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Synergistic Assessment of the Antimicrobial Activities of *Sida acuta, Dioscorea bulbifera* and *Citrus aurantifolia* Extracts on Selected Bacteria

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ABSTRACT

Synergy refers to the combination and interaction of two or more agents which produces a result that is greater than the sum of their individual effects. Hence, the synergistic assessment of the effects of *Sida acuta & Citrus aurantifolia, Sida acuta & Dioscorea bulbifera* and *Citrus aurantifolia & Dioscorea bulbifera* combined extracts on selected bacteria (*Escherichia coli0157:H7, Salmonella typhymurium* and *Vibrio cholerae*) were investigated. The study area was Omuanwa in Ikwerre Local Government Area of Rivers State, Nigeria were the plants were collected. The test organisms were identified by polymerase chain reaction technique, while the antimicrobial effects of the combined plant extracts were examined against the different bacterial species by agar well diffusion method and sensitivity interpreted in accordance with the Clinical and Laboratory Standard Institute. Phytochemicals extracted were Flavonoids, Cardiac Glycosides, Tannin, Phenols, Alkaloids, Steroids, Terpenoid and Saponins, however, the concentrations of phytochemicals extracted by ethanol and aqueous showed statistical significant difference of $p<0.05$. The organisms were more sensitive to *Citrus aurantifolia & Sida acuta* with 91.7%, followed by *Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera* with equal sensitivity of 75.0%, also evident in the mean zones of inhibitions, as the highest mean of 15.500mm was observed in *Citrus aurantifolia & Sida acuta* against all test organisms followed by *Citrus aurantifolia & Dioscorea bulbifera* (12.667mm) and *Sida acuta & Dioscorea bulbifera* (12.417mm). *Escherichia coli0157:H7* was most sensitive to *Sida acuta & Dioscorea bulbifera, Salmonella typhimurium* was most sensitive to *Citrus aurantifolia & Sida acuta*, while *Vibrio cholerae* was most sensitive to *Citrus aurantifolia & Sida acuta.* Considering different mean concentrations of combined extracts of 100%, 75%, 50% and 25% against the test organisms, the mean zone of inhibitions was in the order of increasing concentrations, the higher the concentrations the higher their zones of inhibition. The ability of the combined extracts to have shown cleared zone of inhibitions indicates synergism.

Keywords: *Sida acuta & Citrus aurantifolia, Sida acuta & Dioscorea bulbifera, Citrus aurantifolia & Dioscorea bulbifera, Escherichia coli0157:H7, Salmonella typhymurium, Vibrio cholerae. Synergy, Combined Extracts.*

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INTRODUCTION

The Greek word synergos, which means "working together," is the origin of the word synergy (Berenbaum, 1989). Numerous areas of life, including mechanics, technical systems, human social life, and many more, have been described as having synergies (Breitinger, 2012). Synergy refers to the fact that a system, or the combination and interaction of two or more agents or forces, produces a result that is greater than the sum of their individual effects (Sparreboom *et al.*, 2004). This is true in all instances.

According to this definition, there are three possible forms of this "interaction of agents or forces" (Asante-Appiah & Chan, 1996): These forces could simply add up and have no effect on one another (no interaction), or they could work together to produce a result that is greater than anticipated (synergy), or they could produce a result that is less than the sum of the individual effects. This "negative" summation is called antagonism (Berenbaum, 1977; 1985). Collaborations of naturally dynamic specialists are a significant part of pharmacology and biomedicine. In this context, the biological activity caused by the simultaneous presence of multiple drugs is referred to as interaction (Bijnsdorp *et al.*, 2011). Such circumstances happen in various clinical circumstances:

- In the treatment of infections and cancer, combinations of cytotoxic drugs require lower doses of each to produce better therapeutic effects with fewer side effects.
- Mixes of anti-microbials moreover join improved productivity with less secondary effects also, decreased improvement of opposition.
- Numerous serious clinical circumstances require organization of a few medications basically in light of the fact that of various remedial signs. Albeit in such a case drug blends are not figured out to search for collaborations, the communications of these medications should be surveyed.
- The impact of one medication might be increased by another medication that doesn't deliver such an impact all alone.

In this large number of cases, different medications are regulated, and will show some type of association, synergistic, opposing, or none (Chou, 2006; 2010). Strategies to decide and evaluate drug associations are in this manner a fundamental apparatus in pharmacology. By and large, separates from plants, creatures, or even soils were the main grouped drugs. Instead of a single agent, these were complex mixtures in which some ingredients may have interacted with others. Throughout the long stretches of advancement of drug store, detachment, blend and advertising of single medications turned into the acknowledged norm. Whether an intricate blend or a mix of medications is utilized, the organic connection of all dynamic substances ought to be known. Simple systems can show synergy (Greco *et*

al.,1995): two drugs that only affect one target protein can work together. In such a scenario, we can conduct mechanistic research on the drugs' interactions to ascertain the reasons and mechanisms by which multiple drugs can complement one another (or not). Complex situations, such as patients on multiple medications, may also exhibit synergy. As a rule, more than one natural objective (protein, pathway, or even organ) are associated with such cases, and single unthinking portrayals are not fitting. Extra boundaries to consider are drug retention, tissue dissemination, and leeway (Breitinger, 2012). It could be anticipated that many medications obstruct digestion of different medications. Subsequently, a substance B that dials back leeway of a functioning medication A, say by hindering utilizing chemicals or discharge, may lead to a higher compelling centralization of A that remaining parts in the body for a more extended time frame. As a result, one would see a more prominent impact of medication A when given along with B, albeit the two medications have totally various methods of activity. Although these two drugs would undoubtedly have a "combined effect that is greater than the sum of their individual effects," their combination is synergistic in practice, not strictly speaking (Breitinger, 2012).

