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Antibacterial and Phytochemical Screening of *Calotropis procera* Leaf Extracts against Vancomycin and Methicillin Resistant Bacteria Isolated from Wound Samples in Hospital Patients

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The Phytochemical and antibacterial activities of *Calotropis procera* Leaf Organic Fractions were tested against vancomycin and methicliin resistant bacteria isolated from wound patients in Ondo State Specialist Hospital. The bacterial isolates used are; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Streptococcus pyogenes*. Agar well diffusion method was used to determine the antibacterial activities of the extracts on resistant bacterial isolates. Ethanol extract had the highest zone of inhibition against *Staphylococcus aureus* and *Escherichia coli* with 16.03 mm and 12.05 mm respectively while cold and Hot water extracts recorded the lowest zones of inhibition values of 3.54 mm and 5.53 mm respectively against *Klebsiella pnuemoniae* and *Pseudomonas aeruginosa*. However, the n-Hexane extract had no inhibitory effect against *Streptococcus pyogenes* and *Proteus mirabilis*.

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Phytochemical analysis of the extracts revealed the presence of alkaloids, flavonoids, tannin, saponin, terpenoids, cardic glycoside and phenols. Findings of this research indicate that the leaf extracts of *Calotropis procera* posses antibacterial potency which will assist in the preliminary treatment of wound infections, most especially because of its high inhibitory effect against *Staphylococcus aureus.*

Keywords: Antibacterial; phytochemical; vancomycin; methicillin; wound; bacteria.

1. INTRODUCTION

Nosocomial or hospitalized acquired infections are infections appearing in a patient in a hospital or other health care facility in whom the infection was not present or incubating at the time of admission. Such infection manifest within 72 hrs or more after admission [1]. Nosocomial infections are estimated to cause or contribute to nearly 80,000 deaths annually in the United States [2,3] such infections are serious and public health hazard throughout the world. As a matter of fact, is the fourth leading cause of death [4]. Hospital-associated infection which has a worldwide distribution remains a major cause of deaths among hospitalized patients [5]. In view of that, [1] reported that the hospital is no longer a place where sick people recover from their illnesses but also where illnesses at times get complicated and healthy people get infected. Nosocomial infectious disease is a major problem in many health care systems. It has been reported that 10% of hospital patients will acquire an infection while in hospital [6]. Infections can complicate illness, cause distress to patients and family and can lead to death. Among nosocomial infections, there are main infections that has been reported such as blood stream infections (28%), ventilator-associated pneumonia (21%), lower respiratory infection (12%), urinary tract infection (12%), gastrointestinal, skin, soft tissue and cardiovascular infection (10%), surgical-site infection (7%) and ear, nose and throat infection (7%) [6]. Despite the extensive use of antibiotics and vaccine programs, infectious disease continue to be a leading cause of morbidity and mortality worldwide [7].

The development of drug resistance as well as appearance of undesirable side effects of certain drugs has led to the search of new antimicrobial agents in particular from medicinal plants [8]. Plant extracts, and pure compounds isolated from natural sources have formed the bedrock of modern chemotherapy [9]. Indigeous plants are reservour of various metabolites and provide unlimited source of important chemicals that have diverse biological properties [10]. Over 25% of prescribed medicine in industralized countries are derived directly from plant [11]. Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional medicines, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Plants are reported to have anticancer, antimicrobial, antidiabetic, antiinflammation and antioxidant properties [12].

Recently, attention has been directed towards extracts and biological compounds isolated from medicinal plants. More so, the use of medicinal plants play a vital role in covering the basic health needs in developing countries and these plants may offer new sources of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms [13].

1.1 Description of *Calotropis procera*

Calotropis procera (Ait) R. Br. is member of plant family Asclepiadaceae, a shrub which is widely distributed in the tropics. The plant is erect, tall, large, much branched and perennial with milky latex throughout. It is found in most parts of the world in dry, sandy and alkaline soils and warm climate and is more common in south western and central India and western Himalayas. It is found in waste lands and grows as a weed in agricultural lands. In ancient Ayurvedic medicines, the plant *Calotropis procera* was known as “Rakta arka” [14]. It is a common plant in Nigeria but it is more abundant in the northern part of the country [15].

For many years now, the normadic Fulani women of the Northern Nigeria use the part of the plant in the production of warankasi (a local soft cheese); the practice is still popular today even in almost all the parts of the country where fluid milk is abundant. The importance of this plant locally called “bomubomu” in South-West of Nigeria for use in the country as a cuddling agent

in local cheese production came to limelight many decades ago in countries like India where the latex was used in Indian medicine as a blistering agent.

All parts of plant exude milky latex when cut or broken, which act as a defence strategy against insects, viruses and fungi. A large number of secondary metabolites have been isolated from this plant that include many flavonoids, cardiac glycosides, Triterpenes and sterols [16].

