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Phytochemical Analysis and Antimicrobial Activities of *Calotropis procera* Extracts on Selected Pathogenic Microorganisms

O. S. Asoso[1]*, M. K. Oladunmoye[2], A.O. Ogundare[2]

[1]*Department of Biological Sciences, Afe Babalola University, Ado-Ekiti, Nigeria
[2]Department of Microbiology, Federal University of Technology, Akure, Nigeria

Abstract *In vitro* antimicrobial and preliminary effects leaf and stem extracts of *Calotropis procera* on human pathogens were studied. Twelve pathogenic bacteria and five fungi species were obtained from the Department of Microbiology, Federal University of Technology, Akure, Ondo-State and typed cultures were collected from National Institute of Medical Research (American type culture collection centre ATCC, USA,). Aqueous and solvents like methanol, ethanol and acetone were used in this study. *In vitro* antimicrobial sensitivity of *C. procera* was done using in agar-well method. The methanol-leaf extract revealed the highest inhibitory potency against *E. coli* (15.17mm) among the clinical isolates while among typed isolates; *Shigella dysenteriae* ATCC 24162 was most inhibited the highest inhibition of 20.67 mm. The antifungal activities of the aqueous-stem extract created the highest inhibition of 23.33 mm. The minimum inhibitory concentrations of extracts on bacterial and fungal isolates were between 100 and 400 mg/ml. Minimum bactericidal and fungicidal concentrations (MBC/MFC) of the extracts were mostly between 200 and 400 mg/ml on the employed test microbes. *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 were resistant to all the antibiotics. Alkaloids and carotenoids had the highest values in the stem sample while Alkaloids and Flavonoids have the highest values in the leaf sample. However, Saponin, alkaloids, tannins, steroids, terpenoids, phenol and carotenoids were qualitatively screened from the leaf and stem of *C. procera* plant.

Keywords *Calotropis procera*, phytochemical, antimicrobial, antibiotics

Introduction

Calotropis procera (giant milkweed, apple of Sodom) is a perennial, greyish-green, woody shrub with broad obovate fleshy leaves that grows wild in the tropics and in warm temperate regions. The plant is found in almost all parts of Nigeria but more abundant in the northern part of the country [1], it is either used alone or with other herbs to treat common diseases such as fever, rheumatism, indigestion, cold, eczema and diarrhea. It is a shrub or small tree, which has become a serious weed in pastures and overgrazed rangelands. It is a native to West Africa as far as south as Angola, North and East African, Madagascar, the Arabian Peninsula, Southern Asia, India and China to Malaysia. *Calotropis* was formerly placed in the family of Asclepiadaceous (the milkweed family), which is now considered a subfamily of the Apocynaceae. The stem-bark as a promising antifungal agent, which could be used against dermatophytes and suggests that *Calotropis procera* could be a potential source of chemotherapeutic agents, thus could be used for the treatment of tinea diseases. Several reports in the literature indicate many therapeutic activities of *Calotropis procera* including analgesic, anti-inflammatory, antidiabetics, cytotoxic, and

hepatoprotective effects. The term 'Phytochemicals' refers to a wide variety of compounds made by plants, but is mainly used to describe those compounds that may affects human health. Phytochemicals are found in plant-based foods such as fruits, vegetables, beans and grains. Scientists have identified thousands of phytochemicals. The use of herbal medicine as alternative therapy has prevalent throughout the world due to the growing resistance of pathogens to conventional antibiotics. The need for more potent, safe and affordable drugs has led to intensified research into herbal drugs, the result of which is the introduction of new herbal preparation for therapeutic uses. Medicinal plants are frequently used as remedies for many infectious diseases. The treatment and control of diseases by the use of the available medicinal plants in a locality have been helpful and of a priority to majority of urban and rural dwellers in healing various diseases because of the reliability and stability in plant products for healing [2].

The aim and Objective of this study is to determine the quantitative and qualitative analysis of the named plant and also to determine the antimicrobial effects of the *Calotropis procera* extracts and its fractions on selected pathogenic bacteria and fungi.

Materials and Methods

Plant Collection and Preparation

The plant parts were collected in Elekute quarters, Ado-Ekiti, Ekiti State, Nigeria. The plant parts (leaf and stem) were adequately washed with clean water and air dried at room temperature (25±2˚C). They were then pulverized (crushed) separately with grinding machine to obtain smooth powder.

