 h (bacteria) and 24 h (fungi). The plates were replicated in duplicate and the diameter of zones of inhibition were recorded.

2.7 Minimum Inhibitory concentration

The antibacterial potency of the crude plant extract in variable concentrations was determined in terms of minimum inhibitory concentration (MIC) using agar dilution method [20]. The variable concentrations were prepared in petri dishes as 0.5,1.0,2,4,8,16,32,64,128 and 256 $mgmL^{-1}$ for the experiment. The corresponding volumes of agar required for the dilutions were measured into amber bottles, autoclaved, and was melted in the water bath and allowed to cool before use. The corresponding volumes of extract were poured into the bottles containing the agar to give desired concentrations. The bottles were swirled for proper mixing after which the content was transferred into the pre-labelled petri dishes. This was carried out in the Lamina flow hood and the rest of the procedure was carried out around a Bunsen flame. The agar was allowed to set. The organisms were inoculated by dropping the suspension of organisms on the surface of the agar within the marked segments. The petri dishes were incubated at 37°C and observation was made after 48hr of incubation. Then the concentration at which no growth of a particular organism was observed was taken to be the Minimum inhibitory concentration (MIC) for that organism. A control was also done by inoculating the same organisms on plain Muller-Hinton agar without extract.

2.8 Thin Layer chromatography Characterization of leaf extract

The presence of active metabolites in the extract was evaluated by TLC plates. The analytical normal phase TLC was performed on Aluminium backed pre-coated silica gel TLC plates. , and activated in the oven at 110°C for about 5 min. The solvent system chloroform: methanol (9:7: 0.3) was used. About 10 µL of extract was spotted on the silica plates. The experimental plates were developed in a chromatographic chamber, saturated by 10 mL of the solvent system. Plate development required about 15 to 20 minutes; it was then visualized in daylight and under ultraviolet light at 254 .and 366 nm. Some plates were also sprayed with iodine, 1% vanillin in Conc. sulphuric acid and ferric chloride. Rf values of bands were recorded.

2.9 TLC Bioautography

In order to find out the bioactive phytoconstituents from the TLC plate, TLC Bio-autography by agar overlay method was performed [21] Muller Hinton agar (25 mL) was inoculated with 1 mL of all the organisms separately. The resulting mixture was thoroughly stirred to allow for even distribution of the organisms in the agar. The mixture of the agar and organism was smeared over the already developed TLC plates. The plates were left on the bench overnight in order to allow for diffusion of the active components. The plates were kept in moist chambers and incubated at 37°C for 24 h overnight. Upon incubation, the plates were sprayed with a 1%w/v solution of Methyl thiazolyl tetrazolium salt (MTT). The parts of the plate with growth of microorganisms produce a red colour while cream spots

are seen around the spots with antimicrobial activity.

3. Results

3.1 Extract yield

The percentage yield for the *S. kunthianum* ethanol extract was 6.12% with dark green appearance and gummy texture.

3.2 Phytochemical Analysis

Phytochemical screening revealed the presence of Phenolics and Saponis.

3.3 Antimicrobial preliminary screening

Antimicrobial Activity preliminary screening of the plant extract revealed promising antibacterial effect on 3 organisms, *S. aureus, S. typhi* and *B. subtilis* at 200 and 400 $mgmL^{-1}$ with zones of inhibition in diameter ranging from 13- 29.5 mm and not effective against *P. aeruginosa*. However, the extract was not effective against *A. niger and C. albicans* at the tested concentrations (Table 2).

The average MICs, together with the range, determined using the agar dilution assay, for the ethanol extract of the plant towards selected pathogens, are presented in (Table 3). The antibacterial effects, expressed as MIC of the plant extract against test microorganisms is given in Table 3. The data revealed variability in the MIC against different bacteria, the lowest MIC value 16.0 $mgmL^{-1}$ against *S. typhi* (Table 3).