Each combination is one of a kind; One organism may benefit from the same drugs while another may not. Nonetheless, according to Pharmacy 180 (2019) the Combined Use of Antimicrobial – Pharmacology, the basic rules are:

a. It is rare for two bacteriostatic agents to work together in a synergistic way.
b. Two bactericidal medications are regularly added substance and at some point synergistic assuming the organism is sensitive to both.
c. Blend of a bactericidal with a bacteriostatic medication might be synergistic or antagonistic relying upon the organism. Overall:

Assuming the organism is highly sensitive to the cidal drug - reaction to the mix is equivalent to the static medication given alone (clear antagonism), in light of the fact that cidal drugs act fundamentally on quickly duplicating microorganisms, while the static medication hinders multiplication.

On the off chance that the organism has low sensitivity to the cidal drug - synergism might be seen. As a result, synergistic combinations can be used to treat infections that are typically difficult to treat whenever possible.(Tallarida, 2006; Toews & Bylund, 2005).

However, medicinal plants are the primary source of medication used as complementary or alternative treatments to orthodox medicine (Saganuwan, 2012). The act of Neutraceuticals, cosmeceutical and pharmaceutical industries using medicinal plants is gaining more attention around the globe, hence, the need for the study of antimicrobial activities of *Dioscorea bulbifera* (air potato), *Citrus aurantifolia* (Limes) and *Sida acuta* (wired weed) on some selected bacteria. This study is to find out the synergistic antimicrobial effect of *Sida acuta*,

Dioscorea bulbifera and *Citrus aurantifolia* extracts on *Salmonella typhimurium, Vibrio cholerae* and *Escherichia coli0157:H7* test organisms, which are basically causes of gastroenteritis.

Aim of the study

The aim of the study is to investigate the synergistic assessment of the antimicrobial activities of *Sida acuta*, *Dioscorea bulbifera* and *Citrus aurantifolia* extracts on selected Bacteria (*Salmonella typhimurium, Escherihia coli 0157:H7* and *Vibrio cholerae).*

Objectives of the Study

1. To determine the phytochemistry of the plant extracts
2. To Identify the test organisms by molecular technique (PCR)
3. To determine the antimicrobial effect of *Sida acuta* & *Dioscorea bulbifera* combined extracts on selected organisms (*Salmonella typhimurium, Escherihia coli 0157:H7* and *Vibrio cholerae).*
4. To determine the antimicrobial effect of *Sida acuta* & *Citrus aurantifolia* combined extracts on selected organisms (*Salmonella typhimurium, Escherihia coli 0157:H7* and *Vibrio cholerae).*
5. To determine the antimicrobial effect of *Citrus aurantifolia* & *Dioscorea bulbifera* combined extracts on selected organisms (*Salmonella typhimurium, Escherihia coli 0157:H7* and *Vibrio cholerae).*

Study Area

The study area for plant sampling is Omuanwa Community in Ikwerre local Government Area of Rivers State, it is situated between latitude 4054'0''N and Longitude 6045'0''E and 5012'0''N and 703'0''E (Figure 3.1). The mean annual temperature of the area is 280C. It is predominantly under the influence of the monsoon wind and also record heavy rainfall of 2370.5mm (Wizor, 2012). Generally, Rivers State is characterized by four biodiversity important vegetation zones, namely, the lowland rainforests, freshwater swamp forests, mangrove forests, and barrier island forests while Ikwerre local Government Area has freshwater swamp forests (Wizor, 2012). The Local Government Area is located in the Northern part of Rivers State Nigeria. Rivers State is located on the southern part of Nigeria bounded on the South by the Atlantic Ocean, to the North by Imo, Abia and Anambra States, to the East by Akwa Ibom State and to the West by Bayelsa and Delta States (Ofomata, 1979).

Experimental Design

The study adopted a 3 x 3 (3 by 3) factorial experimental design. This supports the use of three treatments (*Sida acuta, Dioscorea bulbifera* and *Citrus aurantifolia*) and three test organisms (Escherichia coli0157:H7, Salmonella typhimurium and Vibrio cholerae) for the study.