Preparation of Crude Extracts

Aqueous Extraction

One hundred and fifty grammes (150g) of the ground powder of each of the plant parts were soaked separately in 750 ml sterile distilled water for 72 hours at room temperature.

Organic Solvent Extraction

Organic solvents like Methanol, ethanol and acetone were used in this study. One hundred and fifty grammes of the ground powder of each of the plant parts (leaf and stem) were soaked separately with the solvents in 750 ml capacity flask for 72 hours. They were then filtered through a 3-layered sterile muslin cloth. The solutions were concentrated *in vacuo* in a rotary evaporator (RE -52 A Union Laboratories, England), to remove the solvent. This was reconstituted with 5% aqueous $DMSO_4$ before been used for antimicrobial analysis.

Sources of Bacterial Cultures

Test microorganisms: They includes typed cultures of bacteria and fungi (American type culture collection centre (ATCC) USA) and microbial isolates from the stock cultures of the Department of Microbiology, Federal University of Technology, Akure . The microorganisms used in this study include *Shigella dysenteriae, S. dysenteriae* ATCC 24162, *Escherichia coli, E. coli* ATCC 35218, *Staphylococcus aureus, S. aureus* ATCC 25923, *Salmonella typhi, S. typhi* ATCC 22648, *Pseudomonas aeruginosa, P. aeruginosa* ATCC 27853, *Klebsiella pneumonia, and K. pneumonia* ATCC 34089, *Aspergillus flavus, Aspergillus flavus* ATCC 204304, *Malazessia furfur* ATCC 44349, *Candida albicans and C. albicans* ATCC 10231.

Phytochemicals Screening of the Plant Extracts

Determination of Qualitative Phytochemicals

Test for Alkaloids

Five grammes of each plant extract was mixed with 5 ml of 1% (v/v) aqueous hydrochloric acid on a steam bath, 1ml of the filtrate was treated with few drops of Draggendoff's reagent. Blue-black turbidity serves as preliminary evidence of alkaloids presence [3].

Test for Saponins

Five grammes of each plant extracts was shaken with distilled water (5 ml) in a test tube. Frothing which persisted on warming was taken as preliminary evidence of the presence of saponins.

Test for Tannins

Five gramme of each plant extract was added to 100 ml distilled water, stirred and filtered through Whatman No 1 filter paper. Ferric chloride reagent was added to the filtrate. A blue-black or blue green precipitate determined the presence of tannins [3].

Test for Flavonoids

Presence of flavonoids in the plant extracts was tested using $FeCl_3$ and lead ethanoate solutions. A green-blue or violet coloration on addition of $FeCl_3$ solution and appearance of buffcoloured precipitate on addition of lead ethanoate solution indicated the presence of flavonoids in the extract.

Test For the Presence of Cardiac glycosides (Keller-killiani test)

Five grammes of each plant extracts was mixed in 2 ml glacial acetic acid and a drop of ferric chloride solution were added. This was underlayed with 1ml of absolute H_2SO_4. Development of a brown ring at the interface indicates the presence of a deoxy-sugar characteristic of cardenolides. A violet ring might appear below the brown ring, while in the acetic acid layer, a green ring might form which would gradually spread throughout the acetic acid layer.

Test for the Presence of Terpenoids (Salkowski test)

Five millilitre of each plant extract was mixed in 2 ml of chloroform 100% (v/v), and absolute H_2SO_4 (3 ml) was carefully added to form a layer. Formation of a reddish brown layer at the interface showed the presence of terpenoids.

Test for Phenols

One millilitre of each plant extract was mixed with 4 drops of ethanol 100% (v/v) and 3 drops of 1% ferric chloride solution in test tube. Formation of green or red-brown indicates the presence of phenols.

Test for steroids

One gramme of the plant extracts was mixed in a beaker with 5 ml of concentrated acetic acid. It was gently warmed and cooled. One drop of concentrated sulphuric acid was added along the sides of the test tube. Appearance of green colour indicates the presence of steroids.