Table 1:Microbial inhibitory effect of Ethanolic leaf Extract of *S. kunthianum*

	Zones of inhibition(mm) at different Extract Concentration ($mgmL^{-1}$)			**Zones of inhibition (mm) of standard drugs Concentration**	
Organism	**100**	**200**	**400**	**Levofloxacin ($20\ mgmL^{-1}$)**	**Clotrimazole $20\ \mu gmL^{-1}$**
S. aureus	Nil	23.5 ± 1.78	29.5 ± 1.0	23.3 ± 2.33	Nil
S. typhi	Nil	15.5 ± 0.41	18.5 ± 0.9	20.5±0.58	Nil
B. subtilis	Nil	13.0 ± 1.41	14.5 ± 1.8	21.0±0.82	Nil
P. aeruginosa	Nil	Nil	Nil	Nil	Nil
A. niger	Nil	Nil	Nil	Nil	**14.3 ± 1.71**
C. albicans	Nil	Nil	Nil	Nil	**18.0 ± 1.41**

Values reported as mean ± standard deviation (n=4)

Table 2: Minimum Inhibitory Concentration of *S.kunthianum* Extract on Test organisms

Organism	**MIC ($mgmL^{-1}$)**
S.aureus	64.0
S.typhi	16.0
B.subtilis	32.0

3.5 Antimicrobial activity of *S.kunthianum* extract

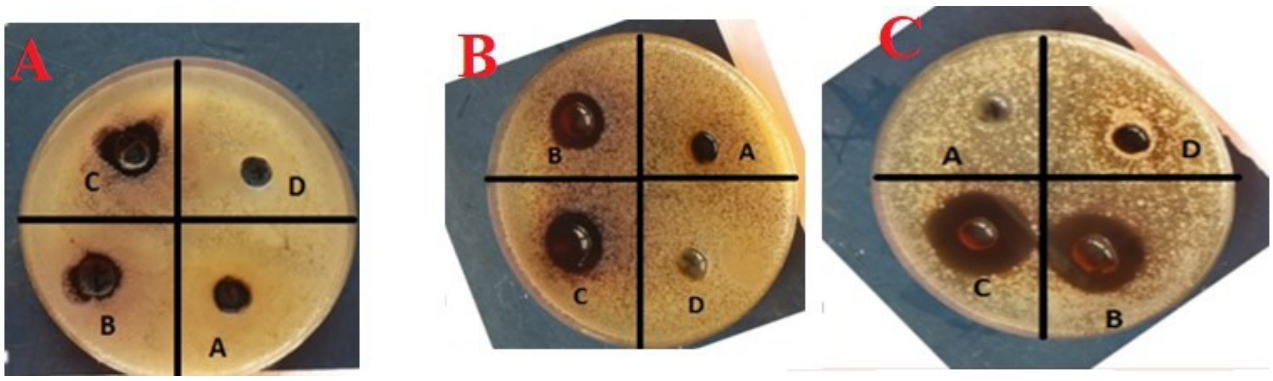

Figure 1 : Antibacterial effect of *S. kunthianum* ethanolic extract against (A) (B) (C) different organisms at various doses
Key- A -100mgmL^{-1}, B - 200mgmL^{-1}, C - 400mgmL^{-1} , D- 5% ethanol (Negative control)

Table 3: Thin Layer Chromatography result of *S.kunthianum* ethanolic extract using Solvent System: Chloroform-Methanol (9.7: 0.3)

Spot	Rf	Colour in Daylight	Colour in UV 254nm	Colour in UV 366nm	Colour in Iodine	Colour in 1%Vanillin in Conc. H_2SO_4	Colour in Ferric Chloride
1	0.07	-	-	-	Dark Brown	-	-
2	0.12	-	-	-	-	-	Pale Green
3	0.13	-	-	-	-	Black	-
4	0.16	-	-	-	Pale Brown	-	-
5	0.20	Pale Yellow	-	-	-	-	-
6	0.26	-	Pale purple	-	Pale Brown	-	-
7	0.30	-	-	-	-	Grey	-
8	0.38	-	Yellow	-	-	Greyish Purple	-
9	0.40	-	-	Orange	-	-	-
10	0.57	Yellow	-	-	-	Greyish Purple	-
11	0.58	-	-	-	Brown	-	-
12	0.65	-	-	-	Pale Brown	-	-
13	0.73	-	-	Orange	-	-	-

