GC-MS profiling and evaluation of antioxidant and antimicrobial properties of methanolic extract and fractions of the leaves of *Solanum dasyphyllum* Schumach and Thonn.

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ABSTRACT

Background: *Solanum dasyphyllum* is a plant with several ethno-medicinal uses and to date, there is little scientific validation of its folkloric claims compared to other species of the Solanaceae family.

Objective: This study determined the antimicrobial and antioxidant potential of methanol extract and fractions of the leaves of *S. dasyphyllum* and identified the phyto-constituents using GC-MS technique.

Methods: Crude methanolic extract of *S. dasyphyllum* leaves were subjected to Phytochemical screening and liquid-liquid fractionation. Crude extracts and fractions were subjected to antimicrobial screening, including minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBC/MFC) determination, time-kill kinetics study, antioxidant activity and Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Results: Phytochemicals present included flavonoids, alkaloids, saponins, cyanogenic glycosides, tannins and reducing sugars. Antimicrobial activities were recorded against all the isolates *(Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Rhizopus spp., Candida albicans and Penicillium spp.)* with MIC between 0.25 and >4 mg/mL, MBC of 1 and >4 mg/mL and MFC of 0.5 and >4 mg/mL. Time-kill kinetics study showed dichloromethane and ethylacetate fractions to be bactericidal. Crude extract displayed moderate antioxidant (IC50 = 425.51µg/mL) and GC-MS analysis showed 29 metabolites, including phenols and polyphenols derivatives, benzofuranone derivative and esters of decanoic acid derivatives, all of which are known to have anti-infective and anti-oxidant properties.

Conclusion: This study therefore elucidates the potentials of the *Solanum dasyphyllum* plant as a good source of bioactive compounds including those with anti-infective and antioxidant properties.

Keywords: *Solanum dasyphyllum*, minimum inhibitory concentration, minimum bactericidal concentration, time-kill kinetics, GC-MS.

Profilage GC-MS et évaluation des propriétés antioxydantes et antimicrobiennes de l'extrait méthanolique et des fractions des feuilles de Solanum dasyphyllum Schumach et Thonn .

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RÉSUMÉ

Contexte : Solanum dasyphyllum est une plante ayant plusieurs usages ethno-curatifs et, à ce jour, il y a peu de validation scientifique de ses revendications folkloriques par rapport à d'autres espèces de la famille des Solanacées.

Objectif: Cette étude a déterminé le potentiel antimicrobien et antioxydant de l'extrait de méthanol et des fractions des feuilles de S. dasyphyllum et identifié les phyto-constituants à l'aide de la technique GC-MS.

Méthodes : L'extrait méthanolique brut des feuilles de S. dasyphyllum a été soumis à un criblage phytochimique et à un fractionnement liquide-liquide. Les extraits bruts et les fractions ont été soumis à un dépistage antimicrobien, y compris la détermination des concentrations minimales inhibitrices (CMI) et des concentrations minimales bactéricides/fongicides (MBC/MFC), l'étude de la cinétique de temps mort, l'activité antioxydante et l'analyse par chromatographie en phase gazeuse et spectrométrie de masse (GC-MS).

Résultats : Les composés phytochimiques présents comprenaient des flavonoïdes, des alcaloïdes, des saponines, des glycosides cyanogènes, des tanins et des sucres réducteurs. Des activités antimicrobiennes ont été enregistrées contre tous les isolats (Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Rhizopus spp., Candida albicans et Penicillium spp.) avec une CMI entre 0,25 et >4 mg/mL, MBC de 1 et >4 mg/mL et MFC de 0,5 et >4 mg/mL. L'étude de la cinétique de temps mort a montré que les fractions de dichlorométhane et d'acétate d'éthyle étaient bactéricides. L'extrait brut a montré un antioxydant modéré (IC50 = 425,51µg/mL) et l'analyse GC-MS a montré 29 métabolites, y compris des dérivés de phénols et de polyphénols, un dérivé de benzofuranone et des esters de dérivés d'acide décanoïque, qui sont tous connus pour avoir des propriétés anti-infectieuses et antioxydantes.

Conclusion : Cette étude élucide donc les potentiels du Solanum dasyphyllum plante comme une bonne source de composés bioactifs dont ceux ayant des propriétés anti-infectieuses et antioxydantes.

Mots-clés : Solanum dasyphyllum, concentration minimale inhibitrice, concentration minimale bactéricide, cinétique de temps mort, GC-MS.

INTRODUCTION

The search for new lead compounds with antimicrobial effects is increasing due to preponderance of strains of microorganisms which exhibits resistance to existing antibiotics.[1] Antimicrobial principles have been in existence since antiquity, and it has been well preserved by nature. This is evident by the existence of microbes before mankind and man's idea that some plants had healing power against the disease caused by the microbes. Since ancient times, man has used plants to treat common infectious diseases and some of these traditional medicines still form part of the habitual treatment of various ailments.[2] Bearberry, known as *Arctostaphylos uva-ursi*, cranberry juice, known as Vaccinium macrocarpon, lemon balm, known as Melissa officinalis, garlic, known as *Allium sativum* and tee tree, known as *Melaleuca alternifolia*, are described as broad-spectrum antimicrobial agents used in the treatment of several infections.[3,4,5] The essential oils of lemon balm, garlic and tee tree have useful application in the treatment of infectious diseases in the respiratory system, urinary tract, gastrointestinal and biliary systems and also on the skin.[6] Herbal remedies are known as worthy sources of novel drugs with antimicrobial activity. Cinchona bark, identified over 300 years ago, resulted in the discovery of notable anti-malarial compound named quinine, and then also, the discovery of artemisinin from sweet wormwood plant, *Artemisia annua*, and the development of its derivatives.[7,8] The thrust in ethnopharmacology is to identify plants traditionally used in the treatment of diseases and derive bioactive compounds which can be developed into novel drugs. Nonetheless, ethnomedicine is the main practice that is used in primary healthcare in rural areas where modern healthcare facilities are lacking.[9]

The increasing prevalence of multidrug resistant (MDR) strains of microorganisms and the appearance of strains with reduced susceptibility to antibiotics originally effective against them raised the specter of untreatable bacterial infections and add urgency to the search for new infection-fighting strategies.[10]

Hypersensitivity and immune suppression are some adverse effects associated with most chemotherapeutic agents against antimicrobial drugs. The search for novel chemotherapeutic substances devoid of this adverse effect should therefore be continued, exploring all possible strategies. Natural products, particularly medicinal plants still remain the major and innovative sources for therapeutic agents.

Currently, research on lead compounds from natural products primarily focuses mainly on plants since they are more readily available and can be selected based on their ethno-medicinal uses. *Solanum dasyphyllum* is a semi-woody under shrub to 1½ m high, hirsute or armed with prickles, naturalized, not known in the wild state, and occurring throughout the region and tropical Africa to South Africa.[11] It is very closely related to *S. macrocarpon* Linn. Solanum is a plant of the Solanaceae family, with about 2000 species, is one of the largest genera of flowering plants, and has a centre of diversity in the New World tropics. The leaves and fruits of *S. dasyphyllum* are being used as food, while leaf-saps are used as medicine in treatment of stomachache and other infections.[12] *S. dasyphyllum* is known to be rich in some phenolic metabolites, which are known antimicrobial and anti-inflammatory phytochemicals present in many plants for a variety of disease causing agents including malaria plasmodium, bacteria and fungi infections and inflammatory disorders. To date there is little scientific validation of the folkloric claims of *S. dasyphyllum* plant. However, there are documented evidence corroborating pharmacological activities of other species of the Solanaceae family in infectious diseases treatment.[13,14] There is therefore, need to validate scientifically the folkloric claims of *S. dasyphyllum* used in traditional medicine. The present study was designed to determine the antimicrobial and antioxidant potentials of the crude methanolic extract (MESd) and four fractions (n-hexane - SdHXF, dichloromethane - SdDMF, ethylacetate - SdEAF and aqueous - SdAQF) of the leaves of *S. dasyphyllum* and also carry out GC-MS analysis of the MESd to evaluate the various bioactive compounds present.