Quantification of Phytochemicals in the Plant Extracts

Determination of Tannin

Fine powdered ground plant sample (0.2 g) was weighed into a 500 ml sample bottle. Then, 100 ml of 70% (v/v) aqueous acetone was added and properly covered with a stopper. The bottles were shaken for 2 hours at 30 ^oC. Each solution was then centrifuged at 1600 rpm for 5 minutes and the sediment was stored on ice. Each solution 0.2 ml was pipetted into test tube and 0.8 ml of distilled water was added. Standard tannic acid solutions were prepared from a 0.5 mg/ml of the stock and the solution was made up to 1 ml with distilled water. Folin-ciocateau reagent (0.5 ml) was added to both the sample and the standard tannic acid solution followed by the addition of 2.5 ml of 20% (v/v) Na_2CO_3. The solutions were then shaken vigorously and incubated for 40 minutes at room temperature (28 ± 2 ^oC. The absorbance was read at 725 nm against a standard tannic acid curve.

Determination of Saponin Concentration

Spectrophotometric method of Obadoni and Ochuko, (2000) was used for the determination of saponin concentration. Two grammes of each plant sample were weighed into a 250 ml beaker and 100 ml of Isobutyl alcohol (100% v/v) was added. The mixture was shaken in a shaker water bath for 5 hours to ensure homogeneous mixture. The mixture was filtered through No 1 Whatman filter paper into 100 ml beaker containing 20 ml of 40% (v/v) saturated solution of magnesium carbonate ($MgCO_3$). The mixture obtained was again filtered with No 1 Whatman filter paper to obtain a clean colourless solution. One millilitre of the colourless solution was pipetted into 50 ml volumetric flask and 2 ml of 5% ferric chloride ($FeCl_3$) solution was added. The mixed solution was made up to the mark of 50 ml with distilled water. It was allowed to stand for 30 minutes for colour (light brown) development. The absorbance was read against blank at 380 nm.

Determination of the Amount of Alkaloid

Five grammes of plant sample was weighed into a 250 ml beaker and 200 ml of 10% (v/v) acetic acid in 200 ml of 100% (v/v) ethanol was added and allowed to stand for 4 minutes. It was filtered through Whatman's No. 1 filter paper and the extract was concentrated on a water bath (50 ^{o}C) for 4 hours to one quarter of the original volume. Absolute ammonium hydroxide (10 ml) was added drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute (2 M) ammonium hydroxide and then filtered. The residue was then considered as alkaloid which was dried and weighed. The formula written below was used to calculate concentration of alkaloid in percentage

$$\text{Alkaloid (\%)}. = (W_3 - W_2 / W_1) \times 100$$

Antibacterial Screening of the Crude Extracts

The antibacterial effect of the extracts was evaluated by agar well diffusion [5]. Inocula of test bacterial isolates were 24 hour culture prepared by inoculating a loopful of test bacteria from stock culture into freshly prepared nutrient both and incubated at 37 ^{o}C for 24 hours. Absorbance of the grown culture was read at 530nm after adjustment with sterile distilled water to match that of 0.5M McFarland standard solution which is equivalent to between 1.0 x 10^6 - 1.0 x 10^7 cfu/ml. One millilitre each of this bacterial suspension was obtained and spread on Mueller-Hinton agar. The plates were allowed to stand for one and half hours for the test bacterial isolates to be fully embedded and properly established in the seeded medium. With a sterile cork borer (No 4 Gallenkamp), wells of equal depth of 0.5 cm (5 mm diameter) were bored inside the agar. Each well was aseptically filled up with 0.5 ml of respective extracts avoiding splashes and overfilling. Sterile 5% aqueous $DMSO_4$ was used as negative control while methecilin and streptomycin (10 mg/ml) were used as the positive control. The plates were incubated at 37 ^{o}C for 24 – 48 hours. The sensitivity of the test organisms to each of the extracts was indicated by clearing around each well. The halo's diameter as an index of the degree of sensitivity was measured with a transparent plastic ruler.

