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By

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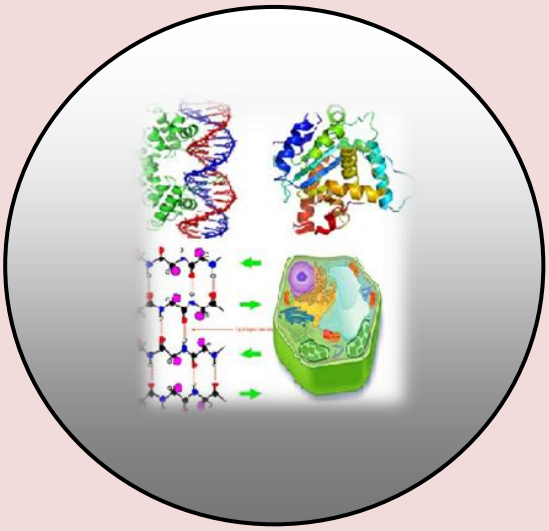
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 37 (1) 2020 Pages No. 1-10



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 37, No. 1: 1-10, 2020
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 37/01/1010/2020

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 30/11/2019 Revised: 01/03/2020 Accepted: 01/03/2020

Antibacterial Activity Studies of *Acacia Nilotica* Leaves and Stem Bark on two Clinical Isolates *Staphylococcus aureus and Escherichial Coli*

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ABSTRACT

Acacia nilotica is a widely used plant in traditional medical practice in northern Nigeria, many African countries and India. The leaves and stem bark samples were collected from birnin Kebbi, Nigeria where the plants grow widely around. Laboratory methods included isolation of isolates of selected organisms, biochemical tests, antibacterial activity tests, MIC and MBC tests. The aim of this study is to compare Acacia nilotica parts through ethanol crude extract and N-Hexane extracts of leaves and stem bark extracts on Staphylococcus aureus and E. coli. The plant extracts exhibited antimicrobial activity against the test microorganisms. The minimum inhibitory concentration for ethanol extract of 200 mg/ml on Staphylococcus aureus and E. coli are 12.0 and 15.0 mg/ml respectively. Acacia nilotica ethanol extracts are found to exhibit more potency than N-Hexane extracts. However, ethanol leave extract shows strong antimicrobial activity against Staph. aureus with MIC 12.5 mg/ml and MBC of 100mg/ml; E.coli with MIC 6.25 mg/ml and MBC 50mg/ml. Therefore, the ethanol leave extracts were found to be safe in single dose administration in the Staphylococcus aureus and E. coli.

Keywords: Acacia nilotica, Leaves, Stem bark, Extracts, Clinical isolates and Antibacteria

INTRODUCTION

Over three quarters of the world population relies mainly on plants and plant extracts for healthcare. More than 30% of the entire plant species at one time or other was used for medicinal purposes. In India drugs of herbal origin have been used in traditional system of medicine such as Unani and Ayurveda since ancient times. The Ayurveda system of medicine uses about 700 species, Unani 700, Siddha 600, Amchi 600 and modern medicine around 30 species. The plant-based traditional medicine system continuously plays an essential role in healthcare (Kamboj, 2000). The World Health Organization (WHO) has listed more than 21,000 plants which are used for many medicinal purposes around the world (Kathe, 2005). They observed that about 74% of 119 plant -derived pharmaceutical medicines are used in modern medicine. It also estimates that 4 billion people estimated 80 percent of the world population presently uses herbal medicine for health care (Mishra *et al*., 2010). Over hundreds of years, herbal medicines derived from medicinal plants, minerals and organic matter are the mainstay of about 75–80% of the world's population for health care marketed and gaining popularity in developed and developing countries (Sekar *et al*., 2010).

Herbs have medicinal property due to presence of different active principles like alkaloids, volatile essential oils, glycosides, resins, oleoresins, steroids, tannins, terpenes and phenols (Anees, 2010). In the last few years there is an exponential growth in the field of herbal medicine because of their natural origin, easy availability, efficacy, safety and less side effects with high efficiency in curing age-related disorders like memory loss, osteoporosis, immune disorders, etc. for which no modern medicine is available (Grover, 2002).

Medicinal plant researchers pursued with several goals like the development of low cost therapeutic compounds and the discovery of prototypic drugs (Elisabetsky, 1991). *Acacia nilotica* is also known as Gum Arabic tree, Babul, Egyptian thorn and Prickly. *Acacia* is multipurpose nitrogen fixing tree legume. It occurs from sea level to over 2000m and withstand an extreme temperature (>50^{o} C) and air dryness but sensitive to frost when it is young (Bargal and Bargali, 2009). It is widely spread in growth in subtropical and tropical Africa, from Egypt to Mauritania southwards to South Africa, and in Asia eastwards to Pakistan and India (Bennison and Paterson, 1994).

Acacia nilotica also known as Bagaruwa in Hausa language has been designated and used as medicinal plants in parts of northern Nigeria, West Africa, North Africa and other parts of the world. The plant is used to treat infections such as diarrhea, dysentery, leprosy, cancer, ulcer, and diabetes (Mukhtar and Tukur, 2000).