Calotropis procera is a well known plant and has been traditionally used for the treatment of a wide range of infections globally [17-20]. The antimicrobial activity of *C. procera* plant extracts against bacteria and fungi is well documented [21-25]. Pharmacological studies of *Calotropis* species showed anti-inflammatory, anti-tumoral [26], antioxidant [9,27,28], antibacterial [29], antidiarrheal [30], antifungal [31] and Nanoparticles Synthesize [32] activities.

1.2 Traditional Uses of *Calotropis procera*

In India or in Subcontinent the use of herbal plants and medicinal plants has been the golden remark of the 21th century. *Calotropis procera* is one of the important member of traditional herbal medicine in every home of India. It strongly recommended in leprosy, hepatic and splenic enlargements, dropsy and worms. The latex is applied to painful joints and swelling, fresh leaves are also use for the same purpose. Oil which the leaves have been boiled is applied to paralyzed part. The milky juice is used as purgative, while flowers are considered as digestive, stomachic, tonic and useful in cough, asthma and loss of appetite. The root bark is said to promote secretion and to be useful in treating skin disease, enlargement of abdominal viscera, intestinal worms, ascites and anasarca. Traditionally the leaves are warmed and tied around any body organ in pain. It is practically useful in backache and in joint pains. Warm leaves also relieve from stomach ache if tied around. Inhalation of burnt leaf cures headache. The traditional folk healers use the milky latex for several ailments. Leaf latex if applied on fresh cut, stops bleeding immediately. Recent investigations have found that the alkaloids, calotropin, calotaxein and uskerin are stimulant to the heart. Flowers and roots are used in Ayurvedic medicine [33].

The plant is used as anthelmintic, the ashes act as an expectorant. The leaves are applied hot to the abdomen to cure the pain inside [34]. The flower is tonic, antisialagogue, used as appetizer and against stomach ache, and cures piles and asthma. Flowers are believed to have detergent properties so they are given in cholera. The fresh roots are used as a toothbrush and are considered to cure toothache. Root bark is useful for treating chronic cases of dyspepsia, flatulence, constipation, loss of appetite, indigestion and mucus in stools. Leaves are used against guinea worms.

Flowers are useful in asthma. Seed oil is geriatric and tonic. Green copra is given in asthma. Plant is used in spleen complaints, rheumatism, epilepsy, hemiplegia, sores, and smallpox and protracted labor. The root skin, latex, flowers, leaves are used for medicinal purpose. It is useful both, internally as well as externally. The poultice of its leaves effectively reduces the pain and swelling in rheumatic joints and filariasis. The medicated oil is beneficial in otitis and deafness. The topical sprinkle of dried leaves powder hastens the wound healing. In glandular swellings the topical application of latex reduces the inflammation. In skin diseases, associated with de-pigmentation, the latex combined with mustard oil, works well. The fomentation with its leaves, slightly warmed with thin coat of castor oil, is beneficial to relieve the abdominal pain. The local application of latex is recommended in hair fall and baldness. It also, is useful in piles. The latex also mitigates the dental aches [34].

1.3 Wound Healing Activity Based on Its Traditional Use

C. procera was selected for evaluation of its wound healing potential in guinea pigs [35] for this purpose; four full thickness excision wounds of 8.0 mm diameter were inflicted on the back of guinea pigs. Topical application of 20 µl of 1.0% sterile solution of the latex of the plant, twice daily was followed for 7 days. The latex significantly augmented the healing process by markedly increasing collagen, DNA and protein synthesis and epithelisation leading to reduction in wound area. Thus the result provided a scientific rationale for the traditional use of this plant in the management of wound healing [34].

1.4 Phytochemicals

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further

than those attributed to macronutrients and micronutrients. They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals [36].

Phytochemicals is any substance that is derived from plant source. Phytochemicals may relate to any one of a numbers of vitamins, minerals or bioactive compounds produced by the plant. Besides vitamins and minerals, there are other pytochemicals which are known to bioactive and may be useful in the fight against various diseases cancer, cardiovascular, arthritis etc. these include the allium compounds, dithiothiones and isothiocyanates, terpenoids, phytoestrogens, flavonoids, phenolic compound, protease inhibitors, phytic acid, glucosinolates and indoles, plant sterols, saponins and chemicals found in various botanicals [37].

The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents [38]. Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoid, alkaloids and phenolic compounds [39].

1.4.1 Classes of phytochemicals

1.4.1.1 Alkaloids

Alkaloids are one of the earliest isolated bioactive compounds from plants, they are heterocyclic nitrogen compounds. They are derived from amino acids, and the nitrogen gives them alkaline properties. The mechanism of antibacterial action is attributed to their ability to intercalate with DNA, inhibition of enzymes (esterase, DNA-, RNA-polymerase), inhibition of cell respiration [40]. They are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid degree of basicity varies considerably, depending on the structure of the molecule, presence and location of the functional groups [41]. They react with acids to form crystalline salts without the production of water [40]. Majority of alkaloids exist in solid such as atropine, some as liquids containing carbon, hydrogen, and nitrogen.

