

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF THE CRUDE EXTRACT AND FRACTIONS OF *TAPINANTHUS GLOBIFERUS* LEAVES ON THE BACTERIAL ISOLATES OF WOUND

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ABSTRACT

Background: *Tapinanthus globiferus* (Mistle toe, *Kauchi*) is a medicinal herb used in the treatment of many ailments ranging from infectious to non infectious diseases. **Objective:** To investigate the effect of the antibacterial activities of the crude leaves extracts and fractions of *Tapinanthus Globiferus* leaves compared against that of Ciprofloxacin and Pefloxacin on seven wound associated bacteria. **Materials and Methods:** This study was conducted as cross sectional experimental study. The susceptibility pattern of the crude extract, fractions and the standard antibiotics were determined using Kirby-Bauer disc diffusion technique. MIC of the extract, fractions and the standard antibiotics were determined using Macro broth dilution techniques. Separation of the crude extract was carried out using

column chromatography and the LD₅₀ of the extract was determined using Lorke's method. Data were analysed using SPSS version 20.0 (California, Inc., USA) and $p < 0.05$ was considered significant. Highly significant activity of the crude extract was observed on *P. aeruginosa* (at 10, 5, 3 and 1mg/disc, $F = 15.2$, $P = 0.0007$) and the lowest was observed on *S. pyogenes* (at 10 and 5mg/disc, $F = 3.3$, $P = 0.0049$). No any significant difference was

observed between the MICs and MBCs of the crude extract when compared with that of Ciprofloxacin. Out of the five fractions of *T. globiferus*, the 5th fraction presented a significant activity on *S. aureus* (at 0.03mg/disc, $P = 0.03$). No any significant difference was observed between the MICs and MBCs of the fractions when compared with Ciprofloxacin. The oral acute toxicity studies with the extract had shown an LD₅₀ of the extract to be >5000mg/kg. **Results:** The overall result of the present study provided evidence that the crude extracts of *T. globiferus* as well as some of the fractions (especially TF5; fifth fraction of *T. globiferus*) could be considered as a potential source of novel antibacterial agents which may be employed to forestall the present antibiotic resistance menace. **Conclusion:** Findings of the present study revealed that the crude methanol leaves extract and fractions of *T. globiferus* possessed an antibacterial activity. Significant antibacterial activities were observed with the crude extract and fractions against some wound associated bacteria despite their multi drug resistance antecedents.

KEYWORDS: Antibacterial activity. Tapinanthus Globiferus leaves. Bacterial isolates of Wound.

INTRODUCTION

Microbial resistance emerged and spread globally threatening the ability of treating common infectious diseases resulting in death and disability of individuals who until recently could continue a normal course of life.^[1] These antibiotic resistance strains are able to withstand most of the current antibiotics, thereby making the standard treatment ineffective, hence given room for the infection to persist, increasing the risk of spreading to others.^[1]

Report of the global surveillance on antimicrobial resistance conducted by WHO in 2014 have shown that, antibiotic resistance is no longer a prediction for the future but happening right now, across the globe and is putting at risk the ability to treat common infections in the community and Hospitals and without urgent, coordinated action the world is heading towards a post antibiotic era in which common infections and minor injuries which have been treatable for decades can once again kill.^[1]

The rise of antibiotic resistance is a global health crisis and governments now recognise it as one of the greatest challenges for public health. It is reaching dangerously high levels in all parts of the world. Antibiotic resistance is compromising the ability of treating infectious diseases and undermining many advances in Medicine.^[1]

Report on a global scale, have shown that, annual deaths resulting from antibiotic resistance is greater than 700,000; this figure is expected to increase to 10 million in 2050 unless an urgent intervention is made.^[2] Of these 10 million deaths, 4.1 million are expected to be from Africa, with Asia having a worst figure of 4.7 million deaths annually.^[2]

It was estimated that, by 2050, 25% of all deaths in Nigeria could be as a result of antibiotic resistance if the trends continue unchecked.^[2]

Preparation and Extraction

On the other hand, plants are traditionally proved to be a rich source of novel drug compound. The herbal mixtures have made large contributions to human health and well being.^[3] A wide variety of secondary metabolites such as tannins, terpenoids, flavonoids, alkaloids, quinines etc. are endowed with antimicrobial properties.^[4,5]

Tapinanthus globiferus (A. Rich) is the most common mistletoe that grows on *Azadirachta indica* tree (host) in West Africa. In Nigeria, *Tapinanthus globiferus* otherwise known as mistletoe is known with different names; *Kauchin darbejiya* (Hausa), *Eme-emi afomo* (Yoruba), and *Osisi/Okwuma osa* (Igbo).^[6]

It is a semi-parasitic plant that mostly grows on the branches of a large number of tree species such as *Azadirachta indica* (neem, *dogon yaro*, *darbejiya*), *Vitellaria paradoxa*, *Kola*, *Citrus*, *Combretum*, *Acacia*, *Aloe* and *Terminalia*.^[7]

In Nigeria, this plant is used traditionally in the treatment of inflammations, malaria, bacterial infections, ulcer, headaches, diabetes mellitus, stroke, stomach problems, as well as convulsions.^[8,9] This work intends to investigate the antibacterial potentials of the crude extract and fractions of *Tapinanthus globiferus* leaves on the bacterial isolates of wound namely; *S. aureus*, *P. aeruginosa*, *E.coli*, *S. liquefaciens*, *S. pyogenes*, coagulase negative *Staph* and *P. mirabilis*.

MATERIALS AND METHODS

Study Area

Specialist Hospital Sokoto is a government owned Hospital located within the Sokoto metropolis. Sokoto metropolis lies between latitude 13°31' 49N, longitude 5°14' 89E and at an altitude of 272 m above sea level. It is located in the extreme north western part of Sokoto

north and Sokoto South Local government areas and also some parts of Kware LGA from the north, Dange Shuni LGA from south and Wamakko LGA to the west.