MATERIALS AND METHODS

Collection of plant samples

The leaves of *Solanum dasyphyllum* were collected from Akungba, Akoko in Ondo State, Nigeria in April, 2017. The plant was authenticated at the Federal Research Institute of Nigeria (FRIN), Ibadan, Oyo-State where it was assigned a voucher number: F.H.I109799 and deposited at the herbarium unit of the Department of Pharmacognosy, Faculty of Pharmacy, and University of Ibadan for future referencing.

Extraction of plant samples

The leaves of *S. dasyphyllum* were air-dried away from direct sunlight after which it was grounded into coarse powder with a mechanical grinder. Two hundred gram (200g) of the dried powdered leave sample was extracted

with a mixture of 80% methanol (1.0L) and 20% water (0.2L) in a Soxhlet extractor apparatus for 72 hours at about a 64^{O}C. The plant extract was filtered with Whatman grade 1 paper and concentrated on rotary evaporator at reduced pressure. The concentrate was then lyophilized (freeze dried), the yield determined, and the crude extract stored in a vial at -20^{O}C for further studies.

Phytochemical analysis of crude extract

Qualitative phytochemical screening of the MESd was done as previously described.[15] The presence of selected phytochemical constituents such as flavonoids, alkaloids, saponins, cyanogenic glycosides, tannins and reducing sugars were investigated.

Fractionation of the crude extract

The lyophilized MESd extracted of *S. dasyphyllum* leaves was reconstituted with 100 mL of methanol (80%v/v) and 50 mL of distilled water, and poured into the fractionating funnel. This was then partitioned separately with 1000mL each of n-hexane first, dichloromethane and then ethylacetate solvent by liquid-liquid fractionation technique. The different fractions of the n-hexane, dichloromethane, ethylacetate and the aqueous were then concentrated on rotary evaporator under reduced pressure and then lyophilized as previously mentioned. The lyophilized powdered fractions were stored at -20^{O}C for further analysis.

Collection and authentication of test clinical isolates

Four clinical multidrug resistant bacteria isolates (Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa) and four clotrimazole resistant clinical fungi (Aspergillus niger, Rhizopus spp., Candida albicans and Penicillium spp.) were collected from the achieve of the Laboratory in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan. The isolates were confirmed for purity and identity using standard microbiological techniques, inoculated on sterile agar slants and stored at 4^{O}C for further study.

Antimicrobial screening of the crude extract and fractions

The MESd and the different fractions (SdHXF, SdDMF, SdEAF and SdAQF) of *S. dasyphyllum* leaves were screened for antimicrobial activities against selected test isolates using agar-well-diffusion method as described previously with some modifications.[16] Briefly, 20 mg of the extract and each of the fractions were weighed separately and dissolved in 10 mL of methanol in a tube each to give the stock concentration of 2 mg/mL. The concentration was serially diluted twice by taking 5mL of the stock concentrate into 5 mL of fresh methanol to give the second and third concentrations of 1 mg/mL and 0.5 mg/mL. Culture plates of the test microorganisms were prepared according to the manufacturer's instructions and test microorganisms prepared into suspension for inoculation on the agar plates. Microbial suspensions were prepared by taking speck of colonies of each bacterium (overnight culture on nutrient agar at 37^{O}C) and fungi (cultured on Sabouraud dextrose agar for 48 - 72 hours) using a sterile wire loop, separately dispersed in normal saline and agitated with vortex mixer to form a homogenised suspensions. The suspensions were further diluted to 0.5 MacFarland standards with sterile normal saline. With the aid of a sterile swab each of the diluted suspensions were inoculated on the dried surfaces of separate Mueller Hinton agar plates for bacteria and Sabouraud dextrose agar plates for fungi by surface spreading. Wells of approximately 6 mm in diameter and 2.5 mm deep were made on the surface of the inoculated media using a sterile 6 mm cup-borer. The number of wells corresponded with the number of the diluted extracts (15) and the controls (2). Each well after labeling was filled with two to three drops of the corresponding diluted extract/fractions. The controls include methanol (80%v/v) as negative and gentamicin (10µg/mL) and ketoconazole (10µg/mL) as positive controls for bacteria and fungi respectively. The plates were first maintained on the bench at room temperature for 2 hours to enable the diffusion of the extracts into the medium after which the bacteria inoculated agar plates were incubated at 37^{O}C for 24 hours, and those for fungi at 25^{O}C for 48 to 72 hours.

All the inoculation procedures were undertaken under aseptic conditions and in duplicates. The zones of inhibition around the wells were measured as diameters in millimeters (mm) with the aid of a transparent ruler for all the two replicates and average measurements recorded.

Determination of Minimum Inhibitory Concentrations (MICs) of the crude extract and fractions

The minimum inhibitory concentration (MIC) of the MESd and the fractions (SdHXF, SdDMF, SdEAF and SdAQF) were determined using the agar-dilution method as previously described.[17] From the powdered MESd and fractions, 800 mg was weighed and dissolved in 10mL of methanol to give a concentration of 80 mg/mL. This was serially

diluted to give five lower concentrations: 40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL and 2.5 mg/mL. The agar-extract media were prepared by adding 1 mL of each of the stock concentrate and the five serially diluted concentrations into 19 mL of pre-sterilized molten Mueller Hinton agar (for bacteria) and Sabouraud dextrose agar (for fungi) to give the final concentrations of 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL of the agar-extract media. The agar-extract media were poured into well labeled Petri dishes and permitted to solidify under laminar flow. Bacteria and fungi cultures and suspensions were prepared from their overnight cultures as described earlier above. With the aid of a sterile swab each of the diluted suspensions were inoculated on the respective sectors on the surface of the agar plates by surface spreading. After streaking of the test isolates to the respective labeled sectors of the agar plates, the plates were incubated at 37^{O}C for 24 hours for bacteria and at 25^{O}C for 48 - 72 hours for fungi. The experiment was carried out in duplicates for each test microorganism. Presence or absence of growth was examined on each plate, and MIC was taken as the lowest concentration that prevented the growth of the test microorganisms.

Assessment of Minimum Bactericidal Concentration (MBC) of the crude extract and fractions

The minimum bactericidal concentration (MBC) for the MESd and the four fractions was determined using the method described by Akinpelu *et al*.[18] Briefly, sterile cotton swabs was used to swab the surface of sectors of the MIC agar plate showing no visible growth (i.e. concentrations >MBC of the extract and each fraction against each isolates) and the cotton swab immediately swab onto the surface of another fresh, well labeled Mueller Hinton agar plates. The plates were then incubated at 37^{O}C for up to 72 hours for both bacteria and fungi. The MBC for the individual isolate is the minimum concentration of the extract/fraction during the MIC assay that did not show any microbial growth on the fresh Mueller Hinton agar plates. This procedure was done in duplicate and the average MBC value recorded.

Determination of the bacterial time-kill kinetics of the extract and fractions

The modified method described by Akinpelu *et al*,[18] was used to determine the time-kill kinetics of the extract and fractions against selected isolates *(S. aureus, E. coli and C. albicans)*. The isolates were initially standardized by dispersing speck of colonies of each bacterium (overnight culture on nutrient agar at 37^{O}C) and fungi (cultured on Sabouraud dextrose agar for 48 - 72 hours) in normal saline with a sterile wire loop and vortexed to form an homogenised suspension. The suspension was further diluted to 0.5 MacFarland standards (approximately106 cfu/mL) with sterile normal saline. Exactly 0.5 mL of the standardized suspension of the culture was added to 9.5 mL of Mueller Hinton broth containing the different concentrations of the extracts relative to their different MBCs (MESd 8 mg/mL, SdEAF 8mg/mL, SdHXF 4mg/mL, SdDMF 4mg/mL and SdAQF 4mg/mL). The microbe-extract-reaction media were held at room temperature and at an interval of time corresponding to 0, 1, 2, 3, 4, 5 and 6 hours, 0.1 mL of each microbe-extract reaction media were withdrawn and immediately transferred to 9.9 mL peptone water recovery medium (10-2 dilution). This was then serially diluted twice with fresh sterile peptone water and the last two dilutions (10-4 and 10-6) plated for viable counts. The bacteria culture plates were incubated at 37^{O}C for 24 hours, while fungi cultured plate was incubated at 25^{O}C for up to 72 hours. Control culture plates containing each of the test isolate without the addition of extract or fractions were also done to determine isolates' growth rate. After the respective period of incubation, the emergent bacteria and fungi colonies were counted and express in cfu/mL. Graph of percentage survivor against time was plotted for the extract and fractions against each of the three isolates to determine the pattern of the kill kinetics.