Plants Sample Collection

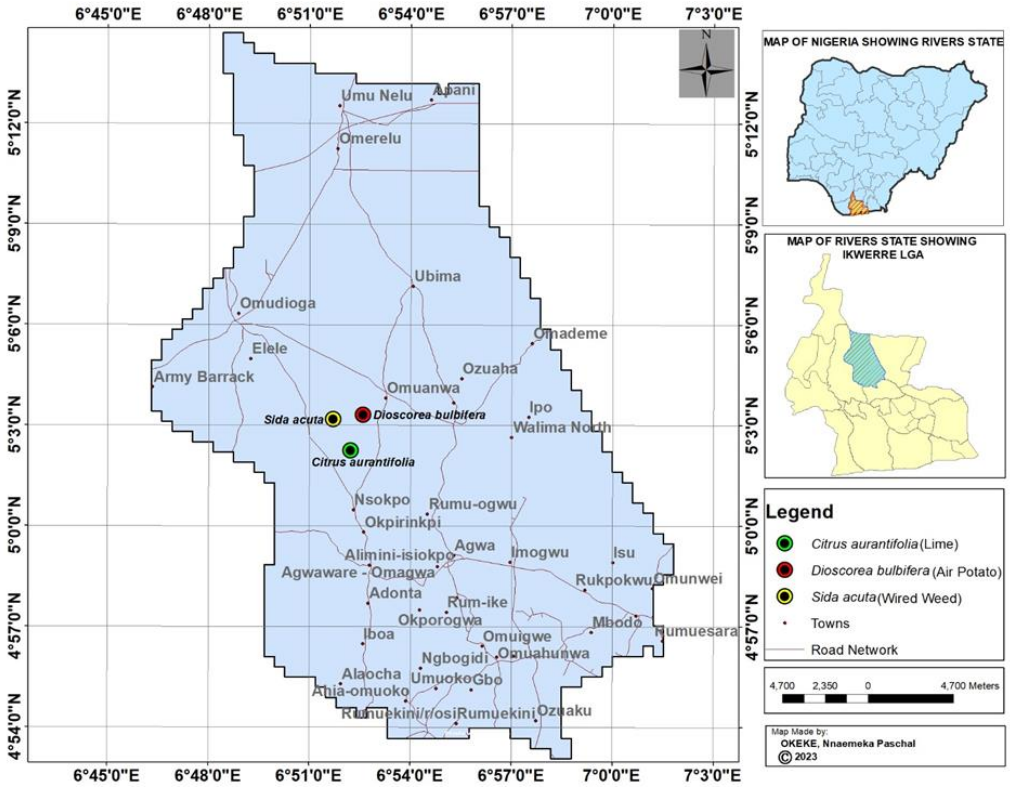
Fresh samples of *Sida acuta* leaves, *Dioscorea bulbifera* bulbs and *Citrus aurantifolia* fruits were collected from different farms in Omuanwa Community, Ikwerre Local Government Area of Rivers State. Thus were identified in the Department of Plant Science and Biotechnology, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt.

Plants Extraction and Phytochemical Analysis

The plants samples (*Dioscorea bulbifera* and *Sida acuta*) were collected, washed with distilled water and dried at room temperature, blended and put in a sterile container for extraction. While fresh Citrus aurantifolia were washed with distilled water about three times and sterilized with 70% ethanol using spray bottle and was ready for extraction.

The extraction for *Dioscorea bulbifera* and *Sida acuta* was done using soxlet extractor for continuous extraction processes and batch extraction method with ethanol and distilled water (Azwanida, 2015). A weighed portion of the pulverized sample (200g) was soaked in 1000ml of absolute ethanol and distilled water (BDHChemicals) for 72hours. The supernatant were decanted and filtered into a 1000ml conical flask through a N0. 1 Whatman filte paper for batch extraction while the soxlet extractor product was sent to the rotary evaporator. The extracts were concentrated using rotary evaporator set at 600C. While 250 ml pure juices of *Citrus aurantifolia* was collected and filtered with N0. 1 whatman filter paper.

It was then prepared in accordance with ISO17025. The following phytochemicals: flavonoids, cardiac glycoside, tannin, phenols, alkaloids, steroids, terpernoids and saponins were determined using the UV visible via scan analysis with the wavelength range of 200-1100nm. At each wavelength, its adsorption were compared with the UV developed standard for phytochemicals to determined the phytochemicals Present and it quantification was done with the help of the dilution factor which gives us the actual concentration. Then the values were subjected to statistical analysis using SPSS.



Map of the Study Area Showing Plants Sample Points

Collection of Test Bacteria (*Escherichia coli0157:H7, Salmonella typhimurium* and *Vibrio cholerae*).

Salmonella enteric sub specie, enteric serova typhimurium derived from ATCC 14028 with lot number 363-543-5, expiry date of 31/01/2023 and *E.coli 0157:H7* derived from ATCC 43888, lot number 795-209-2, expiry date 31/12/2022 were produced, packaged and shipped by Microbiologics 200 Cooper Avenue North, St. Cloud MN 56303, United States (www.microbiologics.com) while *Vibrio cholerae* (coded: VCP004/22) of a male 7years old was collected from Rivers State University Teaching Hospital of an Epidemiology reference laboratory for isolation of Vibrio cholerae.

Cultivation of the Test Organisms

The bacteria (*Salmonella typhimurium* and *Escherichia coli0157:H7*) came in KWIK STIK form which has the same instruction for use or preparations as follows.

The culture plates used to culture Escherichia coli0157:H7 were Nutrient Agar, MacConkey agar, Eosin Methylene Blue (EMB) and Cystein Lactose Electrolyte Deficient (CLED) Agar. The ones used to culture Salmonella typhimurium were Nutrient Agar, Salmonella-Shigella Agar (SSA) and Deoxycholate Citrate Agar (DCA) while the one used to culture Vibrio cholerae are Nutrient Agar and Thiosulphate Citrate Bile Salt (TCBS) Agar.