1.4.1.2 Flavonoids

Flavonoids are important group of polyphenols widely distributed among the plant flora. Structurally, they are made of more than one benzene ring in its structure (a range of C15 Aromatic compounds) and numerous reports support their use as antioxidants or free Radical scavengers [42]. The compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, calchones and catechin and leucoanthocyanidins.

1.4.1.3 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess 'soap like' behavior in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two major groups of saponins and these include: Steroid saponins and triterpene saponins. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they give aglycones. Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning [42]. They possess a bitter and acrid taste, besides causing irritation to mucous membranes. They are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane.

Saponins are also important therapeutically as they are shown to have hypolipidemic and anticancer activity. Saponins are also necessary for activity of cardiac glycosides [41].

1.4.1.4 Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group [42]. They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Common examples of hydrolysable tannins include the aflavins (from tea), daidezein, genistein and glycitein. Tannin rich medicinal plants are used as healing agents in a number of diseases. In Ayurveda, formulations based on tannin-rich plants have been used for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea.

1.4.1.5 Terpenoids

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins [40].

Terpenoids includes hydrocarbons of plant origin and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Terpenoids exhibit various important pharmacological activities i.e., anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities.

1.4.1.6 Anthraquinones

These are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols [43,40]. To test for free anthraquinones, powdered plant material is mixed with organic solvent and filtered, and an aqueous base, e.g. NaOH or NH4OH solution, is added to it. A pink or violet colour in the base layer indicates the presence of anthraquinones in the plant sample [41].

1.4.1.7 Steroids

Plant steroids (or steroid glycosides) also referred to as ‘cardiac glycosides’ are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs [40]. The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids (anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness [43,44]. Caution should be taken when using steroidal glycosides as small amounts would exhibit the much needed stimulation on a diseased heart, whereas excessive dose may cause even death. Diosgenin and cevadine (from *Veratrum veride*) are examples of plant steroids.

2. MATERIALS AND METHODS

2.1 Collection of Clinical Samples

Total of three hundred and forty-seven (347) septic wound swabs were collected from patients at the accidental clinic of Ondo State Specialist Hospital Akure between October 2015 and March, 2016. Patients on antibiotics within 7 days prior to specimen collection were also included. Informed consent was obtained from infected adults and parents or guardians of all patients prior to specimen collection. Ethical approval for the study was obtained from the Ethics and Research Committee of the Hospital (AD.4693/319). Sterile swab sticks were used to collect pus discharges from each patient. The wound swabs were collected strictly under aseptic conditions from both male and female patients at the State Specialist Hospital Akure, by a Certified Medical Laboratory Scientist. All the samples collected were placed in an ice bag and immediately transported to the microbiology Laboratory of the Federal University of Technology, Akure and analyzed within 1 hour of collection.

2.2 Sterilization of Materials Used

All glasswares used were sterilized in hot air oven at 160℃ for one hour, while the culture media used was sterilized in an autoclave at 121℃ for 15 minutes. Inoculating wire loop, forceps and spatula were sterilized by flaming to

red hot on Bunsen burner and then allowed to cool down, after which was then dipped in 75% ethanol before and after investigations. The surface of the work bench, as well as inoculating chamber, was sterilized by swabbing with 75% alcohol before and after each working period. Other aseptic conditions found necessary were adopted during this work.

2.3 Isolation of Bacteria from the Clinical Samples

2.3.1 Isolation of bacteria

The plate streaking technique was used for isolation of bacteria. Swab sticks were used to streak the samples on the already solidified nutrient agar plate, blood agar and chocolate agar, and incubated at 37℃ for 24 hour. Pure cultures of isolate were obtained by sub-culturing unto freshly prepared plates as appropriate [45].

2.4 Identification and Characterization of Bacterial Isolates

The isolated bacteria were identified by using their cultural and morphological characteristics on media. This was followed by microscopic examination of the bacterial isolates under the microscope. The cultural features examined included shape elevation, surface edge and consistency. Physiological and biochemical tests were employed to confirm their identification [46].

2.5 Preparation of Plant Extract

Exactly 300 g each of the dried powdered plant sample were weighted in a beaker and percolated with 3000 ml each of 80% ethanol, distilled cold water, hot water (100℃) and n-Hexane. It were allowed to stand for 3 days at room temperature with agitations at intervals. Afterwards, each extract were sieved through a muslin cloth, filtered through a Whatman (No. 1) filter paper and were concentrated *en vacuo* using rotary evaporator. The dried mass were stored in sterile McCartney bottle and kept in the frigerator at 4℃ at least 24 hrs before subsequently testing [47].

2.6 Phytochemical Screening

The preliminary phytochemical analysis of the extracts were carried out to determine the presence of tannins, flavonoids, saponins, alkaloids, phenols and glycosides using standard procedures [48,49].