Sokoto metropolis was estimated to have a population of 427,760 people^[11] with Hausa and Fulani been the dominant ethnic groups followed by Zabarmawa, Adarawa, Arawa, Kabawa, Nupes with Yorubas, Igbos and others. The major occupations of the inhabitants are; trading, commerce and farming with reasonable proportion of the population working in both public and private sectors.^[10]

Specialist Hospital Sokoto is the referral centre of choice by clients from rural areas and many cases of open wounds are mostly seen there than the other tertiary Hospitals. The Hospital serves as referral centre for almost all the Local government areas (LGAs) across the State; it also serves many clients from Niger republic.

Study Population

The specimens (wound swab and aspirates) were collected from the population of male and female patients with purulent wound seen in surgical outpatient department (SOPD), Female and Male surgical wards of the Specialist Hospital Sokoto after a verbal/written consent of the patients or their relatives was obtained.

Inclusion and Exclusion Criteria

The inclusion criterion covers both male and female patients with purulent wound seen in the surgical out-patient department (SOPD), Male and Female surgical wards of Specialist Hospital Sokoto whose verbal consent were duly obtained. All patients outside the aforementioned category were excluded from this study.

Ethical Issues

Prior to commencement of sample collection an ethical approval was obtained from the ethical committee under the Chairman Medical advisory committee (CMAC), Specialist Hospital Sokoto. Similarly, verbal consents were obtained from patients and their relatives prior to sample collection; this was done via an explanation of the aims and objectives of the study to the patients and their relatives. Patients' privacy and safety are well guarded throughout the period of this study.

Specimen Collection

Specimens (wound swab and aspirates) were collected aseptically using a sterile swab stick and syringes. The wound surfaces were first cleaned with cotton wool soaked in sterile normal saline followed by swabbing the centre of the affected tissues using sterile swab stick. The aspirates were collected by needle aspiration. Prior to the aspiration, the skin around the wound were cleaned with 70% alcohol to get rid of the contaminating microbes and the fluid was collected by inserting the needle deep into the wound changing its angle two to three times to remove fluid from different areas of the wound. The samples were labelled, registered, transported immediately to the Laboratory and processed.

ANALYTICAL METHODS

Bacterial Isolation

Specimens were aseptically cultured onto Blood agar, Chocolate agar and MacConkey agar. Aerobic culture (for the swabs) was carried out on blood and MacConkey agar whereas the anaerobic cultures (for the aspirates) were done on Chocolate agar which was placed inside a candle jar prior to incubation.

The inoculated plates were incubated at 37°C for 24hours. Cultures were identified on the basis of their physical appearance; Gram's staining reaction and biochemical characteristics using Microgen (Microgen Bio-products) biochemical test system for the Gram negative organisms and Catalase, Coagulase, PYR test (pyrolidonyl arylamidase also called pyrolidonyl aminopeptidase), etc for the Gram positive organisms.

Plant Collection and Identification

The leaves of the *T. globiferus* were collected from a neem (*Azadirachta indica*) tree (which serves as the host for this parasitic plant) in the vicinity of the Faculty of Medical Laboratory Science, Usmanu Danfodiyo University Teaching Hospital, Sokoto. The leaves were identified and authenticated in the herbarium of Biological Sciences Department, Usmanu Danfodiyo University Sokoto by Abdulazeez Salihu where voucher specimen (UDUH/ANS/0131) of *T. globiferus* was deposited.

The leaves were shed dried and milled to powder using mortar and pestle in the department of Pharmacognosy, Usmanu Danfodiyo University, Sokoto and stored at room temperature with plastic packaging until use.

Extraction of leaves

Maceration method of extraction was employed in this study for the extraction of the leaves.

Two hundred grams (200g) of the milled powder of *T. globiferus* leaves was dissolved in 1000mL of absolute methanol for 24hrs and filtered using Whatman number 1 filter paper. The filtrate was evaporated to dryness at 50°C in a water bath. The resultant extract was measured and expressed as a % extract yield of the original sample using the formular:

$$\text{Percentage (\%) yield} = \frac{E}{O} \times 100$$

Where: E = Weight of the extract and

O = Weight of the original sample

The dried extracts were placed inside a screw capped sterile containers and kept in a refrigerator at 2-8°C until use.

Preparation of paper Discs

Paper discs of 6mm in diameter were punched from Whatman no. 1 filter paper and labelled T. The prepared paper discs were sterilized with ultraviolet light at room temperature.

Determination of the Antibacterial activity of the Crude Methanol extracts of *T. globiferus* leaves

Kirby Bauer disc diffusion method (devised by^[11] was used to determine the antibacterial activities of the crude methanol extracts of *T. globiferus* leaves.

Ten microliters (10µL) of the prepared concentrations (1000 mg/mL, 500 mg/mL, 300 mg/mL and 100 mg/mL) of the methanol extracts of *T. globiferus* leaves were impregnated to each of the prepared and labelled paper discs to make an extract containing discs of 10mg/disc, 5mg/disc, 3mg/disc and 1mg/disc.

The extracts impregnated discs were placed in an incubator (at 25°C) for 24 hours to dry. After drying, the discs were placed in an appropriate, well labelled screw capped containers and store in refrigerator at 2-8°C.

Preparation of the Inoculum

Direct colony suspension method was the technique employed in the preparation of the inoculum in this study as recommended by.^[12]

Selected colonies from the identified isolates were picked with sterile wire loop and placed in a test tube containing 5mL of sterile normal saline to make a suspension. The turbidity of the inoculum suspension was adjusted to that of 0.5 McFarland standard against a card with a white background and contrasting black lines under an illuminated surface.

Inoculation of Tests Plate

The carefully adjusted inoculum suspension was allowed to stand for 15 minutes and a sterile cotton swab was dipped into the adjusted suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid to remove the excess fluid from the swab.^[12] Thereafter the swab was streaked over the entire sterile surface of the dried Mueller Hinton agar plate. This procedure was repeated twice by rotating the plate at approximately 60° each time to ensure an even distribution of the inoculum.^[12]

Application of Discs to Inoculated Agar Plates

The extract impregnated discs, controls and the standard antibiotics were aseptically placed onto the surface of the inoculated plates. The discs were gently pressed on the agar using a sterile forcep to provide uniform contact with the surface. The discs were distributed at least 22mm away from each other and 14mm away from the edge of the plate. The plates were inverted and placed in an incubator set at $35\pm 2^{\circ}\text{C}$ within 15 minutes after the application of the discs for 18-24 hours.^[12] Antibacterial activity was recorded as a zone of clearing around the discs and was recorded once the zone was greater than 6mm.^[13]

The zones were measured to the nearest millimetre using a ruler held at the back of the inverted petri plate. The petri plates were held a few inches above a black background illuminated with reflected light.