Molecular Identification

DNA extraction (Boiling method)

Five (5) milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^oC for other down stream reactions.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken

to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Sterility Test for the Plant Extracts

Each of the Plant Extracts of *Sida acuta, Dioscorea bulbifera* (aqueous and ethanol) and *Citrus aurantifolia* respectively were tested for sterility by culture before use. A 1g/ml of each of the extracts was plated by spread plate method with the use of a sterilized hockey stick on a freshly prepared nutrient media plates and incubated at 37^0C for 24hours. The plates were observed for growth (Diep *et al.*, 2019).

Qualitative Screening and Determination of Antibacterial Activity

Agar Well

The screening of the antibacterial activities of the plants' extracts and four conventional antibiotics were carried out for the determination of zone of inhibition by agar well as described by the National Committee of Clinical Laboratory Standards (NCCLS, 1993), Clinical and Laboratory Standard Institute (CLSI, 2016; 2019) for antimicrobial disc susceptibility tests. Sterile Mueller-Hinton Agar plates were inoculated with already prepared inoculums of the different test organisms with a sterile cotton swab. Then with 6mm diameter sterile cork borer, different wells were made in the inoculated media plates. A sterile micropipette was used to transfer 50µl of the working solution or suspension of the different concentrations into the well. A control normal saline was also placed in the separate well at the same time and incubated at 37^0C for 24hours.

Statistical Analysis

The results of the quantitative analysis of phytochemical contents were presented as t-test. The frequencies distribution of agar well antimicrobial activities and the different concentration of extracts were all analyzed using percentages and analysis of variance (ANOVA). However, the statistical tool IBM SPSS statistics version 22 was used.

RESULTS AND DISCUSSION

Results in table.1 showed the comparison between the aqueous and ethanolic quantitative phytochemicals analysis of *Sida acuta* extract. Flavonoids, Phenols, Alkaloids, and Steroids extracted by the both solvents, however, Cardiac glycosides, Tannin and Saponins were extracted only by ethanolic while terpenoid was extracted by aqueous only. The mean concentration of phytochemicals extracted by the ethanolic solvent were higher than that of the aqueous solvent. Tannin had the highest ethanolic mean concentration of 50.00 while saponin had the least mean concentration of 0.28. In aqueous extraction, flavonoids had the highest mean concentration of 1.48 and alkaloids had the least mean concentration of 0.25. But anthroquinones and phobatannins were not extracted by the solvent. In the comparison,

p-values (0.00) were significant for flavonoids, cardiac glycosides, tannin, phenols, steroids, terpenoid, and saponins, but not significant for alkaloids as p-value (0.419) is greater than 0.05.

Results in table.2 represent the comparison between the aqueous and ethanolic quantitative phytochemicals analysis of *Dioscorea bulbifera* extract. Flavonoids, Tannin, Phenols, and Alkaloids extracted by the both solvents, however, Cardiac glycosides, and Saponins extracted only by ethanol. In the comparison, p-values (0.00) are significant for flavonoids, cardiac glycosides, tannin, phenols, terpenoid, and saponins, but not significant for alkaloids as the p-value (0.1835) is greater than 0.05. Some of the phytochemicals such as steroid, anthroquinones, terpernoids and phobatanins not extracted by the solvents. Phenols had the highest ethanolic concentration of 18.40 while alkaloids had the least concentration of 0.68. In aqueous extraction, flavonoids had the highest concentration of 6.68 and tannin had the least concentration of 0.07.

Results in table. 3 of the quantitative phytochemicals analysis of *Citrus aurantifolia* extract, shows that the following phytochemicals: Flavonoids, Tannin, Phenols, and Alkaloids, Steroids, Terpenoid and Saponins were extracted. Alkaloids had the highest concentration of 84.00 while terpernoids had the least concentration of 0.53. No solvent was used for the extraction because lime juice is already in the liquid state.

Table. 1: Mean Comparison of Aqueous and Ethanolic Phytochemical Screening of *Sida acuta* Extracts

Phytochemicals	***Sida acuta* Extracts**		**T-test**	**P-value**
	Aqueous(mg/ml)	**Ethanolic(mg/ml)**		
Flavonoids	1.48 ± 0.05	40.00 ± 0.25	151.0879	0.00
Cardiac Glycosides	–	1.48 ± 0.05	29.6000	0.00
Tannin	–	50.00 ± 0.20	250.0000	0.00
Phenols	0.81 ± 00.05	31.00 ± 0.15	190.9383	0.00
Alkaloids	0.25 ± 0.10	0.34 ± 0.00	0.9000	0.419
Steroids	0.70 ± 0.05	1.85 ± 0.04	17.9600	0.00
Terpenoid	0.26 ± 0.01	–	26.0000	0.00
Saponins	–	0.28 ± 0.05	5.6000	0.00

Table. 2. Mean Comparison of Aqueous and Ethanolic Phytochemicals Screening of *Dioscorea bulbifera* Extracts