Determination of antioxidant potential of the crude methanolic extract

The antioxidant potential of the MESd obtained from *S. dasyphyllum* leaves was evaluated by determining the total phenolic content (TPC), ferric ion reducing antioxidant power assay (FRAP), total antioxidant content (TAC) and 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical-scavenging activity.

Total phenolic content (TPC)

The MESd's TPC were determined by the Folin-Ciocalteu assay as previously described.[19] The sample was diluted to different concentrations, 200, 400, 600, 800 and 1000 µg/mL, and 0.5 ml of different dilutions were separately mixed, each with 5 mL of Folin-Ciocalteu reagent (to give a ratio of 1:10 dilution) and left for 5 min before 4 mL 1M aqueous sodium carbonate was then added. The mixture was allowed to stand for another 15 min and the phenols were determined by colorimetric method at 765 nm. The concentrations of phenolic compounds in the MESd were expressed as Gallic Acid Equivalents (GAEs) per gram of the dry extract. All assays were conducted in triplicate

and the mean values were recorded.

Ferric ion reducing antioxidant power assay (FRAP)

The FRAP of the MESd was measured by a method previously described by Phatak and Hendre.[20] The extract was diluted to different concentrations (200, 400, 600, 800 and 1000 µg/mL) and were mixed, each with 2.5 ml of 20 mM phosphate buffer and 2.5 mL 1%, w/v potassium ferric cyanide. The mixture was then incubated at 50^{O}C for 30 min and afterwards, 2.5 mL of 10%, w/v trichloroacetic acid and 0.5 mL of 0.1%, w/v ferric chloride were added to the mixture and kept aside for 10 min. Ascorbic acid was used as positive reference standard and the absorbance was measured at 700 nm. All assays were run in triplicate and the mean values recorded. The relative capability of the plant extract was compared to ascorbic acid.

Total antioxidant content (TAC)

The TAC of the MESd was evaluated by phosphor-molybdenum assay as previously described by Phatak and Hendre.[20] The molybdate reagent solution was prepared by adding 1 mL each of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate into 20 mL of distilled water and made up to 50 mL with distilled water. The MESd was serially diluted to five different concentrations (200, 400, 600, 800 and 1000 µg/mL) and equal volume added to each of the five test tubes individually containing 3 mL of distilled water and 1 mL of molybdate reagent solution. These tubes were incubated at 95^{O}C for 90 min after which the tubes were normalized to room temperature for 20-30 min and the absorbance of the reaction mixture measured at 695 nm. Ascorbic acid was used as positive reference standard at the same concentrations and absorbance as the MESd. The protocol was done in triplicate and the mean values recorded. The antioxidant activity was expressed as equivalent of ascorbic acid.

DPPH free radical-scavenging activity evaluation

The free radical-scavenging activity of the MESd was determined using 1,1-diphenyl-2-picryl hydrazyl radical as previously described by Ebrahimzadeh *et al*.[19] MESd was diluted to different concentrations (200, 400, 600, 800 and 1000 µg/mL) and equal volumes were added to the methanolic solution of DPPH (100 µM). This was left at room temperature for 15 min after which the absorbance was measured at 517 nm. The protocol was carried out in triplicate and the mean value recorded. Ascorbic acid (Vitamin C) was used as standard controls at the same concentrations and measured at the same wavelength with the MESd. The percentage of inhibition was evaluated using the equation below:

$$\% \text{ Inhibition} = \frac{(AB_{cont} - AB_{samp}) \times 100}{Ab_{cont}}$$

Where % Inhibition = percentage of inhibition; AB_{cont} = Absorbance of control; AB_{samp} = Absorbance of test sample.

The IC50 value is the concentration of sample required for scavenging 50% of the radicals and was calculated using linear regression analysis.

Screening for bioactive compounds by Gas Chromatography-Mass Spectrometry (GC-MS)

The MESd was analysed by Gas Chromatograph (Hewlett-Package 5890) coupled to VG Analytical Mass-Spectrometer (70-250S) with a fused silica capillary column (CP-Sil 5CB: 30m x 0.25 mm ID x 1µm of capillary column). Helium gas was used as carrier at flow rate of 1mL/min. The oven was set at a temperature of 90^{O}C initially and sustained for 2 minutes before heating at 10^{O}C/min to 270^{O}C and held isothermally for 15 minutes. A scan was applied to cover a mass range from 36 to 600 amu for 0.6s at 70eV ionization voltage. Important constituents of the analysed crude extract were identified by matching their MS and retention index data with those of the standards using computer search on a NIST version 2.1 MS Library.

RESULTS

Percentage yield and Phytochemical Screening

The percentage yield of the MESd was 12.5%. The result of the preliminary qualitative screening of the MESd is presented in table 1. The phyto-constituents present included: flavonoids, alkaloids, saponins, cyanogenic glycosides, tannins and reducing-sugars (Table 1).

Determination of the antimicrobial activity of the crude extract and fractions

The results of the antibacterial screening of the MESd and the four fractions against the clinical isolates are presented in tables 2 and 3. All the bacterial isolates used in this study were susceptible to the SdDMF and SdEAF fractions at concentrations of ≥0.5 mg/mL. However, MESd and SdHXF are not active against E coli isolate at 0.5mg/mL but active at ≥1 mg/mL concentration (Table 2). Both the MESd and three fractions (SdHXF, SdDMF and SdEAF) were more active on the Gram-positive than

Gram-negative bacteria. The MESd and SdDMF showed highest activity against the *B. subtilis* and *S. aureus* isolates (Gram positives) with zones of inhibition between 20±0.5 and 21±0.3mm. The SdAQF showed relatively highest activity against the Gram negatives (*Escherichia coli* and *P. aeruginosa*) with zones of inhibition of 18±0.3mm and 18±2.5mm respectively. SdEAF showed higher activity against *P. aeruginosa* with zone of inhibition of 20±1.3mm compared to the *E coli*. For both MESd and fractions, the activities against the bacterial isolates were seen to slightly increase with increase in concentrations (Table 2).

The results of the antifungal activities of the MESd and the fractions against the clinical isolates are presented in Table 3. Highest activity was recorded against *Aspergillus* niger by SdEAF with zone of inhibition of 22±3.5 mm followed by SdDMF with zone of inhibition of 21±0.6 mm at 2 mg/mL concentration. The SdHXF was the most active against the *Candida albican and Penicillium sp*. with zones of inhibition of 16±0.1mm and 20±0.5 mm respectively at 2 mg/mL concentration. The MESd was found to be more active against Penicillium specie and Aspergillus niger compared to the other fungi. While the MESd was not active against *C. albican* at concentrations below 2 mg/mL, the SdAQF did not show any activity at <2mg/mL against Aspergillus niger and Rhizopus sp. Activities of the MESd and most of the fractions were seen to slightly increase with increase in concentrations against the fungi (Table 3).

Minimum inhibitory and cidal concentrations determination

The results of the MICs and MBCs/MFCs of the extract and fractions against the bacteria and fungi isolates are presented in table 4. The MICs of the MESd and its fractions against the bacterial and the fungal isolates ranged between 0.25 and >4 mg/mL. The SdDMF was found to have the lowest MIC value (0.5 mg/mL) against the S aureus compared to MESd and other fractions while the MESd had the lowest (0.25 mg/mL) against the B subtilis. For the fungi isolates, the lowest MIC value of 0.25 mg/mL was recorded for SdDMF against Rhizopus sp, A. niger and *Penicillium sp*. The same 0.25 mg/mL MIC value was also recorded for SdEAF fraction against Rhizopus sp. The MBC of MESd and the fractions ranged between 1 and >4 mg/mL while the MFC ranged between 0.5 and >4 mg/mL. The MBC/MIC ratio was >4 for SdDMF against *S. aureus* (ratio 8) and MFC/MIC ratio >4 for SdEAF against all the fungi isolates (ration 8) as presented in Table 4.