Determination of the Minimum Inhibitory Concentrations (MIC) of the Methanolic extracts of *T. globiferus* leaves

The MIC of the Methanolic leaf extracts of *T. globiferus* was determined using CLSI standard.^[12] Thioglycollate broth was prepared and sterilised using an autoclave. Five percent (5%) serum was added after allowing the broth to cool to about 45°C to form a 5% serum enriched Thioglycollate (Thio-S).

One millilitre (1mL) of the prepared broth was dispensed into tubes 1-9, 11 and 12 in a series of 12 test tubes. 2mL of the broth was dispensed into tube 10 to serve as broth control.

The solution of the Methanolic leave extracts of *T. globiferus* was prepared by dissolving 500mg of the dried extract in 5ml of sterile distilled water to make a stock solution of 100mg/ml.

One millilitre (1mL) of the stock solutions (100mg/mL) of the extracts was dispensed into tubes 1 and 2.

Subsequently, from tube 2, doubling dilution was carried out in which 1mL from tube 2 was transferred up to tube 9 and 1mL was discarded. The working inoculum was prepared from an overnight cultures using serum enriched Thioglycollate broth (Thio- S). The broth-cultures were diluted 1:200 by mixing 0.1mL of the inocula and 19.9mLs of the broth.^[13] From this dilution, 1mL of the inoculum was transferred into each tube from tube 1-12 with the exception of tube 10. The final concentrations of the Methanolic leave extracts of *T. globiferus* in each of the test tubes numbered 1-9 after dilutions were; 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39mg/ml respectively. Ciprofloxacin (500µg/ml) was used as a positive control (tube 11) and distilled water as negative control (tube 12). The tubes were incubated at $35\pm 2^{\circ}\text{C}$ for 18-24hrs. At the end of the incubation, the lowest concentration of the extracts showing no growth was taken as the MIC.

Data Analysis

Data was presented in the form of mean \pm SEM. The mean inhibitory zone diametres, MICs and MBCs of the crude extracts of *T. globiferus* leaves were compared to that of the standard antibiotics by one way ANOVA followed by tukey's test. The mean inhibitory zone diametres, MICs and MBCs of the column chromatographic fractions of *T. globiferus* leaves were compared to that of the standard antibiotics by independent sample t test. Mean differences were considered significant when $p < 0.05$. All the statistical analysis were carried out using the Statistical Packages for Social Sciences (SPSS) version 20.0 (California Inc., USA).

RESULT

The Identified Bacterial Isolates

Seventy four (74) organisms were isolated from the 101 specimens analysed. Out of these, 30 (37.8%) were *Pseudomonas aeruginosa*, 24(32.4%) were *S. aureus*, 10(13.5%) were *E.coli*, 4(5.4%) were *S. liquefaciens*, 4(5.4%) were coagulase negative *Staph*, 2(2.7%) were *Proteus mirabilis* and 2(2.7%) were *S. pyogenes* (Table 1.0).

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Determination of the Minimum Bactericidal Concentrations (MBC) of the Methanolic leave extracts of *T. globiferus*.

The MBC of the Methanolic leave extracts of *T. globiferus* was determined by sub-culturing (on solid media) 0.01ml (10µL) of the highest concentrations of the dilutions which showed visible growth and all the tubes showing no visible sign of growth from the MIC tube dilution test.^[15] MBC was the lowest concentration that results in killing 99.9% of the test organisms.^[16]

Phytochemical screening of the crude Methanol extracts of *N. sativa*

The crude methanol extract of *T. globiferus* leaves was tested for the presence of phenols, tannins, terpenoids, alkaloids, saponin, carbohydrate, fixed oil, volatile oil, flavonoid, anthraquinones, cardiac glycosides and protein using the method of Oyeleke and Manga (2008).

Test for Phenols (FeCl₃ test)

To 2mL of the extracts, 5% ferric chloride solution was added. Deep blue black color indicates the presence of phenol.

Test for Tannins (FeCl₃ test)

Some portions of the extracts were dissolved in distilled water. To this solution, 2 mL of 5% ferric chloride solution was added. Formation of blue green indicates presence of tannins.

Test for flavonoids (Schinoder's test)

One millilitre (1mL) of each of the extracts was dissolved in sodium hydroxide solution. Appearance of yellow solution which disappeared on addition of hydrochloric acid indicate the presence of flavonoids.

Test for Alkaloids

Five hundred milligram (500mg) of each of the extracts was dispensed into a test tube. 5mls of 1% aqueous hydrochloric acid was added to the extracts and stirred. The solution was heated on water bath for 20 minutes. It was cooled and filtered. The filtrate were divided into two (a and b) and used for the following tests:

a. Wagner's test

Six (6) drops of Wagner's reagent was added to filtrate a. Formation of orange (or reddish brown) precipitate indicated the presence of alkaloid in the extracts.

b. Meyers's test

One millilitre (1ml) of the filtrate was treated with Meyer's reagent. Appearance of creamy precipitate indicated the presence of alkaloids in the extracts.

Test for Saponins (Frothing or Foaming test)

Some portions of the extracts were diluted with distilled water and shaken in graduated cylinder for 15 minutes. The formation of layer of foam indicated the presence of saponins in the extracts.

Test for Terpenoids**a. Salkowski's test**

The extracts were treated with chloroform and few drops of concentrated sulphuric acid. They were well shaken and allow to stand for some time. Formation of yellow coloured lower layer indicated the presence of terpenoids in the extracts.

b. Libermann Buchard's test

Some portions of the extracts were treated with chloroform and acetic anhydride and few drops of concentrated sulphuric acid were added. Formation of a green-blue coloured product indicated the presence of terpenoids in the extracts.