Determination of Minimum Inhibitory Concentration (MIC) of Extracts

One millilitre each of extract concentration and 1 ml of 24 hours broth culture of each test bacterium (1.0x10^6 cfu/ml) was inoculated into 8 ml of Mueller-Hinton broth in a test tube. The seeded broth was incubated at 37 ^{o}C for 24 hours. A non- test bacteria seeded tube and without extract was used as a control. Turbidity was checked after 24 hours of incubation. The lowest concentration of the extract that produced no visible growth when compared to the control (tube containing no inoculum) was considered as MIC. All experiments were carried out in triplicates

Determination of Minimum Bactericidal Concentration of Each Extract

Pour plate method was carried out on the positive MIC tubes where detectable growth were observed. All experiments were carried out in triplicates.

Minimum Fungicidal Concentration (MFC)

Minimum Fungicidal concentrations (MFC) of the extracts were carried out as described by CLSI (2012).

Results

The phytochemical analysis of the stem of *Calotropis procera* was carried out, where Akaloids, Tannins and Carotenoids content had the highest values of 1476, 233 and 238 mg/100g respectively (Table 1). While the Phytochemical analysis of the leaf was also observed where alkaloids, Saponins, Tannins, Flavonoids and Terprenoids had the highest values of 1343.33, 231.67, 353.33, 666.67 and 941.67 mg/100g respectively (Table 1). The minimum inhibitory concentration and minimum bactericidal concentrations of the leaf extracts of *Calotropis procera* on clinical and typed isolates were carried out; the MIC and MBC were varied from 200-400 mg/ml. Ethanol and methanol extracts were having excellent inhibitory effects on organisms like *Escherichia coli* and *Salmonella typhi* while *Klebsiella pneumonia* ATCC 34089 has a poor inhibitory effect. (Table 2). The Minimum Inhibitory and minimum bactericidal Concentrations of the stem extracts were

analysed using different organisms; where the extracts were having fair antibacterial activities on the organisms. During this study, the plant leaf has a good antimicrobial activities compared to the stem.

Table 1: Phytochemical analysis of the *Calotropis procera* (Stem and Leaf)(mg/100g)

Parameters	**Stem**		**Leaf**	
	Qualitative	**Quantitative**	**Qualitative**	**Quantitative**
Saponins	+	85.00±0.05	++	231.67±10.41
Alkaloids	+++	1476.67±15.28	+++	1343.33±20.82
Tannins	++	233.33±2.88	++	353.33±2.89
Steriods	++	131.67±5.77	+	44.00±1.73
Terpenoids	+	43.33±2.89	+++	666.67±14.43
Flavonoids	+	65.00±0.05	+++	941.67±18.93
Phenolics(GAE/100g)	+	47.83±0.76	+	15.67±0.28
Carotenoids (µg/100g)	++	238.33±8.50	+	16.42.67±9.45

Key +: present in minute quantity, ++: present in medium quantity, +++: present in large quantity, GAE: Garlic acid Equivalent (conventional unit for phenolic)