Time-Kill kinetics determination

The results of the time-kill kinetics of the bactericidal activities of MESd and its fractions against *S. aureus, E. coli* and *C. albicans* showed that the percentage viable count decrease with time at varying degree. The reduction was close to zero count at 4 hour for SdEAF and 5hour for SdDMF fraction against the *S aureus* and *E coli* isolates (Figure 1 and 2). However, the results of the time-kill kinetics against the *C. albicans* showed that the viable count reduced to zero with SdDMF at 5hour and SdEAF at 6 hour (Figure 3).

Antioxidant activity determination

The results of the antioxidant assay of the MESd are presented in table 5 and 6. The TPC of MESd gave a garlic acid equivalent of 1.389 at 200 µg/mL concentration of the MESd and 1.689 at 1000 µg/mL (Table 5). The FRAP of the MESd gave ascorbic acid equivalent of 2.889 at 200 µg/mL of the MESd and 6.021 at 1000 µg/mL, while the TAC of the MESd at 200 µg/mL gave ascorbic acid equivalent of 0.990 and at 1000 µg/mL gave 1.067 ascorbic acid equivalent (Table 5).

GC-MS analysis of the crude extract

The total ion current chromatogram of the MESd is presented in figure 5. The GC-MS analysis showed that the MESd contained 29 metabolites including benzene, eucalyptol, levomenthol, benzofuranone derivative, diethyl phthalate, neophytadiene, 1-Docosene, 17-Pentatriacontene, Phytol, Thunbergol, esters of decanoic acid derivatives such as 6.10.14-trimethyl-2-pentadecanone, 9,12-Octadecadienoic acid, Methyl 11-methyl-dodecanoate, n-Hexadecanoic acid, (Table 7).

Table 1: Preliminary qualitative phytochemical screening of the crude methanolic extract of *S. dasyphyllum* leaves

Phyto-constituents	Qualitative data
Flavonoids	+
Alkaloids	+
Saponins	+
Cyanogenic glycosides	+
Tannins	+
Reducing sugars	+

Table 2: Activities of the crude methanolic extract and fractions of *S. dasyphyllum* against clinical bacterial isolates

Test sample/control	Conc (mg/mL)	*E. coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*
		Zones of growth inhibition (mm)			
MESd1	2	18±1.1	18±0.23	20±1.1	20±1.4
MESd2	1	16±2.1	14±2.4	18±1.4	16±2.2
MESd3	0.5	-	12±0.1	12±0.2	10±0.3
Gentamicin	10(µg/ml)	20±0.2	22 ±2.3	20 ± 0.3	24±2.3
Methanol	(80%v/v)	-	-	-	-
SdHXF1	2	15±0.2	14±1.1	16±1.2	16±0.2
SdHXF2	1	12±1.2	12±0.4	14±0.2	14±1.2
SdHXF3	0.5	-	10±2.1	14±0.3	12±0.5
Gentamicin	10 (µg/ml)	16±0.1	16±1.1	20±3.0	18±2.3
Methanol	(80%v/v)	-	-	-	-
SdDMF1	2	12.1±0.2	18±0.7	20±0.5	21±0.3
SdDMF2	1	9±0.1	12±0.1	20±1.6	16±1.8
SdDMF3	0.5	11±0.1	9±1.6	15±0.4	11±1.8
Gentamicin	10 µg/mL	21±0.4	22.1±0.8	23±1.1	24±0.1
Methanol	(80%v/v)	-	-	-	-
SdEAF1	2	14±0.1	20±1.3	22±0.3	22±0.51
SdEAF2	1	12±0.6	12±0.3	20±1.1	16±1.1
SdEAF3	0.5	12±0.4	10±2.3	16±1.2	12±1.1
Gentamicin	10 (µg/ml)	22±2.1	22±0.5	24±0.2	25±0.7
Mathanol	(80%v/v)	-	-	-	-
SdAQF1	2	18±0.3	18±2.5	15±2.2	14±0.1
SdAQF2	1	16±2,2	14±1.2	14±1.4	12±1.4
SdAQF3	0.5	14±1.3	12±0.2	12±0.2	10±0.3
Gentamicin	10(µg/ml)	22±2.2	18±0.3	20±1.2	18±1.3
Methanol	(80%v/v)	-	-	-	-

Legend: MESd-Methanol Extract of *S. dasyphyllum*, SdHXF-n-hexane fraction, SdDMF-dichloromethane fraction, SdEAF-ethylacetate fraction and SdAQF-aqueous fraction, - =No zone of inhibition**.**

Table 3: Activities of the crude methanolic extract and fractions of *S. dasyphyllum* against clinical fungal isolates

Test sample/control	Conc (mg/mL)	*Candida albicans*	*Penicillum* sp.	*Rhizopus* sp.	*Aspergillus niger*
		Zones of growth inhibition (mm)			
MESd1	2	12±1.2	18±0.2	16±1.3	18±1.2
MESd2	1	-	16±1.4	14±2.2	16±3.2
MESd3	0.5	-	14±2.2	13±3.1	14±0.2
Ketoconazole	10 (µg/mL)	16±0.7	20±0.4	22±5.2	20±.44
Methanol	(80%v/v)	-	-	-	-
SdHXF1	2	16±0.1	20±0.5	16±1.5	14±2.1
SdHXF2	1	14±1.1	16±0.1	14±2.2	12±1.0.1
SdHX3	0.5	12±0.1	14±1.4	14±0.3	10±4.3
Ketoconazole	10(µg/mL)	18±1.4	22±0.1	20±0.4	16±2.2
Methanol	(80%v/v)	-	-	-	-
SdDMF1	2	14.1±0.1	19±0.1	18±3.0	21±0.6
SdDMF2	1	114±0.3	14±1.8	15±2.2	19±0.8
SdDMF3	0.5	9±1.4	13±1.2	14±0.1	13±1.8
Ketoconazole	10 (µg/mL)	16±1.3	23±0.3	18±1.1	22±1.4
Methanol	(80%v/v)	-	-	-	-
SdEAF1	2	14±0.1	20±0.2	18±3.2	22±3.5
SdEAF2	1	12±0.3	14±2.3	16±2.3	20±0.4
SdEAF3	0.5	10±2.3	12±1.4	14±1.1	13±2.2
Ketoconazole	10 (µg/mL)	16±2.2	24±0.4	20±0.2	24±3.4
Methanol	(80%v/v)	-	-	-	-
SdAQF1	2	14±1.4	12±1.5	-	-
SdAQF2	1	12±0.2	10±0.3	-	-
SdAQF3	0.5	-	-	-	-
Ketoconazole	10 (µg/mL)	18±2.2	16±1.1	18±1.1	22±2.2
Methanol	(80%v/v)	-	-	-	-

Legend: MESd-Methanol Extract of *S. dasyphyllum*, SdHXF-n-hexane fraction, SdDM-dichloromethane fraction, SdEAF-ethylacetate fraction and SdAQF-aqueous fraction, - =No zone of inhibition

Table 4: Determination of minimum inhibitory concentrations (MICs), minimum bactericidal/fungicidal concentrations (MB/FC) and the MB/FC/MIC ratio of the crude methanolic extract and fractions against clinical isolates

Test microbes	Extract/Fractions (Concentrations (mg/mL)													
	MESd			SdHXF			SdDMF			SdEAF			SdAQF	
	MIC	MBC or MFC	MBC or MFC/ MIC Ratio	MIC	MBC or MFC	MBC or MFC/ MIC Ratio	MIC	MBC or MFC	MBC or MFC/ MIC Ratio	MIC	MBC or MFC	MBC or MFC/ MIC Ratio	MIC	MBC or MFC	MBC or MFC/ MIC Ratio
S. aureus	2	>4	>2*	4	4	1	0.50	4	8*	4	>4	>1*	4	4	1
B. subtilis	0.25	1	4	1	4	4	0.50	2	4	4	4	1	1	2	2
E. coli	4	>4	>1*	4	4	1	4	4	1	4	>4	>1*	2	4	2
P. earuginosa	2	2	1	1	4	4	0.50	2	4	1	2	2	0.50	2	4
Aspergillus niger	4	>4	>1*	2	>4	>2*	0.25	0.5	2	0.50	4	8*	4	>4	>1*
Rhizopus sp.	4	4	1	2	>4	>2*	0.25	1	4	0.25	2	8*	0.50	2	4
Candida. albicans	4	4	1	>4	>4	1	1	2	2	0.5	4	8*	4	>4	>1*
Penicillium sp.	4	>4	>1*	2	>4	>2*	0.25	1	4	0.5	>4	>8*	1	2	2

Legend: MESd-Methanol Extract of *S. dasyphyllum*, SdHXF-n-hexane fraction, SdDMF, dichloromethane fraction, SdEAF-ethylacetate fraction, SdAQF-aqueous fraction, MIC-minimum inhibitory concentration, MB/FC-minimum bactericidal/fungicidal concentration, *-bacteriostatic action.