Test for Anthraquinones (Borntrager's test)

Five hundred milligram (500mg) of the extracts was taken in a separate test tubes and 10mls of chloroform was added to each. It was shaken for 5 minutes. Formation of bright-pink colour in the upper aqueous layer indicated the presence of anthraquinones in the extracts.

Test for Cardiac glycosides (Kella-kiliani's test)

Five hundred milligram (500mg) of each of the extracts was dissolved in 5mls of FeCl_3 in glacial acetic acid left for 1 minute. One point five millilitre (1.5ml) of conc. H_2SO_4 was added with a pipette by the side of the test tube. Formation of blue colour in the interphase (acetic acid layer) indicated the presence of cardiac glycosides in the extracts.

Test for volatile oil

Some quantities of the extracts were shaken with dil. HCl. A white precipitate was observed which indicated the presence of volatile oils in the extracts.

Test for protein (Xanthoproteic test)

One milliliter each of the extracts was dispensed into test tubes. Concentrated nitric acid was added to the extracts and the mixtures were heated. Formation of yellow colour indicated the presence of proteins in the extracts.

Test for fixed oil (paper test)

A drop of the oily portion of the extracts was placed on the surface of a clean paper. A greasy mass that remained attached to the paper after 5 minutes indicated the presence of fixed oil.

Test for Carbohydrate**a. Molisch's test**

Two millilitres (2mls) each of the extracts solution were dispensed into a test tube. Two drops of the Molisch reagent was added. The solutions were poured slowly into a test tube containing 2ml of concentrated sulfuric acid to form two layers. Formation of purple product at the interface of the two layers indicated the presence of carbohydrate in the extracts.

b. Fehling's test

Two milliliters (2ml) each of the extracts solution was dispensed into test tubes. Equal volume of Fehling's A and B were added to the test tubes containing the solution of the extracts and the test tubes were placed in a boiling water bath for 10 minutes. The content of

the test tubes were observed for colour and precipitate formation. Formation of yellow or brownish-red precipitate indicate the presence of reducing sugars in the extracts.

Separation of the Crude extract using Column Chromatography

Column preparation

The lower end of a glass column 10ml long with an internal diameter of 1.5cm was plugged with cotton wool. Silica gel was poured onto the cotton wool and air bubbles released were trapped with the flat end of a packed rod. The column was packed with a wet silica gel in a step wise manner. The side of the column was tapped gently with a glass rod for compaction of the particles. As the silica gel settles, the column outlet was adjusted. Three grams (3g) of the methanolic extracts of *T. globiferus* leaves was drawn onto the adsorbent and eluted with various dilutions of hexane and ethyl acetate.

Assessment of the Antibacterial activities of the column chromatographic fractions of *T. globiferus* leaves

The antibacterial activities of each of the reconstituted fractions were determined using agar disc diffusion method as described by Kirby Bauer (Bauer, *et al.*, 1966).

Susceptibility testing discs were prepared for each of the column fractions to determine their antibacterial activity. For each of the column fractions 0.03mg/disc (1.5mg/ml) was prepared and used for the antibacterial study.

Determination of the MIC and MBC of the column fractions with the highest antibacterial activity

The same procedure of MIC and MBC determination (as described previously) was used for the determination of the MIC and MBC of the column fractions.

Phytochemical Screening of the most active Column fractions

The same procedure of phytochemical screening (described previously) was used to determine the phytoconstituents of the most active column column chromatographic fractions.

Determination of the Acute toxicity of the Crude Methanol extract of *T. globiferus* leaves.

Twelve (12) albino rats weighing between 123 aand 193 were purchased from the Pharmacology department, Faculty of Pharmacy, Usmanu Danfodiyo University Sokoto.

They were certified healthy by a Veterinary doctor. The animals were kept in the Animal house of the Pharmacology department, UDUS in wire mesh cages. They were maintained under Veterinary supervision and were fed with pellet made from growers mash and water *ad libitum*.

The LD₅₀ of the extracts was determined by the method of Lorke's (1983).

Phase I

In this phase, three groups of three animals each were given the following doses of the extracts; 10, 100 and 1000mg/kg body weight of the extracts. Observation on the adverse effects of the extracts such as tremor, salivation, off feed, time of death were made at regular interval for 24 hours.

Phase II

In this phase, three groups of one animal each were given higher doses of the extracts; 1600, 2900 and 5000 mg/kg body weight of the animals to the groups; I, II and III respectively. Toxic symptoms were observed for 24 hours.

Data Analysis

Data was presented in the form of mean \pm SEM. The mean inhibitory zone diameters, MICs and MBCs of the crude extracts of *T. globiferus* leaves were compared to that of the standard antibiotics by one way ANOVA followed by Tukey's test. The mean inhibitory zone diameters, MICs and MBCs of the column chromatographic fractions of *T. globiferus* leaves were compared to that of the standard antibiotics by independent sample t test. Mean differences were considered significant when $p < 0.05$. All the statistical analysis were carried out using the Statistical Packages for Social Sciences (SPSS) version 20.0 (California Inc., USA).

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Table 1.0: The identified Bacterial isolates and their source.

| Bacteria | No. Isolated (N) | Source | Percentage (%) |
|-------------------------------|------------------|---------------------|----------------|
| <i>Staphylococcus aureus</i> | 24 | Wound swab/pus/asp. | 32.4 |
| <i>Streptococcus pyogenes</i> | 2 | Wound aspirate | 2.7 |
| <i>Serratia liquefaciens</i> | 4 | Wound swab | 5.4 |
| <i>Proteus mirabilis</i> | 2 | Wound swab | 2.7 |
| Coag. negative <i>Staph.</i> | 4 | Wound swab | 5.4 |
| <i>Escherichia coli</i> | 10 | Wound swab/pus | 13.5 |
| <i>Pseudomonas aeruginosa</i> | 28 | Wound swab/pus/asp | 37.8 |

Comparison of the Inhibitory zone diametres of the crude Methanol extracts of *T. globiferus* leaves with standard antibiotics against the Bacterial isolates.