14	0.74	-	Yellow	-	-	Greyish Purple	-
15	0.79	-	-	-	Pale brown	-	-
16	0.80	Yellow	-	-	-	-	-
17	0.83	-	-	-	Brown	-	-
18	0.84	-	-	-	-	-	Pale Green
19	0.87	Green	-	-	-	-	-
20	0.88	-	-	-	Pale Brown	-	-
21	0.90	-	-	Orange	-	-	-
22	0.91	Yellow	Yellow	-	-	-	Dark Green
23	0.92	-	Greenish Brown	Brownish Orange	-	Greyish Purple	-
24	0.93	-	Green	Orange	Greenish Brown	Greyish Purple	Green
25	0.98	-	Greenish Brown	-	Dark Brown	-	-

Figure 2: TLC plates results of *S.kunthianum*

A: Chromatogram of *S.kunthianum* as observed in Daylight B: Chromatogram of *S.kunthianum* as observed under UV 254nm C: Chromatogram of *S.kunthianum* as observed under UV 366nm D: Chromatogram of *S.kunthianum* as observed in iodine tank E: Chromatogram of *S.kunthianum* as observed on spraying with Ferric Chloride F: Chromatogram of *S.kunthianum* as observedon spraying with 1% Vanillin in Conc. H_2SO_4
X: Origin Y: Solvent Front

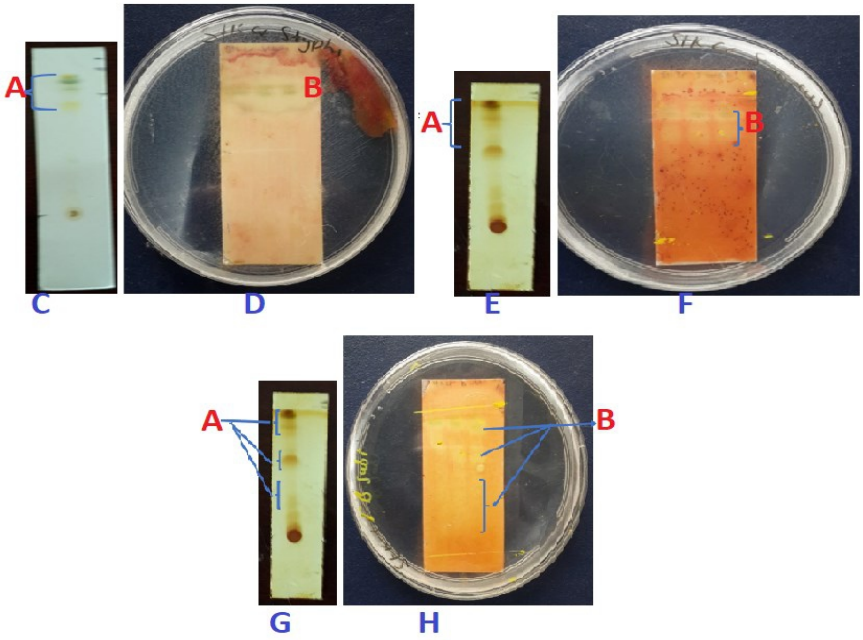

Figure 3: TLC Bioautography by agar overlay assay of bioactive fractions using different oraganism

A: Spots with antibacterial activity B: Cream coloured spots on a red background indicating zone of inhibition
C: TLC plate as observed in dayligh. E &G: TLC plate as observed in iodine tank.
D: TLC bioautography on *S.typhi*, F: TLC bioautography on *S.aureus* H: TLC bioautography on *S.typhi*

4. Discussion

The World Health Organization has reported there are high levels of bacteria resistant infections causing a wide range of deadly infections [22]. *S. kunthianum* is used locally as anti-infective agent in the treatment of venereal diseases e.g. primary syphilis. [15].Antimicrobial activities of medicinal plants have been reported by many researchers [22]. In the present study, antimicrobial activity of ethanol extracts of *S. kunthianum* leaves was evaluated against some microorganisms. It was found to have antibacterial activity against three test bacteria *S. aureus, S. typhi and B. subtilis*. However, *P. aeruginosa* was resistant to the plant extract. The zones of inhibition range from 13.0 ± 1.41mm to 29.5 ± 1.00 mm at test concentrations of 200 $mgmL^{-1}$ and 400 $mgmL^{-1}$ (Table 1). No zones were observed at test concentration of 100 $mgmL^{-1}$. These positive findings correspond with the work of Aliyu and co-workers however there is a disparity in the zones of inhibition and concentrations that produced the zones. Inhibition zones of 35 mm and 25 mm were recorded against *S. aureus* and *Salmonella* spp. respectively[16] at a concentration of 30$mgmL^{-1}$

The antibacterial activity was however lower than that of Levofloxacin and Clotrimazole used as positive controls (Table 1). Isolation of pure antimicrobial compounds from these extracts may result in compounds with comparable activity.