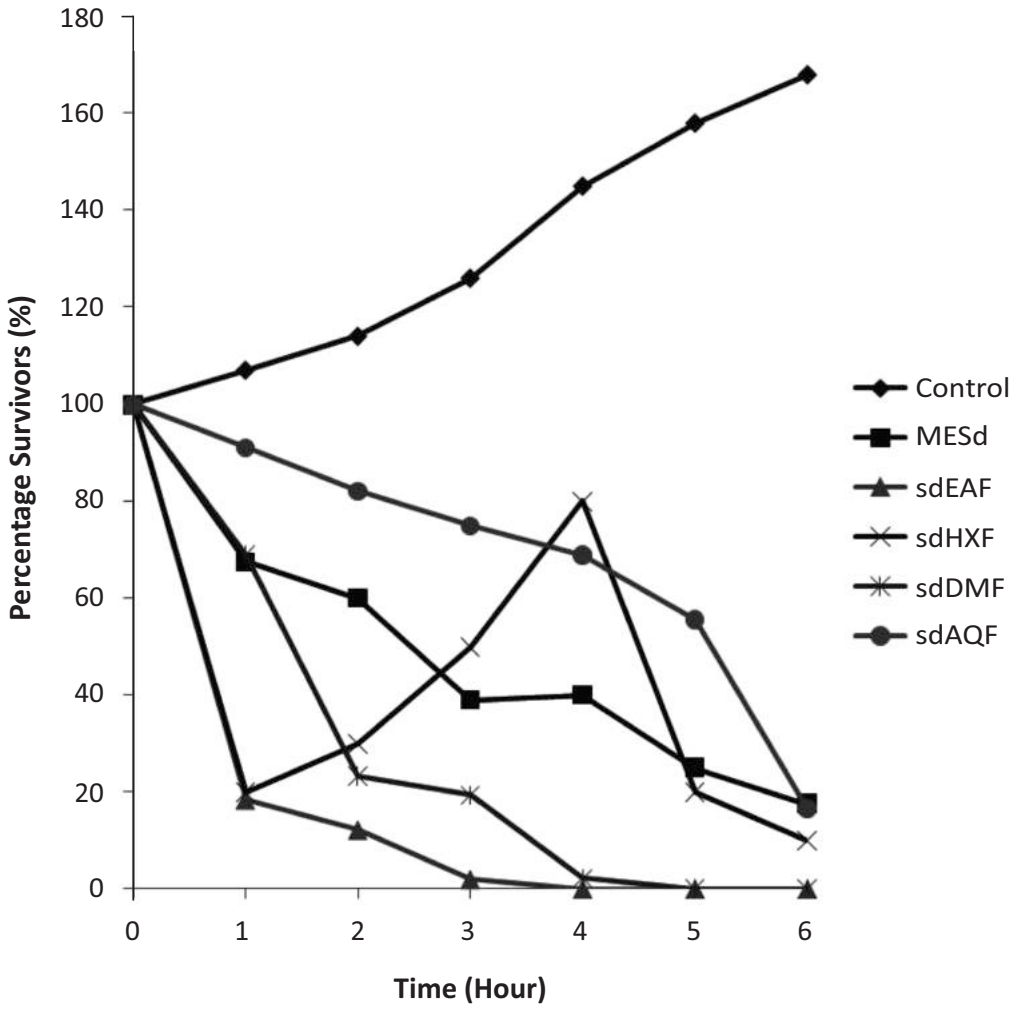

Figure 1: Time-kill kinetics of the extract and fractions of *S. dasyphyllum* leaves against clinical isolate of Staphylococcus aureus

Legend: MESd- Methanolic extract of *S. dasyphyllum*, SdHXF- n-hexane fraction, SdDMF, dichloromethane fraction, SdEAF- ethylacetate fraction and SdAQF- aqueous fraction

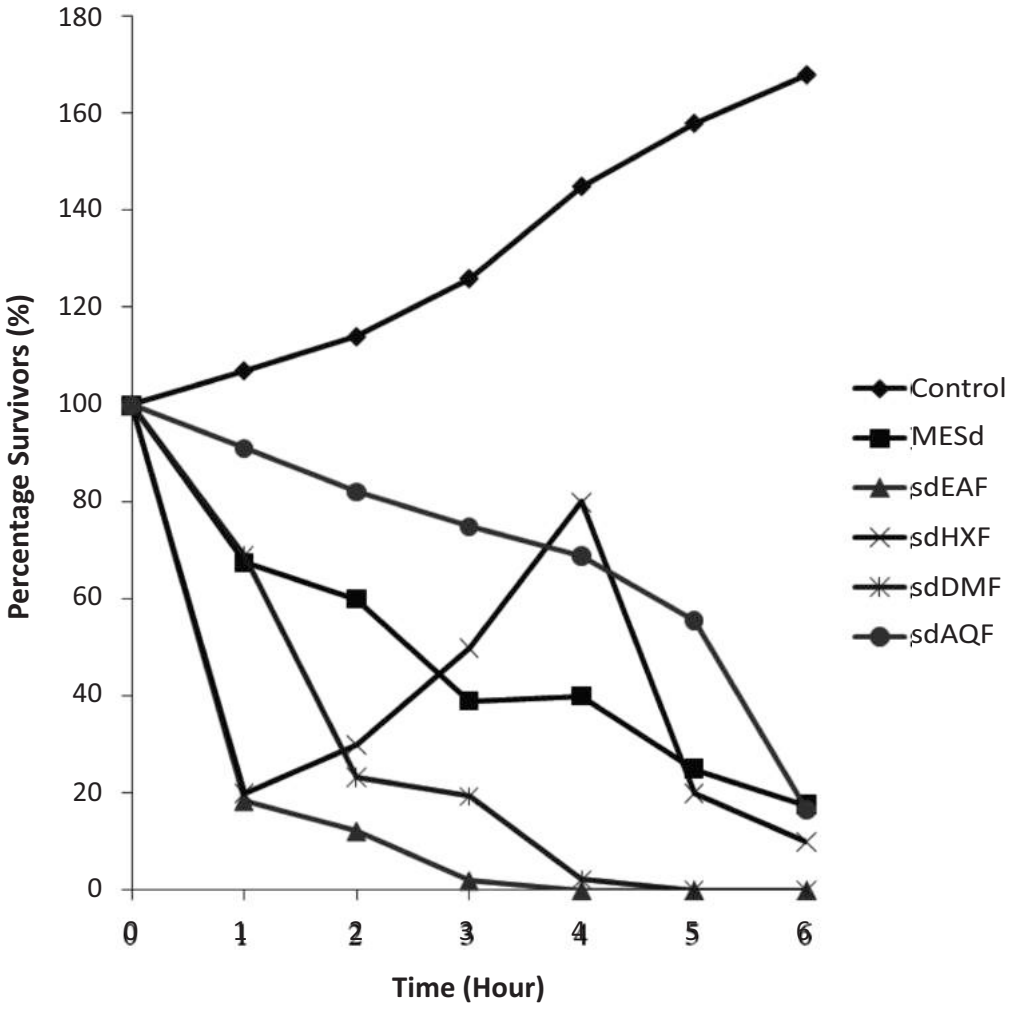

Figure 2: Time-kill kinetics of the extract and fractions of *S. dasyphyllum* leaves against clinical isolate of Escherichia coli

Legend: MESd- Methanol Extract of *S. dasyphyllum*, SdHXF- n-hexane fraction, SdDMF, dichloromethane fraction, SdEAF- ethylacetate fraction and SdAQF- aqueous fraction