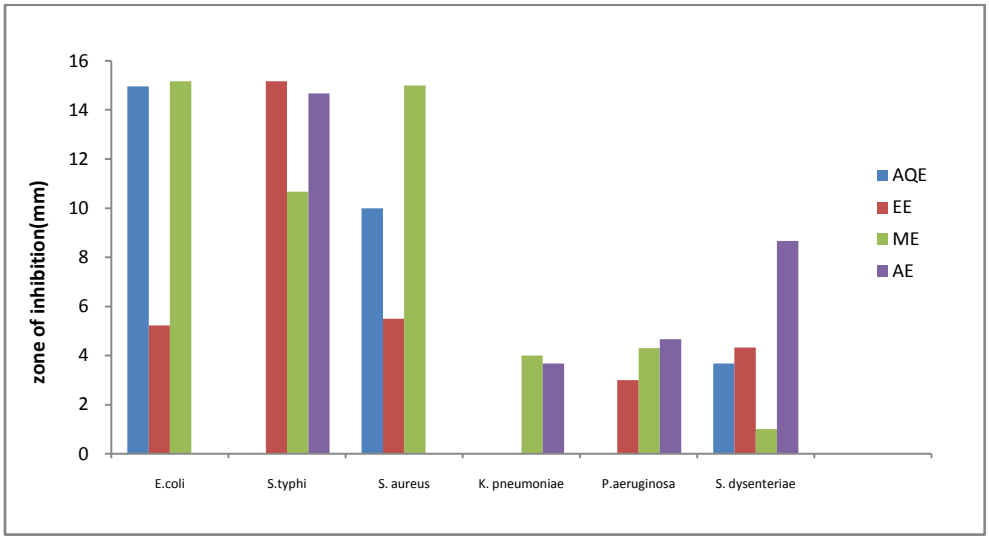

Figure 1: Antibacterial effect of Leaf extracts on Clinical Isolates

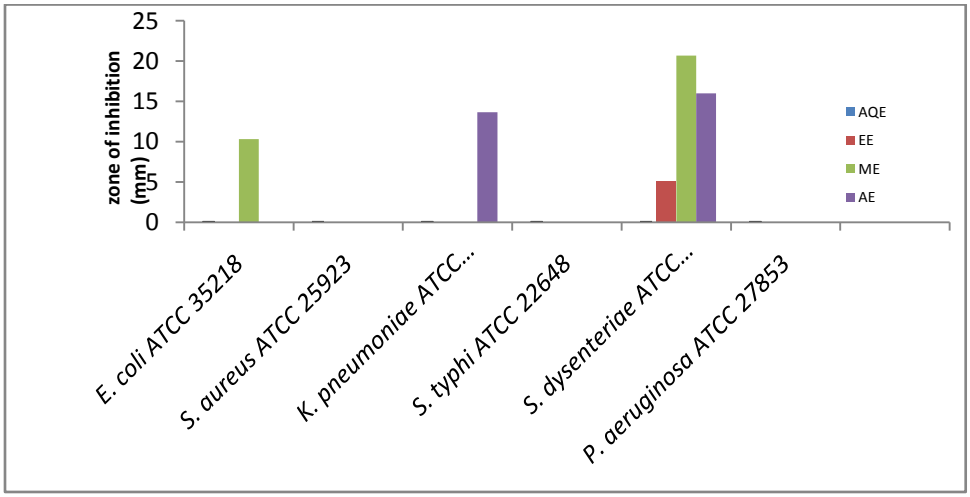

Figure 2: Antibacterial effect of Leaf extracts on Typed Isolates

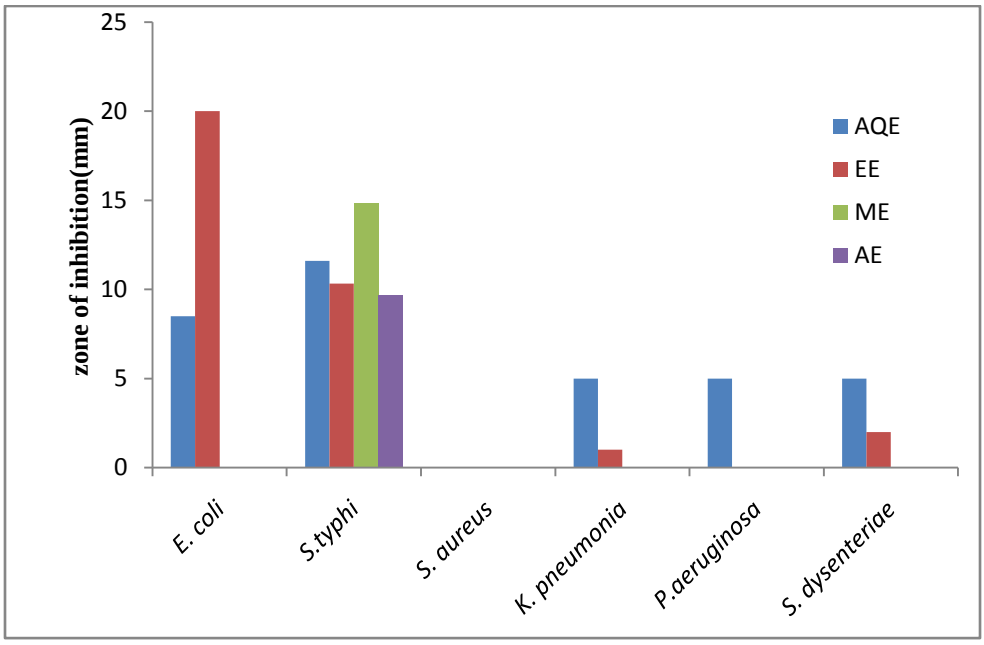

Figure 3: Antibacterial effect of Stem extracts on clinical Isolates Figure

LEGEND: AQE- AQUEOUS EXTRACT, AE-ACETONE EXTRACT, EE-ETHANOL EXTRACT AND ME-METHANOL EXTRACT

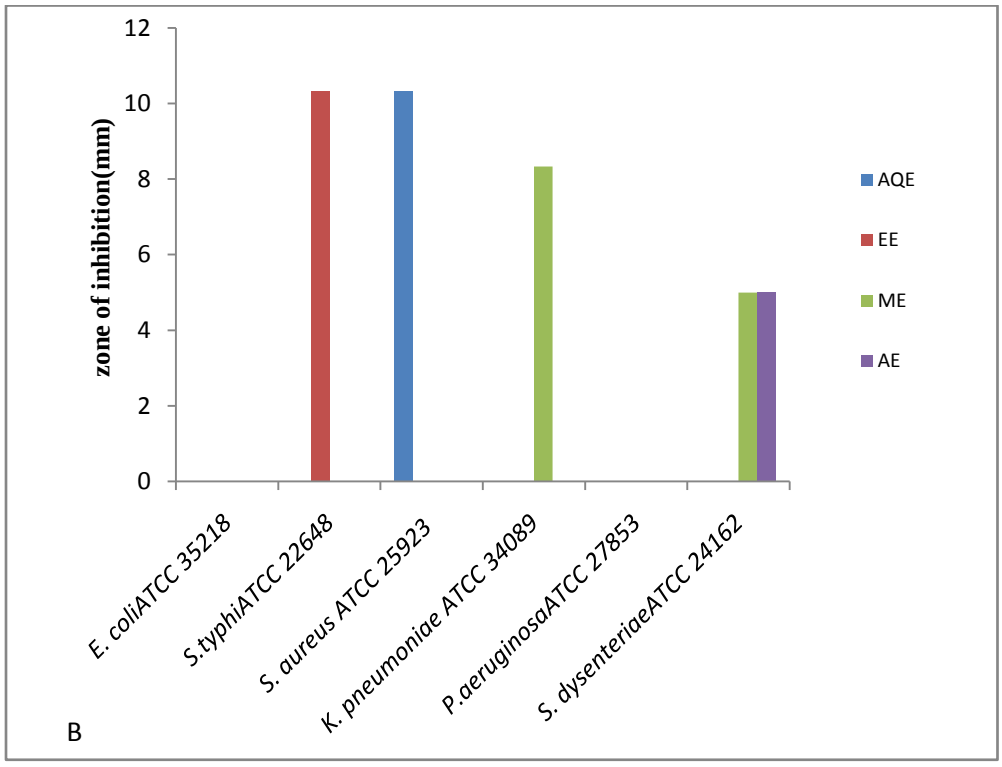

Figure 4: Antibacterial effect of Stem extracts on Typed Isolates

LEGEND: AQE- AQUEOUS EXTRACT, AE-ACETONE EXTRACT, EE-ETHANOL EXTRACT AND ME-METHANOL EXTRACT