Significant differences ($p < 0.05$) were observed between the inhibitory zone diametres of the extract when compared with the standard antibiotics against all the bacterial isolates at varying concentrations. The most highly significant differences ($p < 0.05$) were observed on *P. aeruginosa* [32.0 ± 0.75 , 28.6 ± 0.87 , 25.1 ± 1.12 and 19.3 ± 1.52 mm at 10, 5, 3 and 1mg/disc respectively, in contrast to Ciprofloxacin (21.0 ± 2.0 mm) and Pefloxacin (18.4 ± 1.89 mm), $F = 15.2$, $P = 0.0007$] and *S. liquefaciens* [35.0 ± 1.08 , 32.0 ± 0.71 , 29.3 ± 0.95 and 26.5 ± 1.50 at 10, 5, 3 and 1mg/disc respectively, in contrast to Ciprofloxacin (28.0 ± 2.12 mm) and Pefloxacin (25.5 ± 2.53 mm) $F = 12.4$, $P = 0.0010$]. The lowest was observed on *S. pyogenes* [30.5 ± 4.50 and 27.5 ± 3.50 at 10 and 5mg/disc respectively, in contrast to Ciprofloxacin (26.0 ± 2.00) and Pefloxacin (25.5 ± 5.00), $F = 3.3$, $P = 0.0049$]. No activity was observed with the Methanol and distilled water (Table 2.0).

Table 2.0: Comparison of the inhibitory zone diametres of the crude Methanolic leave extract of *T. globiferus* with standard antibiotics against the Bacterial isolates.

| Isolate | Zones of Inhibition (mm) | | | | | | | | | |
|------------------------|--|--------------|--------------|-------------|--------------|--------------------|--------------------|-------------|------|--------|
| | Methanol extract of <i>T. globiferus</i> (mg/disc) | | | | Neg. Control | | Std drug (mg/disc) | | | |
| | 10 | 5 | 3 | 1 | M | D/H ₂ O | Cipro (0.03). | Pef (0.03). | F | P |
| <i>S. pyogenes</i> | 30.5±4.50** | 27.5±3.50* * | 21.5±5.50 | 17.0±7.00 | 6.0±0.00 | 6.0±0.00 | 26.0±2.00 | 25.5±5.00 | 3.3 | 0.0049 |
| <i>P. mirabilis</i> | 36.0±0.00* * | 34.0±1.00** | 30.5±0.50** | 27.0±0.00 | 6.0±0.00 | 6.0±0.00 | 29.0±1.00 | 29.5±1.50 | 9.3 | 0.0034 |
| CN <i>Staph</i> | 34.3±1.65* * | 30.5±1.44** | 26.8±1.65** | 18.0±1.41 | 6.0±0.00 | 6.0±0.00 | 23.5±1.89 | 18.3±4.27 | 11.2 | 0.0021 |
| <i>S. liquefaciens</i> | 35.0±1.08 * * | 32.0 ±0.71** | 29.3±0.95* * | 26.5±1.50* | 6.0 ±0.00 | 6.0 ±0.00 | 28.0 ±2.12 | 25.5±2.53 | 12.4 | 0.0010 |
| <i>E. coli</i> | 32.5±1.06 * * | 28.9±1.13* * | 24.1±2.41* * | 19.2±2.65 | 6.0±0.00 | 6.0±0.00 | 22.2±2.91 | 23.1±3.17 | 7.2 | 0.0042 |
| <i>S. aureus</i> | 32.7±1.04* * | 29.6±1.21** | 26.0±1.30 ** | 21.3±1.39 | 6.0±0.00 | 6.0±0.00 | 25.1±1.54 | 22.1±1.24 | 5.2 | 0.0044 |
| <i>Pseudomonas</i> | 32.0 ±0.75** | 28.6 ±0.87** | 25.1±1.12** | 19.3±1.52 * | 6.0±0.00 | 6.0±0.00 | 21.0 ±2.04 | 18.4 ±1.89 | 15.2 | 0.0007 |

Data are presented as mean ± SEM. Values with the superscript (**) are significantly greater than the two standard antibiotics on the right while values with superscript (*) are significantly greater than one of the antibiotics on the right by using ANOVA (at p <0.05). Values > 6 ± SEM indicate some activities.

Key: M = Methanol, D/H₂O = Sterile distilled water, Std = standard, Cipro. = Ciprofloxacin, Pef. = Pefloxacin, CN Staph = Coagulase negative *Staph.*

Comparison of the MICs and MBCs of the crude methanol extracts of *N. sativa*, *T. globiferus* and the combined extract of the two plants.

No any significant difference ($p > 0.05$) was observed between the MICs and MBCs of the extracts when compared with that of Ciprofloxacin (Table 3.0). The most highly insignificant difference was observed on *P. aeruginosa* [MIC (1.83 ± 1.68 in contrast to Ciprofloxacin (0.040 ± 3.37) and MBC (4.75 ± 4.46 in contrast to Ciprofloxacin (0.085 ± 7.05), $F = 0.14$, $P = 2.32$].

Table 3.0: Comparison of the MICs and MBCs of the crude Methanol extracts of *T. globiferus* leaves with Ciprofloxacin against the Bacterial isolates.

| Isolate | MIC (mg/ml) | | MBC (mg/ml) | | | |
|------------------------|----------------------|-------------|----------------------|--------------|------|------|
| | <i>T. globiferus</i> | Cipro | <i>T. globiferus</i> | Cipro | P | F |
| <i>S.pyogenes</i> | 2.34 ±7.84 | 0.023±7.84 | 6.25 ±0.00 | 0.047 ±15.60 | 0.20 | 2.01 |
| <i>P. Mirabilis</i> | 0.78±0.00 | 0.009 ±0.00 | 1.56±0.00 | 0.016±0.00 | 0.65 | 1.09 |
| CN <i>Staph</i> | 0.78±0.00 | 0.014±1.96 | 3.13±0.00 | 0.047 ± 9.01 | 0.56 | 1.24 |
| <i>S. liquefaciens</i> | 0.78±0.00 | 0.019 ±3.92 | 1.96 ±3.92 | 0.039 ±7.68 | 0.84 | 0.92 |
| <i>E. coli</i> | 0.87±0.87 | 0.026±2.61 | 3.13±6.36 | 0.052±5.20 | 0.42 | 1.50 |
| <i>S. aureus</i> | 1.01±1.23 | 0.025±1.73 | 3.20±3.14 | 0.054±4.89 | 0.32 | 1.70 |
| <i>Pseudomonas</i> | 1.83±1.68 | 0.040±3.37 | 4.75 ±4.46 | 0.085±7.05 | 0.14 | 2.32 |

Data are presented as mean ± SEM. No significant difference ($p > 0.05$) was observed between the MICs and MBCs of the crude extracts when compared with ciprofloxacin by using ANOVA.