Phytochemicals	***Dioscorea bulbifera* Extracts**		**T-Test**	**P-value**
	Aqueous(mg/ml)	**Ethanolic(mg/ml)**		
Flavonoids	6.68 ± 0.01	8.84 ± 0.08	26.7915	0.00
Cardiac Glycosides	–	1.20 ± 0.01	120.0000	0.00
Tannin	0.07 ± 0.01	1.03 ± 0.17	5.6373	0.00
Phenols	1.72 ± 0.01	18.40 ± 0.05	327.1217	0.00
Alkaloids	0.23 ± 0.01	0.68 ± 0.28	1.6061	0.1835
Steroids	–	–	–	–
Terpenoid	–	–	–	–
Saponins	–	1.15 ± 0.02	57.5000	0.00

Table. 3. Mean Phytochemicals Screening of *Citrus aurantifolia* Extract

Phytochemicals	***Citrus aurantifolia* Extracts (mg/ml)**
Flavonoids	19.5 ± 1.5
Cardiac Glycosides	$-$
Tannin	28.5 ± 6.80
Phenols	27.14 ± 0.23
Alkaloids	84.0 ± 0.20
Steroids	1.00 ± 0.01
Terpenoid	0.53 ± 0.20
Saponins	3.22 ± 0.10

Figure 1 represents the results of the agarose gel electrophoresis of the selected bacterial isolates. Lanes 1 – 3 represent 16SrRNA gene bands (1500bp) of *Escherichia coli0157:H7*, *Salmonella typhimurium* and *Vibrio cholerae* respectively. Lane L represents the 100bp Molecular ladder indicated at 500bp.

In Figure 2 and 3, the obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate M1 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates A1, A2 and VC within the *Escherichia*, *Salmonella,* and *Vibrio* spp. and revealed a closely relatedness to *Escherichia coli0157:H7*, *Salmonella typhimurium* and *Vibrio cholerae* respectively.