2.6.1 Determination of saponin content

2 g of sample was weighed into a 250 ml beaker and 100 ml of Isobutyl alcohol or (But-2-ol) was added shaker was used to shake the mixture for 5 hours to ensure uniform mixing. The mixture was filtered with No 1 Whatman filter paper into 100 ml beaker containing 20 ml of 40% saturated solution of magnesium carbonate ($MgCO_3$). The mixture obtained was filtered again using No 1 Whatman filter paper to obtain a clean colourless solution. 1 ml of the colourless solution was taken into 50 ml volumetric flask using pipette and 2 ml of 5% iron (III) chloride ($FeCl_3$) solution was added and made up to the mark with distilled water. It was allowed to stand for 30 minutes for the colour to develop. The absorbance was read against the blank at 380 nm [48].

2.6.2 Determination of flavonoid content

0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50l 10% A1C13, 50l 1 M potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 minutes. The absorbance of the reaction mixture was subsequently measured at 415 nm: The total flavonoid content was subsequently calculated. The non flavonoid polyphenols were taken as the difference between the total n-phenol and total flavonoid content [49].

2.6.3 Determination of tannin content

About 1.0 g of sample was weighed into a 50ml of sample bottle. 10 ml of 70% aqueous acetone was added and properly covered. The bottle were put in an ice bath shaker and shaken for 2 hours at 30℃. Each solution was then centrifuged and the supernatant stored in ice 0.2 ml of each solution was pipette into the test tube and 0.8 ml of distilled water was added. Standard tannin acid solutions were prepared from a 0.5 ml of Folin–ciocateau reagent was added to both sample and standard followed by 2.5 ml of 20% Na_2CO_3. The solution were then vortexed and allowed to incubate for 40 minutes at room temperature, its absorbance was read at 725 nm against a reagent blank concentration of the same solution from a standard tannic acid curve that was prepared [49].

2.6.4 Determination of terpenoid content

2.0 g of soap sample was soaked in 50 mL of 95% ethanol for 24 hrs. The extract was filtered and the filtrate extracted with petroleum ether (60

to 80℃) and concentrated to dryness. The dried ether extract was treated as total terpenoids [48,49].

2.6.5 Determination of cardiac glycosides content

10 ml of the soap solution water extract was pipette into a 250 ml conical flask. Chloroform was added and shaken on vortex mixer for 1 hour. The mixture was filtered into 100 ml conical flask.10 ml of pyridine and 2 ml of 29% sodium nitroprusside were added and shaken thoroughly for 10 minutes. 3 ml of 20% NaOH was added to develop a brownish colour. Glycosides standard (Digitoxin), concentrations which ranged from 0-50 mg/ml were prepared from stock solution and the absorbance was read at 510 nm [49].

2.7 Reconstitution and Sterilization of Plant Extracts

Each of the extracts (ethanol, distill cold water, hot water and n-hexane) were reconstituted using 0.01% Tween 20 as described by [50]. This was done by dissolving 0.5 g of the extract in 10 ml 0.01% Tween 20. The resultant solution was filtered using sterile Millipore membrane filter (0.45 µm).

2.8 Antibacterial Activity

2.8.1 Determination of antibacterial activities by leaf extract

Agar well diffusion technique as described by [51] was used to determine the *in-vitro* antibacterial activity of the crude extract. A 1ml aliquot of 18hrs broth culture that had been adjusted to turbidity equivalent of 0.5 McFarland standards was dispensed into sterile Petri dishes previously labeled with the test bacteria. Molten sterile Muller-Hinton was aseptically poured into the plates and gently rotated for the bacteria to be homogenously distributed in the medium. The agar plates were allowed to solidify, after which a sterile cork borer of 6 mm in diameter was used to cut uniform wells in the agar plates. The wells were later filled with 0.5 ml of the each extracts. In addition, 20% Tween 20 was used as the negative control while Ciprofloxacin served as the positive control. The experiment was conducted in triplicates. All plates were incubated at 37℃ for 24 hours. Clearance zones around the wells were noted and measured in millimeters.

2.8.2 Determination of MIC of the extract

Minimum inhibitory concentration is defined as the minimum concentration of the extract that will not allow any visible growth or turbidity of the organism in the broth [46]. Broth dilution technique was used to determine the MIC of the extract [51]. The four extracts that showed antibacterial activities were reconstituted by diluting 2.5 g of each in 10 ml of Tween 20 and filtered with a sterile Millipore membrane filter (0.45 mm). Different concentrations of the extracts (250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, and 15.625 mg/ml) were used. The reconstituted extracts were serially diluted in sterile broth culture and 0.1ml of the 18 hours broth culture of each of the test organisms that have been adjusted to turbidity equivalent to 0.5 McFarland standard was introduced to each test tube containing the serially diluted extracts and incubated for 24 hours at 37℃. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration (MIC).

2.9 Data Analysis

All the experiments were carried out in triplicate and data obtained from the study were subjected to analysis of variance. Treatment means were compared using Duncan's New Multiple Range Test (DNMRT) at 5% level of significance using SPPS version 21.