Key:

Cipro = Ciprofloxacin.

CN staph = Coagulase negative *Staphylococcus*.

Results of the preliminary phytochemical screening of the crude Methanolic leave extracts of *T. globiferus*.

The result of the preliminary phytochemical screening of the crude Methanolic leave extracts of *T. globiferus* is shown in Table 4.0. The extract revealed the presence of; tannins, saponins, cardiac glycosides, flavonoids, carbohydrate, terpenoids, and protein.

Table 4.0: Preliminary phytochemical screening results of the crude Methanolic leave extract of *T. globiferus*.

| <u>Constituents</u> | <u>Type of test</u> | <u>ME of TGs</u> |
|---------------------|---------------------------|------------------|
| Alkaloids | Meyer's test | - |
| | Wagner's test | +++ |
| Tannins | FeCl ₃ test | +++ |
| | Strong lead sub | +++ |
| | metals test | |
| Saponins | Froathing test | ++ |
| Cardiac glycoside | Kella-Killiani's test | +++ |
| Anthraquinones | Borntrager's test | - |
| Flavonoids | NaOH test | +++ |
| | Schinoda's test | ++ |
| Carbohydrate | Mollisch's test | + |
| | Fehling's test | + |
| Terpenoids | Liebermann-Buchard's test | + |
| Fixed oil | Paper test | + |
| Protein | Xanthoproteic test | + |

Key:

+++ and ++ = Present, + = trace, - = Not detected, ME = Methanol extract, TGs = *T. globiferus*.

Residues obtained from column chromatography

Residues obtained from the column chromatography of the methanol extracts of *T. globiferus* leaves are shown in Table 5.0. Five fractions labeled TF1-TF5 were obtained from the column chromatography with the highest residues recovered from the first fraction (TF1) and fifth fraction (TF5) (0.09 and 0.06g respectively) followed by the 4th (TF4) and 2nd (TF2)

fractions (0.04 and 0.03g respectively). The lowest residue was obtained from the 3rd fraction [(TF3) 0.02mg].

Comparison of the Inhibitory zone diametres of the column fractions of *T. globiferus* leaves with the standard antibiotic on the two bacterial isolates.

Significant difference was observed between the inhibitory zone diametres of TF5 when compared with the standard antibiotics on *S. aureus* [20.3±3.13 in contrast to ciprofloxacin (18.0±3.06) and Pefloxacin (15.0±3.46), $P = 0.03$] (Table 6.0). No significant difference was observed with TF5 when compared with the standard antibiotics on *P. aeruginosa* [12.3±0.67 in contrast to Ciprofloxacin (15.7±1.87) and Pefloxacin (12.7±1.33), $P = 0.092$]. Similarly, no significant differences were observed with the remaining *T. globiferus* fractions when compared with the standard antibiotics. No activity was recorded with TF3 on the two bacterial isolates (Table 6.0).

Comparison of the MICs and MBCs of the most active column fractions of *T. globiferus* leaves with Ciprofloxacin on the two Bacterial isolates.

No any significant difference was observed between the MICs and MBCs of the two most active column fractions of *T. globiferus* leaves when compared with Ciprofloxacin (Table 7.0). The most highly insignificant difference was observed on *P. aeruginosa* [MIC and MBC (0.188±0.00 and 0.750±0.41 of TF5, in contrast to 0.031±0.01 and 0.063±0.01 of Ciprofloxacin), $P = 1.02$].

Phytochemical screening of the most active column fractions of *T. globiferus* leaves

The result of the phytochemical screening of the most active column fractions of *T. globiferus* leaves (TF4 and TF5) are shown in Table 8.0. The 4th fraction of *T. globiferus* (TF4) revealed the presence of cardiac glycoside and steroid whereas the 5th fraction (TF5) revealed the presence of cardiac glycoside, tannins, terpenoid, saponins and flavonoid.

Determination of the LD₅₀ of the crude Methanolic leave extract of *T. globiferus*.

The oral acute toxicity studies showed an LD₅₀ of *T. globiferus* to be greater than 5000mg/kg body weight of the wistar albino rats (Table 9.0).

Table 5.0: Amount of residues recovered from Column chromatography of the Methanolic leave extracts of *T. globiferus*.

| Extract | Fractions | Amount recovered (g) |
|---------|-----------|----------------------|
|---------|-----------|----------------------|

| | | |
|----------------------|-----|------|
| <i>T. globiferus</i> | TF1 | 0.09 |
| | TF2 | 0.03 |
| | TF3 | 0.03 |
| | TF4 | 0.04 |
| | TF5 | 0.06 |

Key:

TF = *T. globiferus* fraction.

1, 2, 3,.. = Different fractions obtained.

Table 6.0: Comparison of the Inhibitory zone diametres of the column chromatographic fractions of *T. globiferus* with standard antibiotics on the two Bacterial isolates.

| Isolate | Zones of Inhibition (mm) | | | | | | | | |
|------------------|---------------------------------|-----------|----------|-----------|-------------|--------------------|-----------|-----------|-------|
| | TF1 | TF2 | TF3 | TF4 | TF5 | D/H ₂ O | Cipro | Pef | P |
| <i>Pseudo.</i> | 8.0±0.58 | 8.3±0.33 | 6.0±0.00 | 9.3±0.88 | 12.3±0.67 | 6.0±0.00 | 15.7±1.87 | 12.7±1.33 | 0.092 |
| <i>S. aureus</i> | 13.7±0.33 | 12.7±1.20 | 6.0±0.00 | 13.0±0.88 | 20.3±3.13** | 6.0±0.00 | 18.0±3.06 | 15.0±3.46 | 0.03 |

Data are presented as mean ± SEM, (n = 3). Values with the superscript (**) are significantly greater than the two standard antibiotics on the right by using independent sample t test (at p < 0.05). Values > 6 ± SEM indicate some activities.