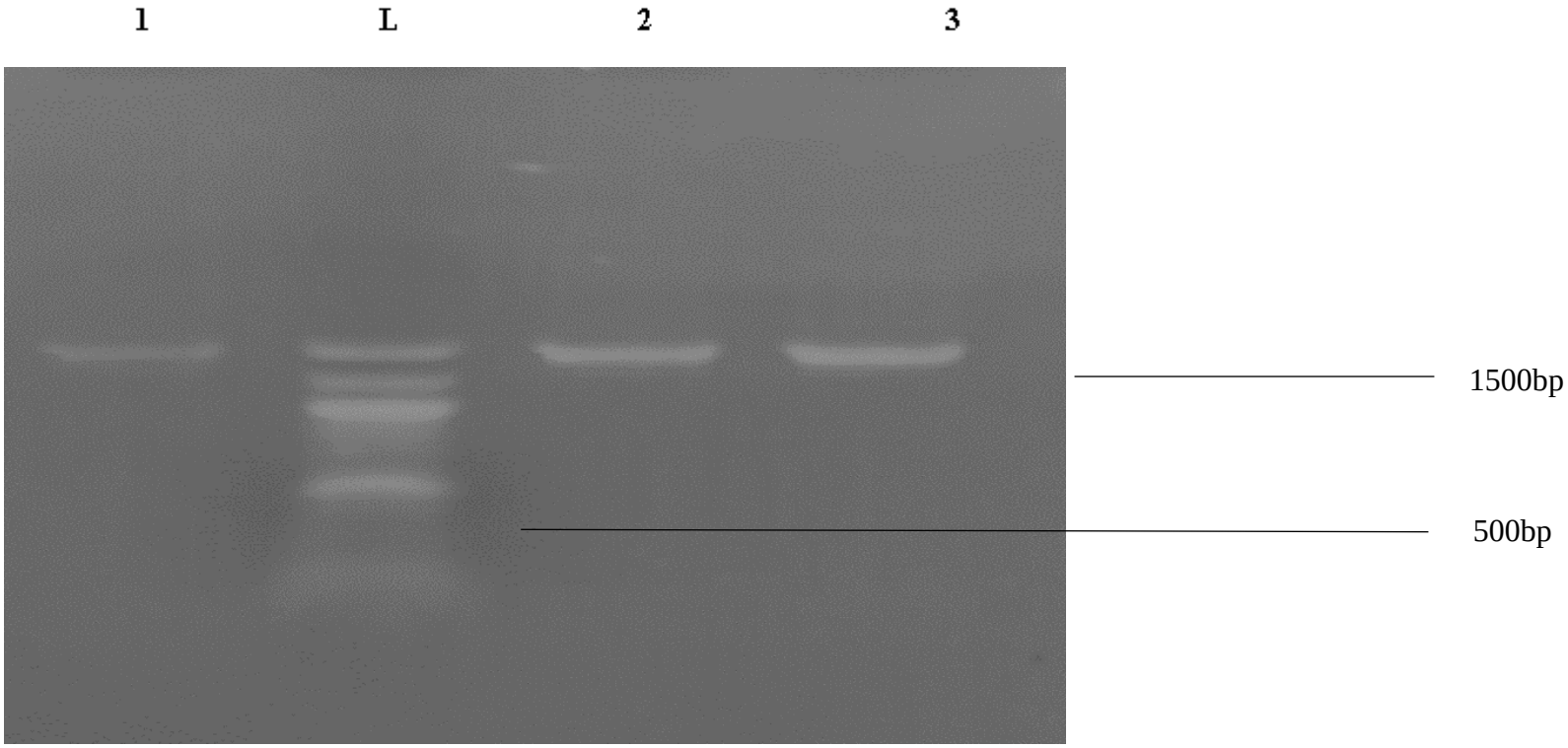


Figure 1:Agarose Gel Electrophoresis of Test Organisms

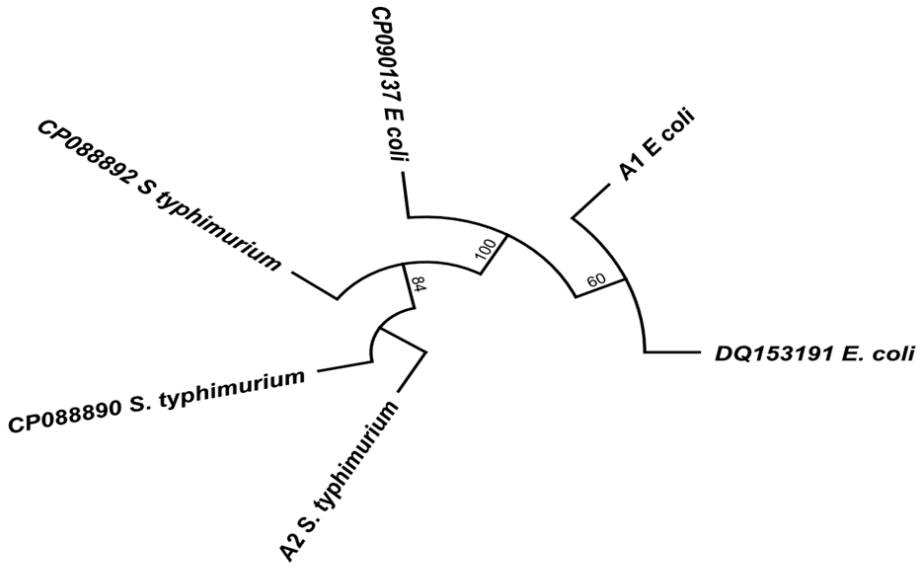


Figure 2: Phylogenetic Tree Showing the Evolutionary Distance Between the Bacterial Isolates of *Escherichia coli0157:H7* and *Salmonella typhymurium*.

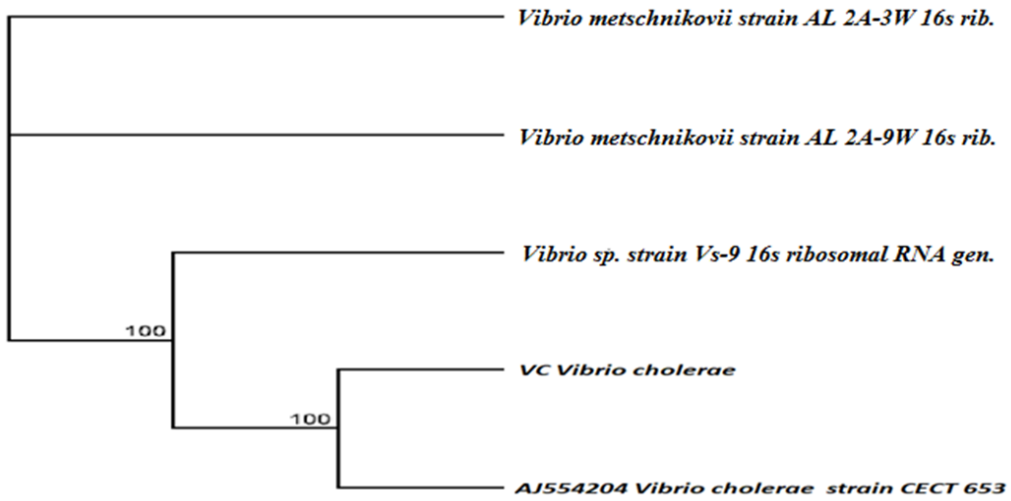


Figure 3: Phylogenetic Tree Showing the Evolutionary Distance between the Bacterial Isolates of *Vibrio cholerae*

Table 4. shows the frequency distributions of the antibiogram (extracts) based on the percentage sensitivity and resistance irrespective of the concentrations. The organisms showed higher level of sensitivity to *Citrus aurantifolia & Sida acuta* combination with 91.7%, while *Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera* yielded 75.0%. On the other hand, *Citrus aurantifolia & Sida acuta* showed least percentage resistance of 8.3% followed by *Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera* that yielded 25%.

Table 5. presents percentage distributions of the antimicrobial activity of combined extracts on the test microorganisms based on their percentage sensitivity and resistance. *Escherichia coli0157:H7* was 100% sensitive and 0% resistant to *Sida acuta & Dioscorea bulbifera*, 75%

sensitive and 25% resistant to *Citrus aurantifolia & Sida acuta* and also *Citrus aurantifolia & Dioscorea bulbifera*. *Salmonella typhimurium* showed 100% sensitive and 0% resistance to *Citrus aurantifolia & Sida acuta*, 75% sensitive and 25% resistance to *Citrus aurantifolia & Dioscorea bulbifera* and 50% sensitive and 50% resistance to *Sida acuta & Dioscorea bulbifera.* Similarly *Vibrio cholerae* showed 100% sensitive and 0% resistance to *Citrus aurantifolia & Sida acuta*, 75% sensitive and 25% resistance to *Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera.*

Table 6. describes the overall mean antibiogram activity (zone of inhibitions) of the combined extracts, *Citrus aurantifolia & Sida acuta* had the highest mean of 15.500(mm), followed by *Citrus aurantifolia & Dioscorea bulbifera* 12.667 (mm) and *Sida acuta & Dioscorea bulbifera* with the least mean of 12.417(mm).