3. RESULTS

Table 1 shows the percentage yield of the extracts by different solvents. 12.37% was the highest yield obtained from Cold water, 11.90% from Hot water, 9.13% from Ethanol and 5.43% from n-Hexane.

The result of the antibacterial activities of *Calotropis procera* leaf extracts at 500 mg/ml as shown in Tables 2-5 reveals the highest inhibition by the Ethanol extract had the highest zone of inhibition against *Staphylococcus aureus* with 16.03 mm and lowest zone of inhibition against *Klebsiella pnuemoniae* with 8.03 mm. For Cold water extract, the highest zone of inhibition was recorded against *Escherichia coli* with 13.30 mm and lowest zone of inhibition against *Pseudomonas aeruginosa* with 4.60 mm. For Hot water extract, the highest zone of inhibition was recorded against *Escherichia coli* with 12.27 mm and lowest zone of inhibition against *Klebsiella pnuemoniae* with 3.49 mm. For n-Hexane

extract, the highest zone of inhibition was recorded against *Pseudomonas aeruginosa* with 10.77 mm and lowest zone of inhibition against *Staphylococcus aureus* with 7.70 mm.

The minimum inhibitory concentration (MIC) tests of *Calotropis procera* leaf extracts on the bacterial isolate as shown on Fig. 1 at 250 mg/ml indicates that for Ethanolic extract, the MIC values for *P. aeruginosa* was 31.25 mg/ml*, K. pneumoniae was* 62.5 mg/ml, *S. auerus was* 15.625 mg/ml, *E. coli* was 15.625 mg/ml, *P. mirabilis* was 15.625 mg/ml and *S. pyogenes* was 31.25 mg/ml. Cold water extract, MIC values were recorded for *K. pneumoniae* was 125 mg/ml, *P. mirabilis* was 62.5 mg/ml, *S. auerus* was 15.625 mg/ml, *P. aeruginosa* was 125 mg/ml, *S. pyogenes* was 62.5 mg/ml and *E. coli* was 15.625 mglml. Hot water extract showed MIC values for *K. pneumoniae* was 125 mg/ml, *P. mirabilis* was 31.25 mg/ml, *S. auerus* was 31.25 mg/ml, *P. aeruginosa* was 62.5 mg/ml, *S. pyogenes* was 62.5 mg/ml and *E. coli* was 15.625 mglml. n-Hexane extract showed MIC values for *K. pneumoniae* was 125 mg/ml, *S. auerus* was 31.25 mg/ml, *P. aeruginosa* was 31.25 mg/ml*, E. coli* was 15.625 mgl/ml while *S. pyogenes* and *P. mirabilis was* not determined.

Table 6 reveals the qualitative analysis of the phytochemical constituents of *Calotropis procera* Leaf Extracts. Phenolics, saponins, tannins, cardic glycoside, flavonoids and alkaloids were present in Ethanol, Cold water, Hot water and n-Hexane extract while flavonoids, phenolics, tannins and cardic glycoside were absent in n-Hexane extract.

Table 1. Physical characteristics and percentage (%) yield of the extracts obtained from *Calotropis procera* leaves

Solvent (3L)	Input: Dried leaf	Output: (extract)	% recovery	Colour	Odour	Texture
Cold water	300 g	37.1	12.37	Dark-Brown	Pleasant fruity	Oily
Hot water	300 g	35.7	11.90	Dark-brown	Pleasant fruity	Oily
Ethanol	300 g	27.4	9.13	Black	Slightly repulsive	Oily
n-Hexane	300 g	16.3	5.43	Yellowish-black	Slightly repulsive	Gummy

Table 2. Antibacterial activity of *Calotropis procera* ethanol leaf extract on resistant bacterial isolates

Plant extract	Zone of inhibition (diameter in mm)					
	E. coli	***S. aureus***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. pyogenes***
CPX (25 mg/ml)	24.12±0.00^{b}	20.45±0.58^{a}	25.61±0.58^{c}	21.67±0.33^{a}	25.33±0.33^{a}	19.00±0.00^{b}
50 mg/ml	1.00 ±0.15^{b}	2.00±0.12^{c}	0.00±0.00^{a}	0.00±0.00^{a}	3.06±0.15^{d}	0.00 ±0.00^{a}
100 mg/ml	5.13±0.21^{c}	5.60 ±0.15^{c}	2.30±0.10^{a}	2.00±0.15^{a}	6.40±0.15^{d}	4.12±0.25^{b}
250 mg/ml	10.47±0.25^{b}	13.40±0.36^{c}	8.40±0.44^{d}	7.39±0.10^{c}	12.11±0.06^{c}	8.60±0.45^{d}
500 mg/ml	12.50±0.17^{b}	16.03±0.15^{d}	10.37±0.15^{d}	8.03±0.25^{b}	14.73±0.38^{d}	9.17±0.12^{d}