The minimum inhibitory concentration (MIC) of *S. kunthianum* extract for the various microorganisms were obtained which were 64, 16 and 32 $mgmL^{-1}$ for *S. aureus, S. typhi and B. subtilis* respectively (Table 2). This deviates from the work of Aliyu and co-workers, who reported that the MIC on *S. aureus* was 2.09$mgmL^{-1}$ and 4.17$mgmL^{-1}$ on *Salmonella* spp. [16]. This disparity may be due to the difference in the strain of the test organisms. It may also be due to seasonal and geographical variation in the secondary metabolytes. Considering the fact that` Aliyu and co-workers collected the plant in Zaria in the Northern part of

Nigeria in the month of December. Whereas the plant used in this study was collected in Ibadan in the South-West zone of Nigeria in the month of August.

The phytochemical screening revealed that the ethanolic extract of *S. kunthianum* leaves contains saponins and polyphenolic compounds. This corresponds with the work of Falodun and co-workers. An example of a flavonoid that has been isolated from the leaves is sterospermin [17]. Alkaloids were found to be absent, and this corresponds with the work of Aliyu and co-workers [16]. Free and bound anthraquinones were also found to be absent (Table1). However, two anthraquinones Sterekunthal A and Pyranokunthone B have been isolated from the lipophilic extract of the leaves of the plant [23]. The presence of these secondary metabolites in *S. kunthianum*, gives the reason for the antimicrobial activity of these plants. Thin layer Chromatography was also used as a technique to identify the phytoconstituents present in the crude extract of *S. kunthianum*. Upon running the TLC different detection reagents and methods were used viz UV 254 and 366 nm, iodine vapour, ferric Chloride solution and 1% Vanillin in conc. H_2SO_4 solution. The best solvent system for *S. kunthianum* was chloroform-methanol (9.7: 0.3). The TLC separation revealed the presence of polyphenolic compounds with chromophores and unsaturation. Spots with black (one spot) and purplish colours (5spots) on spraying with vanillin in Conc. H_2SO4 were observed indicating saponins and steroidal compounds may be present (Table 3, Fig. 2).

Bioautography is a useful technique for detecting bioactive compounds as well as indicator for separation technique during bioassay-guided isolation of active compounds.

The principle behind the detecting agent tetrazolinium salt is that the dehydrogenase enzyme in living organisms converts the salt to a deep red coloured formazan. As such zones of inhibition appear as cream-coloured spots on a red background of the plate [22].

The bioautography results confirmed the presence of different antimicrobial compounds in the extract. Some of the spots on TLC plates showed good activity against the test organisms' ith zones of inhibitions evident from the clearing of the spots (Figure 3). The bioactivity against the test organisms was observed to be attributable to the effects of the compounds separated on the TLC plate. It is indicative of the effects of the components of the extract against the test organisms. The separated compounds might have found their way into the cells of the test organisms and ensured the production of efflux pump inhibitors which may have facilitated the penetration of more phytochemicals into the microbial cells. Studies have shown that medicinal plant compounds have resistance modifying activities in vitro [24], these may serve as lead compounds to expand portfolio of bioactive agents[25]. This has been shown to increase the effectiveness of antimicrobial compounds plants against both Gram positive and Gram-negative bacteria.

5. Conclusion

The ethanolic leaf extract of *Stereospermum kunthianum* contains phytochemicals which have shown activities against *S. aureus*, *S. typhi* and *B. subtilis*. The bioactivities observed against the test organisms were due to the combined effects of the compounds separated on the TLC plates. From the results obtained we can conclude that the antimicrobial compounds are likely to be in the mid polar solvent fraction of the crude extract. Investigation of their independent bioactivity could produce potential novel drug leads for the development of new antibacterial drugs to forestall the global burden of antibiotic resistance.

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