Table 4: Overall Antibiogram Susceptibility of the Combined Extracts on Organisms

Extract Category	Extract	No. Resistant n(%)	No. Sensitive n(%)
Combined extracts	*Citrus aurantifolia & Sida acuta*	1 (8.3)	11 (91.7)
	Citrus aurantifolia & Dioscorea bulbifera	3 (25.0)	9 (75.0)
	Sida acuta & Dioscorea bulbifera	3 (25.0)	9 (75.0)

(N = 12)

Table 5: Percentage Distributions of Antimicrobial Activity of Combined Extracts on Test Organisms

Combined Extracts	Organisms	No. Resistant n(%)	No. Sensitive n(%)
Citrus aurantifolia & Sida acuta	*Escherichia coli0157:H7*	1 (25.0)	3 (75.0)
	Salmonella typhimurium	0 (0.0)	4 (100.0)
	Vibrio cholerae	0 (0.0)	4 (100.0)
Citrus aurantifolia & Dioscorea bulbifera	*Escherichia coli0157:H7*	1 (25.0)	3 (75.0)
	Salmonella typhimurium	1 (25.0)	3 (75.0)
	Vibrio cholera	1 (25.0)	3 (75.0)
Sida acuta & Dioscorea bulbifera	*Escherichia coli0157:H7*	0 (0.0)	4 (100.0)
	Salmonella typhimurium	2 (50.0)	2 (50.0)
	Vibrio cholerae	1 (25.0)	3 (75.0)

(N = 4)

Table 6: Overall Mean Antibiogram Activity of Combined Extracts

Extract Category	Extracts	Mean(mm)	SD(mm)
Combined Extracts	*Citrus aurantifolia & Sida acuta*	15.500	4.7386
	Citrus aurantifolia & Dioscorea bulbifera	12.667	5.1405
	Sida acuta & Dioscorea bulbifera	12.417	4.8328

(N=12)

The table 7. below describes the mean zone of inhibition of combined extracts concentrations, the combined extracts of *Citrus aurantifolia & Sida acuta* had highest mean

of 20.333mm and least mean of 8.667mm in concentrations of 100% and 25% respectively, *Citrus aurantifolia & Dioscorea bulbifera* had highest mean of 18.333mm and least mean of 6.000mm in the concentrations of 100% and 25% respectively, similarly, *Sida acuta & Dioscorea bulbifera* had the highest mean of 17.667mm and least mean of 7.333mm in the concentrations of 100% and 25% respectively. There were varying mean values across different concentrations of the combined extracts with statistical significant $p<0.05$. Finding implies that the higher the concentration, the higher the zone of inhibitions.

Table 7: Mean Zone of Inhibition of Combined Extracts Concentrations

Extracts	Concentration (%)	Mean (mm)	SD (mm)	F-value	Df	p-value	Remark
	Combined extracts						
Citrus aurantifolia & Sida acuta	25.0	8.667	2.3094	35.333	11	.000	Sig
	50.0	15.000	.0000				
	75.0	18.000	1.7321				
	100.0	20.333	.5774				
Citrus aurantifolia & Dioscorea bulbifera	25.0	6.000	.0000	14.184	11	.001	Sig
	50.0	11.667	2.8868				
	75.0	14.667	2.5166				
	100.0	18.333	2.8868				
Sida acuta & Dioscorea bulbifera	25.0	7.333	2.3094	8.038	11	.008	Sig
	50.0	10.000	4.0000				
	75.0	14.667	2.5166				
	100.0	17.667	2.0817				

DISCUSSION

The major objectives of this research was to find out the antimicrobial activities of plant extracts combinations of *Dioscorea bulbifera* & *Sida acuta*, *Dioscorea bulbifera* & *Citrus aurantifolia* and *Sida acuta & Citrus aurantifolia* on selected diarrhea causing bacteria (*Escherichia coli0157:H7, Salmonella typhimurium* and *Vibrio cholerae*). This was achieved by ascertaining the phytochemical analysis and antimicrobial activities of the plant extracts by the evaluation of the zones of inhibitions of the plant extracts combinations. The reason for this investigation is to curb the random consumption of these extracts in speculation for the management of stomach disorders without specifications, as these tests organisms are most of the causes of gastroenteritis. The results obtained were analysed statistically and compared with standard limits.