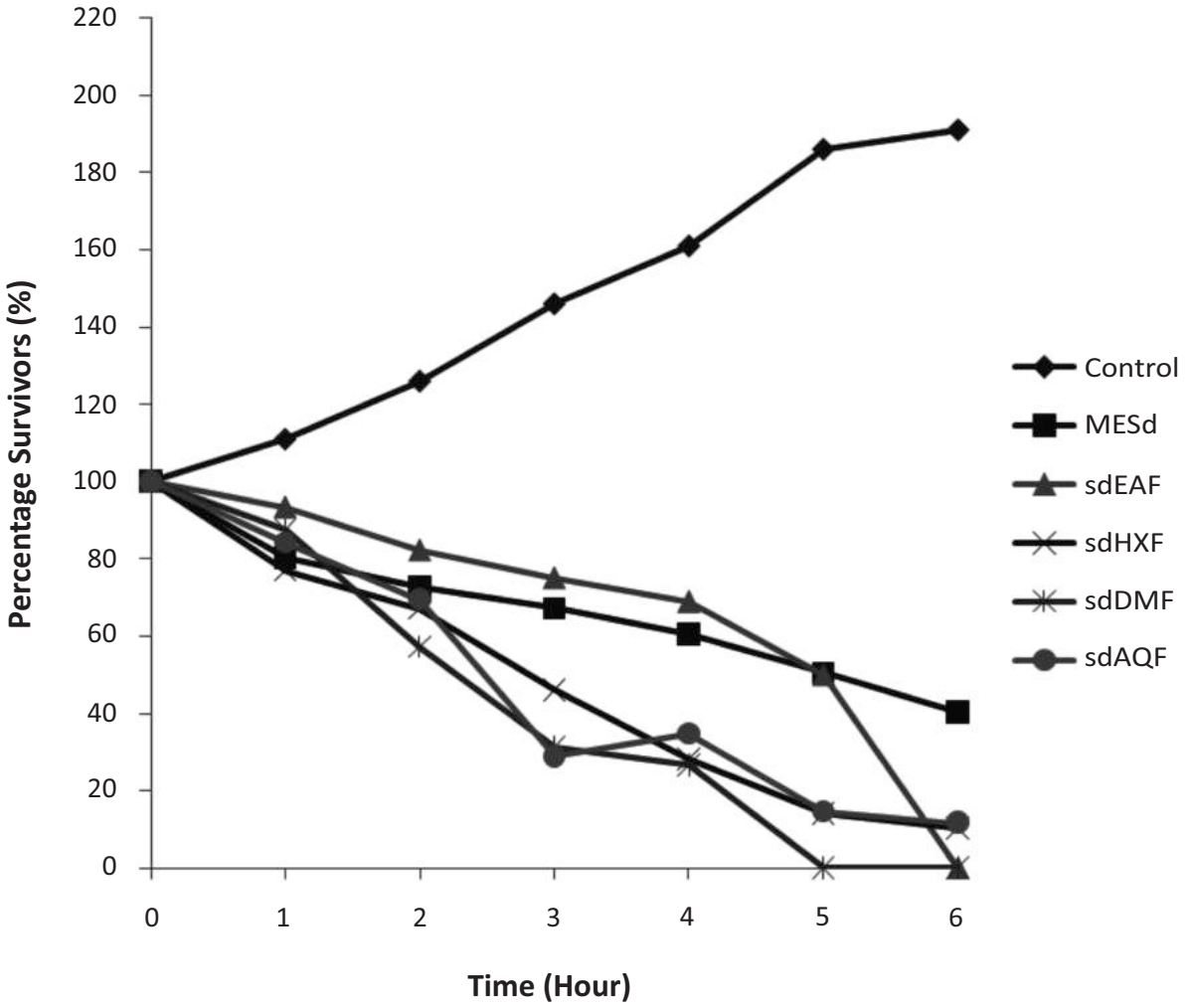

Figure 3: Time-kill kinetics of the extract and fractions of *S. dasyphyllum* leaves against clinical isolate of Candida albicans

Legend: MESd- Methanol Extract of *S. dasyphyllum,* SdHXF- n-hexane fraction, SdDMF, dichloromethane fraction, SdEAF- ethylacetate fraction and SdAQF- aqueous fraction

Table 5: Determination of total phenolic content, ferric ion reducing antioxidant power and total antioxidant capacity of the methanolic extract of *S dasyphyllum* leaves in comparison with standards

MESd Concentration (µg/mL)	200	400	600	800	1000
TPC (GAE)	1.389±0.000	1.444±0.000	1.512±0.000	1.629±0.000	1.689±0.001
FRAP (ASAE)	2.889±0.000	4.744±0.001	5.274±0.001	5.400±0.000	6.021±0.001
TAC (ASAE)	0.990±0.002	1.003±0.000	1.044±0.001	1.063±0.001	1.067±0.000

Legend: MESd – Methanol Extract of *S. dasyphyllum*, ASAE – Ascorbic acid equivalent, GAL – Garlic acid equivalent, TAC – Total Antioxidant Capacity, TPC – Total Phenol Content, FRAP - Ferricion reducing antioxidant power

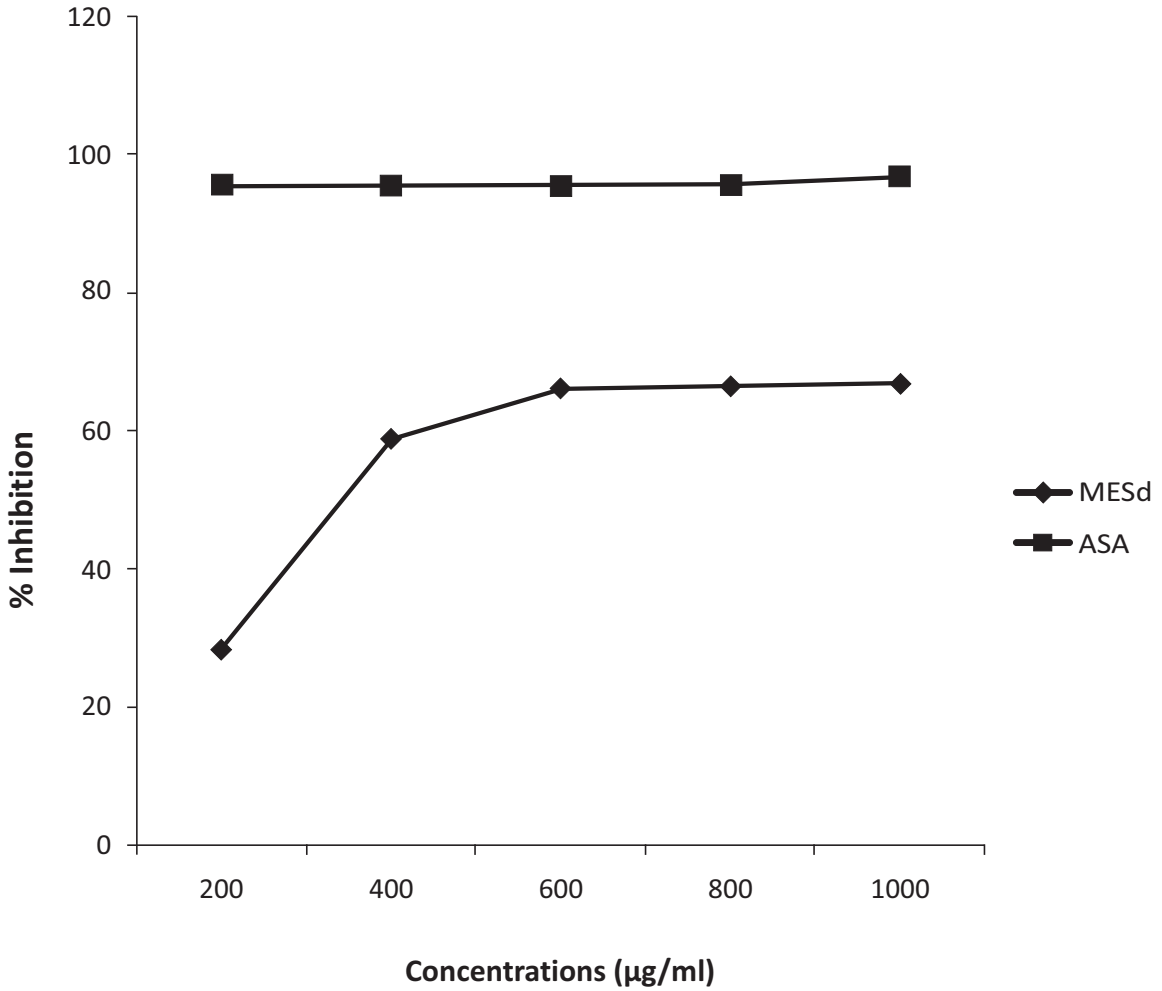

Figure 4: DPPH free radical inhibiting activity of crude methanolic extract of *S. dasyphyllum* leaves and ascorbic acid