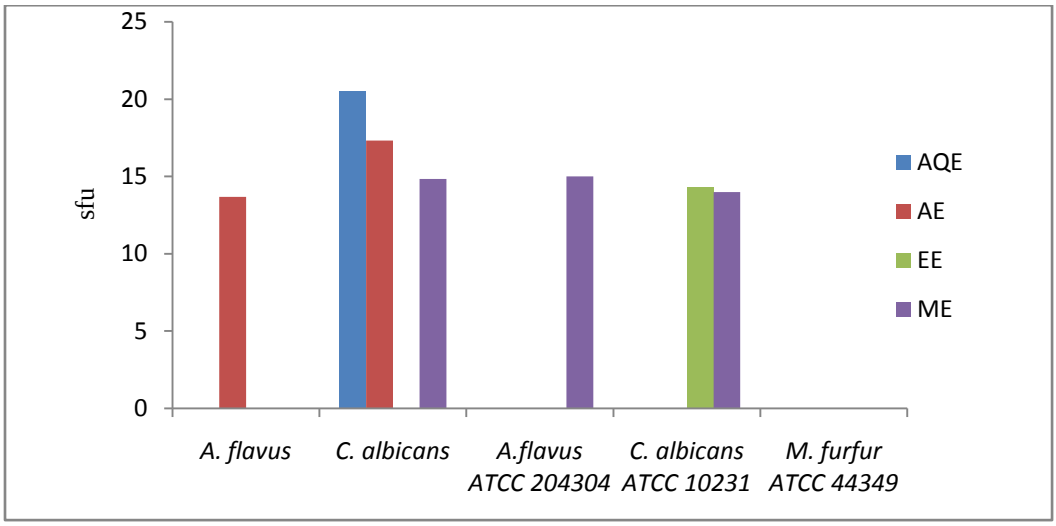

Figure 5: Antifungal effect of Leaf extracts on the selected Isolates

KEY: AQE- AQUEOUS EXTRACT, AE-ACETONE EXTRACT, EE-ETHANOL EXTRACT AND ME-METHANOL EXTRACT

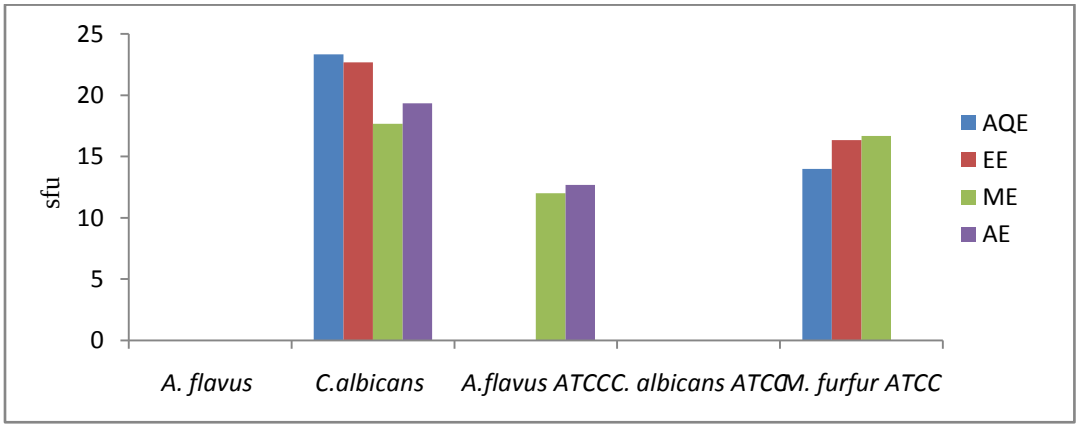

Figure 6: Antifungal effect of Stem extracts on some selected Isolates

KEY: AQE-AQUEOUS EXTRACT, AE-ACETONE EXTRACT, EE-ETHANOL EXTRACT AND ME-METHANOL EXTRACT

Table 2: Minimum inhibitory and Minimum bactericidal concentrations of leaf extracts *(Calotropis procera)* on clinical and typed Isolates using different solvents (mg/ml)

Organisms	**MIC (mg/ml)**				**MBC (mg/ml)**			
	ME	**EE**	**AE**	**AQE**	**ME**	**EE**	**AE**	**AQE**
E. coli	200	300	0	300	300	0	0	400
K. pneumoniae	300	0	300	0	400	0	0	0
S. typhi	300	200	200	0	400	400	0	0
S. dysenteriae	400	300	300	400	0	0	0	0
S. aureus	300	300	0	300	400	0	0	400
E. coli ATCC 35218	200	0	0	0	400	0	0	0
K. pneumonia ATCC 34089	0	0	300	0	0	0	0	0
S. dysenteriae ATCC	200	300	200	0	300	0	400	0

Key: MIC- minimum inhibitory concentration, MBC-Minimum bactericidal concentration, ME-methanol Extract, EE-ethanol Extract, AE-acetone Extract, AQE-aqueous Extract

Table 3: Minimum inhibitory and Minimum bactericidal concentrations of stem extract of *(Calotropis procera)* (mg/ml) on clinical and typed Isolates using different solvents