Results of phytochemical analysis carried out on the different plant extracts showed different secondary metabolites such as Flavonoids, Cardiac Glycosides, Tannin, Phenols, Alkaloids, Steroids, Terpenoid, Saponins comprising of both aqueous and ethanolic extracts. In the comparative phytochemical analysis of the extract of *Sida acuta* and *Dioscorea bulbifera,* it was shown that ethanolic extraction had the highest numbers of phytochemicals extracted

and the highest mean concentrations of the phytochemicals than the aqueous extraction. The quantitative comparison of the two forms of the extracts proved the ethanolic extracts to be better than the aqueous extracts based on the concentration and number of phytochemicals extracted, judging from the results of the p-values< 0.05 obtained, which justified the choice of the ethanolic extracts over aqueous extracts of *Sida acuta* and *Dioscorea bulbifera* in the research, except for Alkaloid that had p-values (0.419 and 0.184 respectively), which were not significantly different. However, *Citrus aurantifolia* was not involved in ethanolic and aqueous extraction because of its original liquid state which does not support any further liquid-liquid extraction. On the basis of concentration, Alkanoid of *Citrus aurantifolia* showed the highest mean concentration of 84.0 across all phytochemical extracted, followed by Tannin (50.00) of *Sida acuta* then Phenol (18.40) of *Dioscorea bulbifera*. However, the mean concentrations of the phytochemicals influenced the antimicrobial performances of the plant extracts in the hierarchy of *Citrus aurantifolia, Sida acuta* and *Dioscorea bulbifera.*

Identification of the test organisms was based on the most reliable identification technique known as the Polymerase Chain Reaction (PCR) and sequencing of the test organisms. In the agarose gel electrophoresis of the selected bacterial isolates, Lanes 1 – 3 represents 16SrRNA gene bands (1500bp) of *Escherichia coli0157:H7*, *Salmonella typhimurium* and *Vibrio cholerae* respectively. Lane L represents the 100bp Molecular ladder indicated at 500bp. The obtained 16SrRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (nr/nt) data base,the 16SrRNA of the isolates (*Escherichia coli0157:H7*, *Salmonella typhimurium* and *Vibrio cholerae*) showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates A1, A2 and M1 within the *Escherichia*, *Salmonella* and *Vibrio sp* revealed a closely relatedness to *Escherichia coli0157:H7*, *Salmonella typhimurium* and *Vibrio cholera* respectively.

Antimicrobial susceptibility test of the plant extracts on the test organisms showed that the three plant extracts combinations had varied degree of sensitivity and resistance. The organisms were most sensitive to *Citrus aurantifolia & Sida acuta* combination, whereas they expressed equal strength of sensitivity to *Dioscorea bulbifera & Sida acuta* and *Citrus aurantifolia & Dioscorea bulbifera* combinations. In contrast, the organisms showed the highest-equal level of resistance to *Dioscorea bulbifera & Sida acuta* and *Citrus aurantifolia & Dioscorea bulbifera* combinations and least to *Citrus aurantifolia & Sida acuta* combination (see table 4). However, the overall antimicrobial susceptibility test of the combined plant extracts on test organisms was supported by the mean zone of inhibitions of

extracts, with *Citrus aurantifolia & Sida acuta* having highest zone (synergized), followed by *Citrus aurantifolia & Dioscorea bulbifera* and *Dioscorea bulbifera & Sida acuta* having lowest zone (no interaction or antagonistic to each other) in the combined extracts. The order in the antimicrobial performance of the plant extracts is evident in the concentrations of the phytochemicals extracted, hence, the higher the concentration of phytochemicals the higher the antimicrobial activity. However, *Citrus aurantifolia* had the highest phytochemical concentration in Alkaloid, followed by *Sida acuta* in Tannin and the least phytochemical concentration was *Dioscorea bulbifera* in Phenol (See Table .1, .2 and 3).

Percentage distributions of the antimicrobial pattern of combined extracts on the test organisms showed the response of each of the test organisms to the extracts. Among the combined extracts, *Escherichia coli0157:H7* responded highest sensitive to *Sida acuta & Dioscorea bulbifera* and least equal sensitive to *Citrus aurantifolia & Sida acuta* and *Citrus aurantifolia & Dioscorea bulbifera* respectively. *Salmonella typhimurium* responded highest, moderate and least sensitive to *Citrus aurantifolia & Sida acuta, Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera* respectively. Whereas, *Vibrio cholerae* showed highest response to *Citrus aurantifolia & Sida acuta,* then least-equal percentage sensitivity to *Citrus aurantifolia & Dioscorea bulbifera* and *Sida acuta & Dioscorea bulbifera* respectively. However, *Citrus aurantifolia & Sida acuta* combination was most active on *Vibrio cholerae* and *Salmonella typhimurium* followed by *Escherichia coli0157:H7*. While *Citrus aurantifolia & Dioscorea bulbifera* combination had equal antimicrobial activity on *Salmonella typhimurium, Vibrio cholerae* and *Escherichia coli0157:H7*. *Sida acuta & Dioscorea bulbifera* was most active on *Escherichia coli0157:H7*, followed by *Vibrio cholerae* and *Salmonella typhimurium* (see table 5).

Considering different percentage concentrations of plant extracts of 100%, 75%, 50% and 25% against the test organisms, the mean zone of inhibitions was in the order of increasing concentrations. Thus, findings implied that the higher the concentrations the higher their zones of inhibition as supported by the findings of Munoz *et al*., (1995).

CONCLUSION

Nature nurtures every creature with green plants and also provided medicinal properties in these plants in form of phytochemicals in the treatment of diseases. Recently attention is being given on a substitute for a safe natural bioremedies for the therapy of infectious diseases because of their less or no harmful effects and resistance in microbes against them. The results of this study revealed that the different combinations of plants extracts were found to be very effective against the test organisms.

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