Legend: MESd - Methanol Extract of *S. dasyphyllum*, ASA - Ascorbic acid

Table 6: Determination of free radical scavenging property and IC_{50} value of the methanolic extract of *S. dasyphyllum* leaves in comparison with Ascorbic acid

Concentration of MESd (µg/ml)	% Inhibition (MESd)	% Inhibition (ASA)
200	28.44±0.001	95.60±0.001
400	58.86±0.002	95.68±0.001
600	66.19±0.001	95.68±0.000
800	66.55±0.000	95.82±0.000
1000	66.91±0.001	96.99±0.002
IC_{50}	425.51µg/mL	97.49µg/mL

Legend: MESd – Methanol Extract of *S. dasyphyllum*, ASA – Ascorbic acid

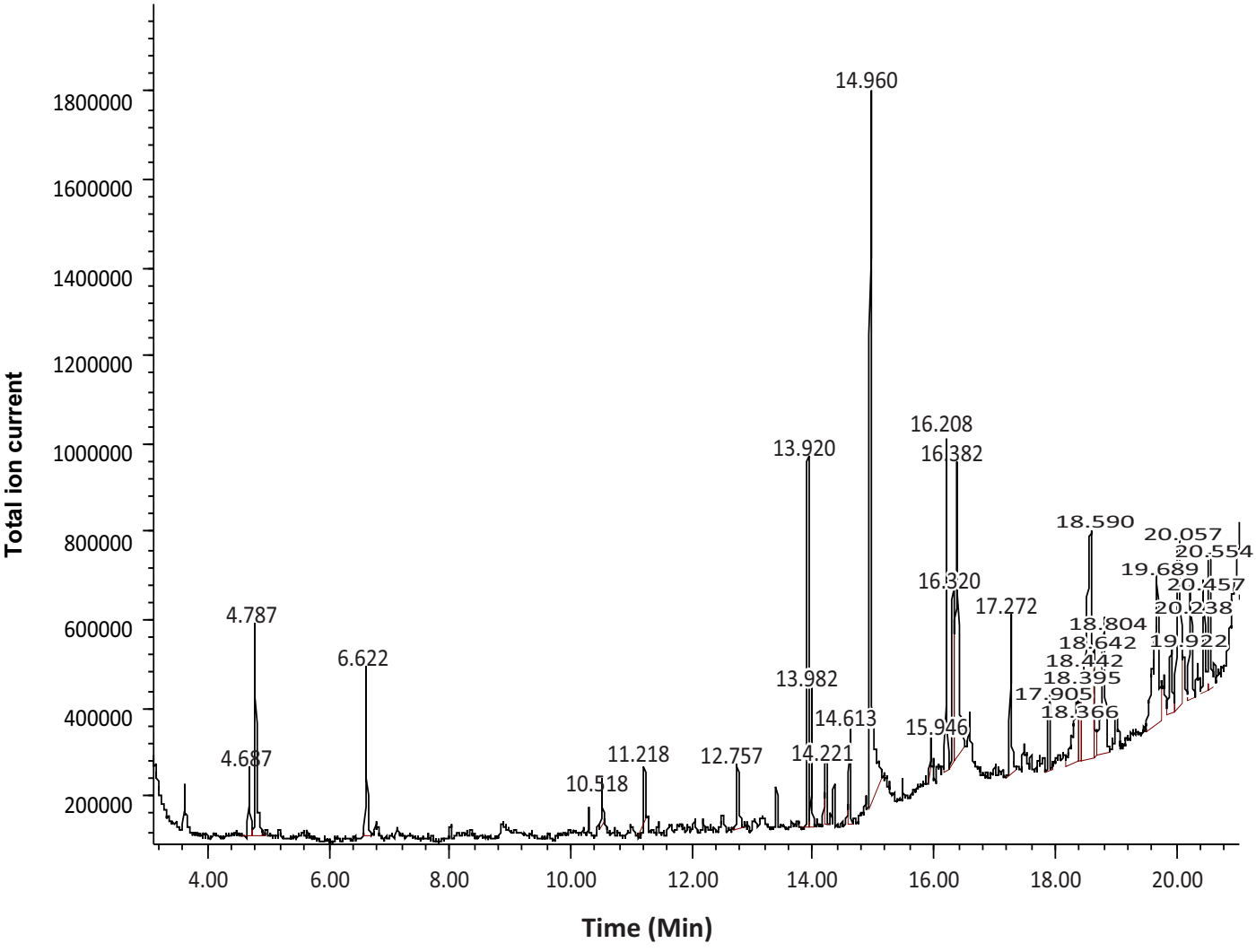

Figure 5: Total ion current chromatogram of the crude methanolic extract of *S. dasyphyllum* leaves

Table 7: GCMS analysis showing the chemical constituents of the crude methanolic extract of *S. dasyphyllum* leaves

Serial No.	Constituents	Retention time (min)	Peak area	Total (%)	m/z
1	Benzene, 1-methyl-3-(1-methylethyl	4.687	3369974	1.202	134.218
2	Eucalyptol	4.787	11349796	4.047	154.249
3	Levomenthol	6.622	7400539	2.639	156.265
4	2(4H)-Benzofuranone, 5,6,7,7a-tetr ahydro-4,4,7a-trimethyl-, (R)-	10.518	1967511	0.702	180.244
5	Diethyl Phthalate	11.218	1358949	0.485	222.237
6	Paradrine	12.757	4212600	1.502	151.206
7	2-Pentadecanone, 6,10,14-trimethyl	13.920	12897177	4.599	268.478
8	Neophytadiene	13.982	4793467	1.709	278.500
9	Cyclotetradecane	14.221	2438364	0.870	196.372
10	Methyl 11-methyl-dodecanoate	14.613	4181829	1.491	228.371
11	n-Hexadecanoic acid	14.960	41749276	14.888	256.424
12	1-Docosene	15.946	1206020	0.430	308.585
13	Phytol	16.208	11567479	4.125	296.531
14	9,12-Octadecadienoic acid (Z,Z)-	16.320	10934469	3.899	280.400
15	9-Octadecenoic acid	16.382	21205470	7.562	282.500
16	17-Pentatriacontene	17.272	7189211	2.564	490.900
17	delta.-Dodecalactone	17.905	2699731	0.963	198.300
18	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	18.366	7910832	2.821	154.250
19	Thunbergol	18.395	2604394	0.929	290.500
20	3-buten-2-one, 4-(5,5-dimethyl-1-oxaspiro[2.5]oct-4-yl)	18.442	3936300	1.404	208.300
21	trans-Sesquisabinene hydrate	18.590	36539798	13.030	222.370
22	Caryophyllene oxide	18.642	4095689	1.461	220.351
23	cis-11-Hexadecenal	18.804	13638063	4.863	238.410
24	9,17-Octadecadienal, (Z)-	19.689	18339012	6.540	264.400
25	Dicyclopentyl-	19.922	5794112	2.066	166.300
26	1,2-Pentanediol, 5-(6-bromodecahyd ro-2-hydroxy-2,5,5a,8a-tetramethyl-1-naphthalenyl)-3-methylene-	20.057	17905812	6.385	403.400
27	Z,E-2,13-Octadecadien-1-ol	20.238	7790870	2.778	266.500
28	Decane, 1,10-dibromo-	20.457	5150169	1.837	300.070
29	1H-Indene, 5-butyl-6-hexyloctahydro-	20.554	6198656	2.210	264.500