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05). Key: CPX= Ciprofloxacin

Table 3. Antibacterial activity of *Calotropis procera* cold water leaf extract on resistant bacterial isolates

Plant extract	Zone of inhibition (diameter in mm)					
	E. coli	***S. aureus***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. pyogenes***
CPX (25 mg/ml)	24.12±0.00^{b}	20.45±0.58^{a}	25.61±0.58^{c}	21.67±0.33^{a}	25.33±0.33^{a}	19.00±0.00^{b}
50 mg/ml	4.12±0.35^{b}	2.01±0.07^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
100 mg/ml	5.50±0.10^{b}	7.20±0.16^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	2.22±0.27^{b}
250 mg/ml	12.50±0.27^{b}	9.37±0.31^{b}	2.30±0.35^{a}	2.16±0.14^{a}	8.73±0.25^{b}	6.97±0.21^{c}
500 mg/ml	13.30±0.11^{c}	10.57±0.58^{c}	4.60±0.10^{a}	3.54±0.25^{a}	10.17±0.15^{c}	7.20±0.10^{b}

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05). Key: CPX= Ciprofloxacin

Table 4. Antibacterial activity of *Calotropis procera* hot water leaf extract on resistant bacterial isolates

Plant extract	Zone of inhibition (diameter in mm)					
	E. coli	***S. aureus***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. pyogenes***
CPX (25 mg/ml)	24.12±0.00[b]	20.45±0.58[a]	25.61±0.58[c]	21.67±0.33[a]	25.33±0.33[a]	19.00±0.00[b]
50 mg/ml	5.44±0.33[c]	2.60±0.33[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
100 mg/ml	8.12±0.33[d]	5.21±0.33[c]	3.50±0.33[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
250 mg/ml	10.63±0.12[b]	9.34±0.40[b]	4.43±0.41[b]	2.00±0.33[a]	8.37±0.31[b]	5.90±0.20[b]
500 mg/ml	12.27±0.25[b]	9.57±0.34[b]	5.33±0.47[b]	3.49±0.25[a]	8.87±0.15[b]	7.63±0.21[c]

Table 5. Antibacterial activity of *Calotropis procera n*-hexane leaf extract on resistant bacterial isolates

Plant extract	Zone of inhibition (diameter in mm)					
	E. coli	***S. aureus***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. pyogenes***
CPX (25 mg/ml)	24.12±0.00[b]	20.45±0.58[a]	25.61±0.58[c]	21.67±0.33[a]	25.33±0.33[a]	19.00±0.00[b]
50 mg/ml	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
100 mg/ml	3.60±0.20[b]	0.00±0.00[a]	0.00±0.00[a] 0[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
250 mg/ml	9.37±0.29[a]	6.37±0.25[a]	7.43±0.35[c]	4.50±0.26[b]	0.00±0.00[a]	0.00±0.00[a]
500 mg/ml	9.63±0.34[c]	7.70±0.20[a]	10.77.0.08[a]	8.47±0.12[c]	00.00±0.00[a]	0.00±0.00[a]

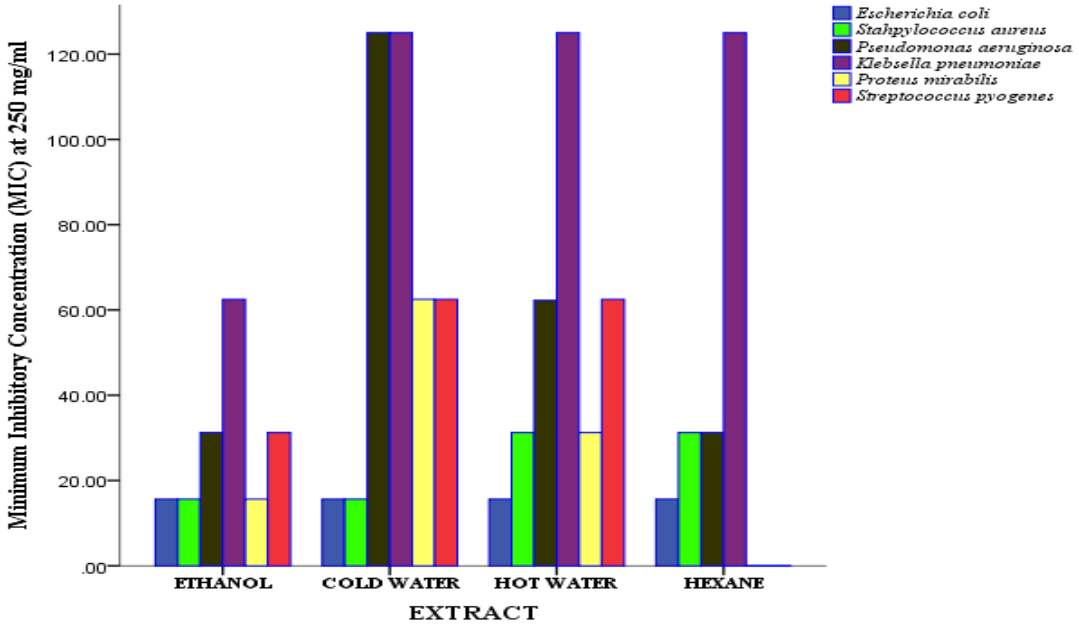

Fig. 1. Minimum inhibitory concentration of *Calotropis procera* leaf extracts on resistant bacterial isolates at 250 mg/ml