Organisms	**MIC (mg/ml)**				**MBC (mg/ml)**			
	ME	**EE**	**AE**	**AQE**	**ME**	**EE**	**AE**	**AQE**
E. coli	0	200	0	400	0	300	0	0
S. typhi	300	300	200	300	400	400	300	400
S. dysenteriae	0	400	0	400	0	0	0	0
S.aureus ATCC 25923	0	0	0	300	0	0	0	400
K. pneumoniae ATCC 34089	0	300	0	0	0	400	0	0
S. dysenteriae ATCC 24162	400	0	400	200	0	0	0	400
S. typhi ATCC 22648	300	0	0	0	400	0	0	0

LEGEND: AQE- AQUEOUS EXTRACT, EE-ETHANOL EXTRACT, ME- METHANOL EXTRACT, AE-ACETONE EXTRACT, MIC- MINIMUM INHIBITORY CONCENTRATION and MBC-MINIMUM BACTERICIDAL CONCENTRATION

Discussion and Conclusions

The qualitative and quantitative phytochemical analyses were carried out and antimicrobial activities of extracts were determined, where Alkaloids, carotenoids and tannins had the highest values of 1476, 238 and 238mg/100g in leaf extracts while alkaloids flavonoids and terprenoids had the highest yield in stem extracts with values of 1343, 941, 666 mg/100g respectively. The antibacterial activity of *Calotropis procera* leaf extracts on the test clinical bacterial species is presented in Figure 1. The aqueous leaf extract of *C. procera* inhibited *E. coli* with value of 14.96 mm while methanol leaf extract of *S. aureus* and *E. coli* shows the most inhibitory effects with halo of 15.00 and 15.17 mm respectively. Other reference typed bacterial species were evaluated and fewer organisms were susceptible compared to the clinical isolates, methanol leaf extract of *S. dysenteriae* ATCC 24162 was the only organism that has the highest inhibitory activity with halo of 20.67mm which is in support with Vadlapudi *et al.,* 2012 [6] which worked on the methanolic extracts of *C. procera* exhibit considerable antimicrobial activity against tested microbial strains. The antibacterial activity of stem extracts on clinical and typed isolates showed in Figures 3 and 4 has inhibitory activity on ethanol extract on *E. coli* and followed by the methanolic extract on *S. typhi* at values of 20.00 mm and 14.83 mm respectively which is in agreement with Komathi *et al.,* 2012 [7]. Aqueous stem extract on *S. dysenteriae* ATCC 24162 had the highest inhibitory activity of 12.67mm among the typed isolates. Generally, typed organism like *P. aeruginosa* ATCC 27853 was resistant to all the extracts. The antifungal activity of the aqueous leaf extract of *C. procera* was potent against *C. albicans*. *Malazessia furfur* ATCC 44349 was resistant to leaf extract but susceptible to the stem extract. Antibiotics susceptibility test was carried out on the selected bacteria using the extracts. Some organisms such as *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35218 were resistant to all the antibiotics but *E. coli* ATCC 35218 was susceptible to methanol extract of the leaf with halo of 10.33mm. Minimum Inhibitory concentration of the extract was varied from 50-400 mg/ml while the typed Isolates have fewer values only *E. coli* ATCC 35218, *S. dysenteriae* ATCC 24186 and *K. pneumonia* ATCC 34089 have a MIC values. The MBC values of the leaf extracts were found between 300-400mg/ml. In this study, the MIC and MBC of the stem extracts using clinical and typed Isolates were revealed but it was observed that the leaf extracts were more active than the stem extracts and during my findings which I observed that the leaf and latex of the plant possess more antimicrobial activities than any other part of the plant. The phytochemical screening of the stem showed the presence of terpenoids, saponins, alkaloids, phenolics where ethanol has the best solvent in phytochemical analysis from the stem (Table 2). The leaf showed the presence of saponins, alkaloids, Tanins,

Steroids, Flavonoids and Cardiac glycosides and Glycosides where methanol has the best solvent in phytochemical analysis from the leaf (Table 3).

The present study has investigated the phytochemical components of *Calotropis procera* as a good plant for medicinal purposes. However, the antimicrobial activities of the extracts of *Calotropis procera* revealed that the plant using aqueous and extracts like methanol, ethanol and acetone can be use for the killing or inhibition of some diseases. Due to the antimicrobial activities of the *Calotropis procera* extracts, it may be said that it can be effectively used in curing the diseases that could be manifested by the human pathogenic bacteria employed in this study and will be of hepatoprotective effects.

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