DISCUSSION

The phyto-constituents detected in crude MESd included: flavonoids, alkaloids, saponins, cyanogenic glycosides, tannins and reducing-sugars. This correlated with previous reports of some other authors who worked on *S. dasyphyllum*[12] and other species of the Solanaceae family such as *S. incanum L., S. microcarpon L.* and *S. melongena L.*[21,22,23]

The activities of the MESd can be referred to as broad-spectrum antibacterial action since it has activity against all the bacteria that were tested. This broad-spectrum antibacterial activity has been previously reported among some other species of the Solanaceae family by some authors. Akanmu *et al*[22] reported activities of the aqueous and methanol leaf extracts of *S. incanum* against S. aureus*, P. aeruginosa, S. pyogenes and K. pneumoniae. Indhumathi* and *Mohandass*[13] also reported similar broad-spectrum antibacterial activity of the ethanolic extract of *S. incanum against S. aureus, B. subtilis, P.*

aeruginosa, Salmonella paratyphi and *Vibro cholerae. Amutha*[24] worked on the antibacterial activity of extract of *S. melongena* seed against human bacterial pathogens. According to the report, activity was observed against *S aureus* and *E coli*. Manal *et al*[21] reported the antimicrobial activity of the methanolic extract of *S. incanum* ripe fruit against bacterial (*B. subtilis* and *E. coli*) and fungal (*C. albicans and A. niger*) isolates. In this study, the different genera of the fungi isolates were susceptible at varying degrees to the crude extract and the different fractions and thus also maintained the broad-spectrum action against the fungi isolates. *Prakash* and *Jain*[25] similarly studied the antifungal activity of the crude extract of *S. nigrum* and reported activity against A. niger, *A. flavus* and *C. albicans*. The broad-spectrum of activities exhibited by the crude methanolic extract and the different fractions in this and previous studies suggests that the extracts and fractions may contain multiple antimicrobial constituents or single constituent with broad-spectrum property. Previous reports have demonstrated that some secondary metabolites present in plant extracts possess antimicrobial properties.[26,27] Phytoconstituents such as saponins, alkaloids, tannins, flavonoids, glycosides, etc, have been tested and confirmed for antimicrobial property.[27,28,29] The presence of these phytoconstituents in this study further suggests that these metabolites might have contributed to the broad-spectrum antimicrobial activity observed.

The minimum inhibitory concentration (MIC) of the MESd and fractions against both the bacteria and fungi isolates falls within the range 0.25 and >4 mg/mL while the MBC and MFC falls within the range 0.5 and >4 mg/mL. Akanmu *et al*[22] in their study on the methanolic and aqueous extract of *S. incanum* had slightly higher MIC values between 5 and 7.5 mg/mL and much higher MBC values between 20 and >80 mg/mL against the bacteria isolates with the methanolic extract showing the greatest activity. To consider an antimicrobial agent as either static or cidal, the ratio of the MBC or MFC to MIC is usually calculated. For the agent to be considered cidal in action the ratio of the MBC or MFC to MIC must be less than or equal to four.[30] In this study, the activity of MESd is considered bactericidal against *B. subtilis and P. aeruginosa,* and *fungicidal against Rhizopus sp.* and *C. albicans*. The SdAQF and SdHXF both exhibited cidal effect against all the bacterial isolates while SdDMF exhibited cidal action against all the fungi isolates used in this study. This was evident from the results of the time-kill kinetic study and thus reflects the strong antimicrobial properties of the Solanaceae family and their potential as possible source of novel therapeutic agents.

The time-kill kinetics of the methanolic extract of *S. dasyphyllum* and its fractions against *S. aureus, E. coli* and *C. albicans* showed that the percentage viable count decrease with time at varying degree. To the best of our knowledge, this is the first time that the time-kill kinetics study on *S. dasyphyllum* will be reported. However, in a similar study carried out by Oladosu *et al* [31] but with a different plant (Acacia nilotica), complete killing of *S. aureus* and *P. aeruginosa isolates* was recorded after 8 hours with the methanolic extract at 1 mg/mL and 2 hours at 2 mg/mL concentration of the extract. This thus corroborates the microbicidal potentials of extracts from medicinal plants such as *S. dasyphyllum* extract and fractions in this study.

The results of the TPC, FRAP and TAC obtained from this study showed that the antioxidant activity of MESd increases with increase in concentration, but the antioxidant activity is relatively weaker than the respective standards. The DPPH assay showed an increase in the percentage inhibition of the free radical scavenging property with increase in the concentration of MESd. However, the MESd demonstrated weaker DPPH radical scavenging activity compared to the ascorbic acid standard for each of the concentration. This suggest that MESd may contain low level of phytoconstituents such as the flavonoids and polyphenols etc, known to possess strong antioxidant property and DPPH radical scavenging activity.[19,32] Similar report was documented by Sodeinde *et al* [12] in their study on the antioxidant activity of *S. dasyphyllum* leaves and fruit extracts. The IC50 value of DPPH for the MESd was 425.51µg/mL while that of ASA standard was 97.49µg/mL (Figure 4). It has been reported that extracts with IC50 within the range 150 - 500µg/mL are considered to have moderate antioxidant activity.33 This further confirmed that MESd may not be rich in flavonoids, polyphenols and other phytoconstituents with strong antioxidant activity.[12,19,32]

The 29 metabolites determined by the GC-MS analysis of the MESd have useful medicinal benefits. While Phytol is an acyclic diterpene usually used as precursor for production of vitamin E and K1, 1-methyl-3-(1-methylethyl)-benzene (p-Cymene) is a monoterpene with an antioxidant, anti-inflammatory, antinociceptive, anxiolytic, anticancer and antimicrobial properties.[34] Eucalyptol is a cyclic ether and monoterpenoid with anti-inflammatory, antioxidant and analgesic effects in

various diseases, including respiratory disease, pancreatitis, colon damage, cardiovascular and neurodegenerative diseases.[35] Levomenthol has antipruritic, anti-inflammatory, antibacterial and antifungal properties and it's useful in the treatment of upper respiratory diseases and superficial skin infections associated with inching.[36] Dihydroactinidiolide (5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- 2(4H)-Benzofuranone) is a volatile terpene. Paradrine (4-hydroxyamphetamine) is a sympathetic nervous system stimulant useful in a medical process called mydriasis. Hexahydrofarnesylacetone (2-Pentadecanone, 6,10,14-trimethyl) is a member of the class of compounds known as sesquiterpenoids. Caryophyllene oxide is one of the major sesquiterpenes suitable for medicinal uses such as an anti-inflammatory, local anesthetics, antioxidant, and perhaps in cancer treatment.[37] To our knowledge, this is the first time that the GC-MS analysis of S. dasyphyllum crude extract will be described. Previous studies have focused on other species of the Solanaceae family such as S. nigrum, S. incanum etc. Jasim et al[38] carried out GC-MS analysis to characterize the alkaloid constituents and antimicrobial activity of S. nigrum. Sundar and Pillai[14] also carried out GC-MS profiling of petroleum ether, methanol and ethanol extract of S. incanum leaves. In another comprehensive study carried out by El-Shaboury *et al*,[39] the diversity of eleven species of Solanum (*S. villosum Mill, S. villosum L, S. nigrum, S. incanum L, S. coagulans, S. schimperianum, S. macracanthum, S. glabratum, S. torvum, S. sisymbriifolium and S. dulcamara*) from southwest Saudi Arabia was assessed. The assessment was based on the differences in the secondary metabolites of the ethanol extracts of the different species using GC-MS analysis. Their findings revealed 87 different phyto-constituents at six different retention times. Most of the metabolites identified by the GC-MS analysis in the different species were also described in this study.

CONCLUSION

From the findings in this study, it is very clear that S. dasyphyllum, like most other medicinal plants, is a good source of active metabolites that may be of clinical value. The various bioactive compounds revealed by the phyto-compound screening and GC-MS analysis suggest that S. dasyphyllum is a reservoir of various bioactive metabolites with excellent anti-infective but moderate anti-oxidant potential that may be useful in the development of better and effective natural and semi-synthetic drug products.

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