The quantitative analysis of phytochemical constituents of *Calotropis procera* Leaf Extract is shown in Fig. 2. The Cold water extract had the highest quantity of phenolic (13.38 g/ml) while the least of phenolic was recorded in n-Hexane Extract (0.03 g/ml). Cold water extract recorded (11.89 g/ml) has the highest quantity of flavonoid while n-Hexane extract recorded the least with quantity of (0.06 mg/ml). The highest quantity of tannins (4.72 mg/ml) and saponins (10.34 mg/ml) was recorded in Cold water extract while the least of tannins (0.07 mg/ml) was recorded in n-Hexane extract and saponins (7.24 mg/ml) recorded in Hot water extract. Cold water extract recorded the highest quantity of alkaloid (12.27 mg/ml) while the least (10.06 mg/ml) was recorded in Hot water extract.

Table 6. Qualitative phytochemical constituents of *Calotropis procera* leaf extract

Constituents	Crude extract	Cold extract	Hot extract	Ethanol extract	n-hexane extract
Alkanoids	++	++	+	++	++
Flavonoid	++	++	+	++	-
Phenolics	++	++	+	++	-
Saponin	+	+	+	+	+
Tannin	+	+	+	+	-
Terpenoids	+	+	+	+	+
Cardic glycoside	+	+	+	+	-

Key: + = Positive; - = Negative; ++ = Strongly positive

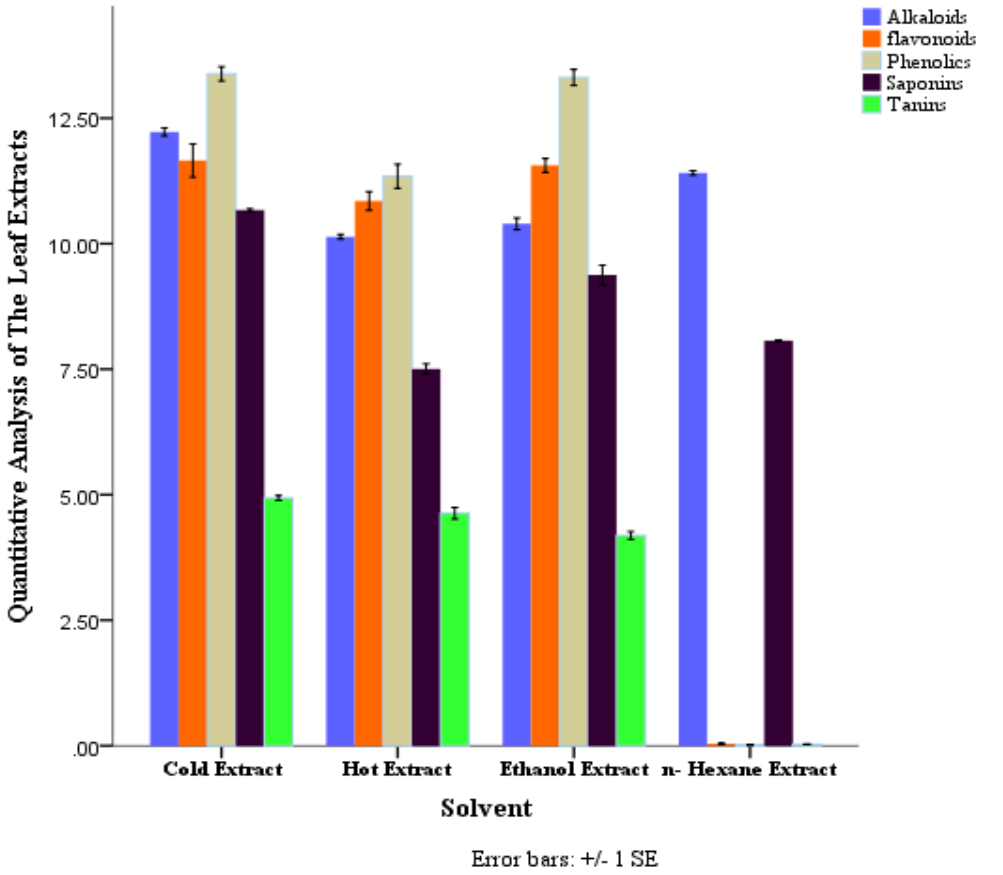

Fig. 2. Quantitative phytochemical constituents of *Calotropis procera* leaf extract

4. DISCUSSION

In this study, the bacteria; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Streptococcus pyogenes* were isolated from wound infections. This is in agreement with previous study of [52] where the named bacterial isolates were the mostly implicated in wound infections.