Key:

TF= *Tapinanthus globiferus* fractions

Figures; 1,2,3 etc represent the number of fractions obtained, D/H₂O=Sterile distilled water,

Cipro = Ciprofloxacin, Pef = Pefloxacin,

Pseudo = *P. aeruginosa*

Table 7.0: Comparison of the MICs and MBCs of the most active column fraction of *T. globiferus* with Ciprofloxacin on the two Bacterial isolates.

| Isolate | MIC (mg/ml) | | MBC (mg/ml) | | P |
|--------------------|-------------|---------------|-------------|---------------|-------|
| | TF5 | Ciprofloxacin | TF5 | Ciprofloxacin | |
| <i>S. aureus</i> | 0.047±0.17 | 0.0156±0.01 | 0.380±0.00 | 0.031±0.01 | 0.073 |
| <i>Pseudomonas</i> | 0.188±0.00 | 0.031±0.01 | 0.750±0.41 | 0.063±0.01 | 1.02 |

Data are presented as mean ± SEM, (n = 3). No any significant difference (p > 0.05) was observed between the MICs and MBCs of the fractions when compared with Ciprofloxacin by using independent sample t test.

Key:

TF5 = 5th fraction of *Tapinanthus globiferus*

Table 8.0: Results of the phytochemical screening of the most active column fractions of *T. globiferus*.

| Constituents | Type of test | TF4 | TF5 |
|--------------|---------------------------|-----|-----|
| Alkaloids | Meyer's test | - | - |
| Flavonoids | Schinoda's test | - | ++ |
| Tannins | FeCl ₃ test | - | ++ |
| CG | Kella-Killiani's test | + | + |
| Terpenoid | Liebermann-Buchard's test | + | + |
| Saponins | Froathing test | - | + |
| Fixed oil | Paper test | - | - |

Key:

++ = Present

+ = Trace

- = Not detected

NF3 and NF4 = 3rd and 4th fractions of *N. sativa*, TF4 and TF5 = 4th and 5th fractions of *T. globiferus*

CG = Cardiac glycoside

Table 9.0: Results of oral acute toxicity of the methanolic leave extract of *T. globiferus*

| Phase | Groups | no. of animals | Weight (g) | Dose (mg/kg) | O.P | B.C |
|-------------------------|--------|----------------|------------|--------------|-------|----------|
| <u>Mortality</u> | | | | | | |
| I | I | 3 | i. 134 | 10 | 24hrs | No |
| | | | ii. 125 | | | |
| | | | iii. 126 | | | |
| | II | 3 | i. 168 | 100 | 24hrs | No |
| | | | ii. 162 | | | |
| | | | iii. 161 | | | |
| | III | 3 | i. 193 | 1000 | 24hrs | No |
| | | | ii. 187 | | | |
| | | | iii. 179 | | | |
| II | I | 1 | 149 | 1600 | 24hrs | No |
| | II | 1 | 142 | 2900 | 24hrs | off feed |
| | III | 1 | 138 | 5000 | 24hrs | Calm |

The oral LD₅₀ of the methanolic leave extract of *T. globiferus* was found to be > 5000mg/kg.

Key:

O.P = Observation period

B.C = Behavioural changes

DISCUSSIONS

In this study, the antibacterial activities of the crude extract and fractions of *T. globiferus* leaves were tested against seven wound associated bacteria viz; *S. aureus*, *P. aeruginosa*, *E.coli*, *S. liquefaciens*, *S. pyogenes*, coagulase negative *Staph* and *P. mirabilis* (Table 1.0). The antibacterial activity of the extracts was recorded when the inhibition zone was greater than 6mm.

Significant differences were observed between the mean inhibitory zone diameters of the crude methanol extracts of *T. globiferus* leaves when compared with the standard antibiotics on all the clinical bacterial isolates (Table 2.0). The most highly significant difference was observed on *P. aeruginosa* ($F = 15.2$, $P = 0.0007$) at all concentrations of the extract and the lowest was observed on *S. pyogenes* ($F = 3.3$, $P = 0.0049$). However, no significant difference was observed between the mean inhibitory zone diameters of the crude extract when compared with the standard antibiotics on some of the isolates at lower concentration (Table 4.4). The most highly insignificant difference was observed on *S. pyogenes* ($F = 0.93$, $P = 0.29$) (Table 2.0).

These findings are in line with the work of [16] who reported the broad spectrum antibacterial activity of *T. globiferus* leaves against; *S. aureus*, *B. subtilis*, *E. coli* and *S. typhi* at dose dependent manner. [17] have also reported the wide spectrum antibacterial activity of the extract against multi drug resistance *E.coli*, *Proteus spp*, *Pseudomonas spp*, *Bacillus spp* and *Salmonella spp* isolated from farm animals.

These observed activities may be due to the phytoconstituents present in the crude methanol extract of *T. globiferus* leaves (tannins, flavonoids, saponins etc) (Table 4.0). These observations were supported by the work of [18] who reported tannins to exert an antibacterial effect by the inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through the inhibition of oxidative phosphorylation and iron deprivation.[19] reported that tannins have the ability to interfere with the bacterial cell wall synthesis where they formed complex with polysaccharide through a non-specific forces such as hydrogen bonding and hydrogen effects as well as by covalent bond formation. Thus, tannin irreversibly inhibits the enzyme trans-

peptidase by reacting with serine residue in the trans-peptidase. This reaction is irreversible and so the growth of the bacterial cell is inhibited. [20] also reported that the ability for the tannin compounds to cause the bacterial colonies to disintegrate probably results from their interference with the bacterial cell wall, thereby inhibiting the microbial growth.