Findings in this research work indicate that the percentage yield of the extract using different solvent varied among the extracts used. The findings are in accordance with the work of [53,54] that worked on the antibacterial activities of *Calotropis procera* leaves extracts on pathogenic microorganisms but slights differences in solvents used for extraction. It is reported that the percentage recovery was dependent on the solvent used. This result

corroborates with those obtained by [53] in which percentage yield of *Calotropis procera* varied with different extraction solvents. It has been shown that polar solvent have ability to extract organic and inorganic materials from natural sources [55]. Polar solvents have been proved by [53] to give a higher yield of active components in plants than the non polar solvents.

In this research, it was observed that *Calotropis procera* cold water extract gave the highest yield of 12.37%. This was followed by Hot water extract with 11.90%. This may be due to high level of polarity of water which makes it easier to dissolve chemical component of the leaves that have been shown to be organic in nature and slightly polar. Ethanolic extract showed percentage yield of 9.13% while the lowest percentage yield is observed by n-Hexane extract 5.43% due to low polarity of the solvent to chemical component of the leaves. The slight variation between hot water extract and cold water extract yield might be due to the fact that heat applied has helped hot water extract to degrade and oxidize the chemical compounds from the plant which pull the soluble chemicals easily and makes them more volatile. This means they evaporate into the air more easily giving cold water extract higher percentage yield. In addition, it was deduced from this investigation that the antibacterial activities of the extracts against bacterial isolates varied. According to this observation it was ethanolic extract that had the highest antibacterial activities in comparism to other solvents used for extraction. The results of this study reveal that Ethanolic extract had highest zone of inhibition against *Staphylococcus aureus* which is in conformity with the work done by [47,56] but in contrast to the work done by [57] which reveal that the aqueous extract has the highest zones of inhibition against *Staphylococcus aureus*.

The commercial antibiotics were observed to be more effective in inhibiting the bacterial isolates. [58] reported that the state of administration of an antimicrobial agent affects the effectiveness of such agent, and that antibiotics being in a refined state and plant extracts in crude state, may record higher antimicrobial activity. Also, the small molecular size possessed by antibiotics as reported by [59] aids their solubility in diluents as this could enhance their penetration through the cell wall into the cytoplasm of the organism.

In this study, the Qualitative and Quantitative analysis of the *Calotropis procera* leaf extracts as shown on Table 6 and Fig. 2 respectively, reveals the presence of active constituents such as alkaloids, flavonoids, tannin, saponin, terpenoids, cardic glycoside and phenols in all the extracts, except hexane where only alkaloids, terpenoids saponin. [54,60] performed phytochemical screening of the ethanol extracts of flowers, young bud, mature leaves and stems of *C. procera* (Ait) R. Br. (Asclepiadaceae) and found that alkaloid, cardiac glycoside, saponin, phenolics, triterpenoids and tannins were present in almost all partswhich was in agreement with present result. The different phytochemical constituents present in the different extracts may be attributed to the different solvents used for extraction as reported by [56] that different solvents have different spectrum of solubility for the phytoconstituents of plants.

Antibacterial activity of medicinal plants and drugs varies in their inhibitory effect depending on the concentration, temperature, nature of organism, size of inoculums [61]. The presence of tannin is also an indication that there are phenolic acids present in the plants which may be responsible for its antibacterial activities [34, 63]. The result from this study has shown that the leaf extracts of *Calotropis procera* has antibacterial properties. Thus the leaf extracts of *Calotropis procera* exhibited a broad spectrum activity, against the test organisms in this work, and hence can be employed in treatment of diseases caused by such. Also the susceptibility of *Staphylococcus aureus* to the plant extracts justifies the traditional use of the leaf in wound healing. This is in agreement with the findings of [34,62,33] which reported that the plant is useful in treatment of wounds, ulcers, spleen complaints, sores, rheumatism, smallpox, epilepsy, hemiplegia, gumboils and skin infections.

The observed variation in the values of Minimum inhibitory concentration (MIC) could be as a result of the nature and level of antimicrobial agent present in the extracts.

5. CONCLUSION

In this study, the microbiological investigation done on *Calotropis procera* leaves obtained in Nigeria have shown activity coherent with the use of this plant in folk medicine. The extracts could serve as useful sources for new antibacterial agent owing to the increase rate of resistance of bacteria to vancomycin and methicillin antibiotics. Therefore the rational use

of antibiotics must be a priority. Public health policy on appropriate prescribing and use of antibiotics must be instituted and affected based on recent antibiogram tests. Findings of this research indicate that the leaf extracts of *Calotropis procera* posses antibacterial potency which will assist in the preliminary treatment of wound infections, most especially because of its high inhibitory effect against *Staphylococcus aureus.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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