[21] reported that saponins inhibit bacterial RNA synthesis, protein synthesis and cell division. [22] reported that flavonoid cause damage to the bacterial cell membrane, leading to the inhibition of macromolecular synthesis by depolarization of the membrane and inhibition of DNA, RNA, and proteins synthesis leading to cell lysis. Studies on the antibacterial activity of different flavonoids by [23] had shown that quercetin (flavonoid) inhibits DNA gyrase, sophoraflavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function and licochalcones A and C inhibit energy metabolism. These (activities of saponins, tannins and flavonoids) and the activity of other phytoconstituents present in the crude methanol extract of *T. globiferus* leaves might be responsible for the observed significant antibacterial effects.

The non significant findings with the extract at lower concentrations are in conformity with the work of [16] who reported the broad spectrum antibacterial activity of *T. globiferus* against; *S. aureus*, *B. subtilis*, *E. coli* and *S. typhi* at dose dependent manner which increase with increase in concentrations of the extract and decrease with decrease in the concentration of the extracts.

The decrease in the antibacterial activity observed with the lowest concentrations of the extract (3 and 1mg/disc) may be due to the decrease in the bioactive components responsible for the antibacterial activity possibly due to the decrease in the quantity of the extracts.

No any significant difference was observed between the MICs and MBCs of the extract when compared with Ciprofloxacin (Table 3.0). The most highly insignificant difference was observed on *P. aeruginosa* [MIC (1.83±1.68 in contrast to Ciprofloxacin (0.040±3.37) and MBC (4.75 ±4.46 in contrast to Ciprofloxacin (0.085±7.05), F = 0.14, P = 2.32].

There is paucity of literature on the MICs and MBCs of *T. globiferus* leaves, however the non significant findings between the MICs and MBCs of the crude extracts in contrast to that of the standard antibiotics may be due to the differences in the purity of the active compounds.

Fractionation of the crude methanol extract of *T. globiferus* by column chromatography yielded five fractions (TF1-TF5) (Table 5.0). Significant difference was observed between the inhibitory zone diameters of TF5 when compared with that of the standard antibiotics on *S. aureus* [20.3 ± 3.13 at 0.03mg/disc, $P = 0.03$ in contrast to ciprofloxacin (18.0 ± 3.06) and Pefloxacin (15.0 ± 3.46)] (Table 6.0). No significant difference was observed between the inhibitory zone diameters of all the *T. globiferus* fractions when compared with the standard antibiotics on *P. aeruginosa* ($P = 0.092$). No activity was recorded with TF3 (Table 6.0).

Phytochemical screening of the fractions revealed flavonoids, tannins, cardiac glycosides and terpenoids in TF5 and cardiac glycoside and fixed oil in TF4 (Table 8.0).

It could be suspected that the significant activity shown by the 5th fraction of *T. globiferus* (T5) when compared with the other fractions and the standard antibiotics may be due to the phytoconstituents present in the fraction. Previous studies have reported the antibacterial activities of the phytoconstituents. Studies have reported the activities of these constituents [24]; [25]; [26]; [27] and [28].

However, the less or inactivity observed on the other column fractions could be due to; the loss of antimicrobial components on fractionation and the eluting solvents used during fractionation.

These findings conform with the work of [29] who attributed the loss of antimicrobial activity of fractions to the elimination of inorganic constituents that stabilize and activate the antimicrobial substances in the plant extract and loss of some labile constituents during separation. Similarly, [30] reported that other compounds of the neutral fractions also contribute to the antibacterial activity of an extract.

No significant difference was observed between the MICs and MBCs of the fractions when compared with ciprofloxacin on the two Bacterial isolates. The most highly insignificant difference was observed on *P. aeruginosa* ($P = 1.02$) (Table 7.0).

The non significant differences observed between the MICs and MBCs of the fractions in contrast to that of the standard antibiotics may be due to the differences in the purity of the active compounds.

The overall result showed less activity of the fractions in contrast to their crude extracts counterpart.

These findings are in agreement with the work of [31] and [32] who reported higher antibacterial activity for crude extracts when compared with fractions. These findings suggest a possible interaction between some inorganic substances and the phytoconstituents present in the crude extract which may be responsible for the increase in the activity of the crude extracts in contrast to that of the fractions.

However, the fractions are more potent than the crude extracts, because the fractions were found to be active at minute quantity when compared with the crude extract (in the assessment of MIC, 1mg/ml of the fraction was used as against 100mg/ml of the crude extract). The increase in the potency of the fractions may be due to the fact that, bioactive substances are present along with other impurities in the crude extracts which may reduce the contact between the test organisms and the bioactive compound through masking, however, fractionation is thought to expose these active substances to have a direct contact with the test organisms. This may be responsible for the increase in the activity seen with the minute quantity of the fractions when compared with their crude extract counterparts.

The acute toxicity of *T. globiferus* was found to be greater than 5000mg/kg body weight of the wistar albino rats (Table 9.0).

These findings are in conformity with the work of [33] and [34] who reported an oral LD₅₀ of *T. globiferus* leave extracts as greater than 5000 mg/kg in both chicks and mice. They also reported significant effect of the extract on serum levels of aspartate-transaminase, alanine amino-transferase, alkaline-phosphatase and total protein.

The low toxicity observed suggests a wide margin of safety for therapeutic doses of this extract, which means that they may be relatively safe even at higher doses.

CONCLUSIONS

The findings from this study revealed that the crude methanol leaves extract and fractions of *T. globiferus* possessed an antibacterial activity. Significant antibacterial activities were observed with the crude extract and fractions against some wound associated bacteria despite their multi drug resistance antecedents.

The antibacterial potency of the fractions demonstrated in this study could be an important step towards the purification and structural elucidation of the components responsible for antibacterial activity in *T. globiferus* leaves

Due to their broader antibacterial activity coupled with less or non toxic effects, the leaves of this plant may become an alternative source of antibacterial agents that would complement the effort of the existing antibiotics or provide a novel or lead compound that may be employed to forestall the antibiotic resistance menace.

Further studies are recommended to isolate and characterize the respective bioactive components of these extracts and correlate their action to specific phytoconstituents which would enhance possible drug development.

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