This research study is based on the use of extracts of parts of *Acacia nilotica* tree such as leaves and stem bark collected from Birnin Kebbi, northwestern Nigeria (Fig. 1). This was carried out through the use of extractors such as ethanol crude and N-Hexane to determine their efficacy on bacteria isolates such as *Staphylococcus aureus* and *Escherichia coli.*

MATERIALS AND METHODS

Plant sample collection and Preparation

The leaves and stem bark of *Acacia nilotica* were collected from the Old Town area of Birnin Kebbi, Kebbi State, Nigeria. The freshly collected leaves, and stem bark of *Acacia nilotica* were washed thoroughly with running tap water and then dried in the laboratory at room temperature for a period of one week until they were completely dried up. They were then pounded into powdered form.

Preparation of Aqueous extracts

The extraction of the leaves and stem bark of *Acacia nilotica* sample was done in accordance to the method proposed by Ogunjobi and Elizabeth (2011)**.** 10g of each powdered extracts of leaves and stem bark of *Acacia nilotica* were weighed, soaked in 100cm3 of ethanol for 72 hours and the mixtures were agitated at intervals for proper mixing, whereby, the mixtures were filtered using whattman No. 1 filter paper and the filtrate was concentrated using water bath at 40^{0}C to obtain the crude ethanol extract. 2.0g of the prepared powder extracts of the leaves and stem bark of *Acacia nilotica* were measured separately using weighing balance and poured into 10ml of distilled water. The same procedure was repeated for 4.0g, and 6.0g, to obtain various varying concentrations of 20mg/ml, 40mg/ml, and 60mg/ml respectively for each of the extracts.

Test organisms

The micro-organisms used for this research work were *Staphylococus aureus* and *Escherichia coli* .These clinical isolates were obtained from Microbiology Laboratory of Federal Medical Center, Birnin Kebbi, Kebbi State, Nigeria after seeking for ethical approval from the hospital management.

Biochemical tests

The following biochemical tests were carried out as described by Oyeleke and Manga (2008).

Catalase Test: Colonies were picked using sterile wire loop and place in the centre of clean glass slides; three (3) drops of hydrogen peroxide were added. Thereafter, presence of bubbles indicates positive while absence of bubbles indicate negative.

Coagulase Test: Two colonies were emulsified in 0.5ml of saline contained in a clean stereological tube; 1ml of attracted human plasma was checked after 2 hours and 4 hours of incubation for signs of increased viscosity or complete clothing of the plasma in the absence of increased viscosity of clothing of the plasma after 4hours of incubation. The tube was left over night in the incubation at **3^{0}c** and observed the following day**.** Increased viscosity or complete clothing indicates a positive tube coagulase test while absence of viscosity or clothing indicates a negative coagulase test.

Citrate Test: Simmons citrate agar was inoculated with the isolate, using sterile wire loop and incubate for 47 hours at 37^0C; a deep blue color indicate a positive result.
Motility Test: This contains low concentration of agar (0.2-5%) and motion organisms are able to move away from the line of inoculation through sloppy agar.
Indole Test: The organisms were grown in 5ml of peptone waters, nutrients broth for 24 hours. After 4 hours of incubation, kowac's indole reagent was added (eight drops). It was shaken gently. A positive reaction is indicated by the development of a red color in the reagent layer above the broth within 1 minute. In negative, the indole reagent is yellow color.
Urease Test: The prepared urease medium was inoculated with the test isolated and incubated at 36^0C for 4-48 hours. A bright pink or red color indicated a positive reaction.
Triple Sugar Iron test (TSI): colonies from fresh culture were picked and streaked on the surface of the slope and stabled on the bottom by using sterile wire loop and incubated at 37^0C for 48 hours. Tripe sugar iron media are composite media and several reaction can be read each after 24 hours incubation. Gas information is determined by the appearance of one or several bubbles. These can results in crack in the butt or the butt may be pushed from the bottom.
Formation of hydrogen sulphate is determined by the blackening at the slant butt junction. Glucose information is indicated by butt becoming yellow. In addition to glucose formation, lactose or sucrose or both sugar are fermented in TSI both the butt and the start would become yellow. In TSI, it means that either glucose or lactose has been fermented or glucose has been fermented or glucose, lactose and sucrose have been fermented.
Methyl Red Test (MR): Colonies from culture were inoculated and the period of incubation shows positive result as indicated by bright red color while negative test shows yellow color.
Spore Detected: Smear was prepared and flood with malachite green and brought to steaming. It was allowed to remain for 2 to 3 minutes and washed with tap water. Satranin solution was applied and allowed to remain for 30 seconds; it was washed, dried and examined under the oil immersion objective, the spore stain green.
Voges Proskauer (VP): The prepare MRVP medium inoculation with test isolate and incubated. Five drops of 40% potassium hydroxide (KOH) was added followed by 15 drops at 5% Naphthol in ethanol. It was shake and the cap of the tubes loosed and placed in a sloppy position. The development of a red color indicated VP positive while red color change indicated VP negative (Oyeleke and Manga, 2008).

Antibacterial activity

Disc diffusion techniques was used to determine the antibacterial activity of leaves and stem of *Acacia nilotica* . Nutrient agar was prepared and poured into a plate, Agar wells were prepared using sterilized syringe. The selected strains of bacteria were inoculated into each plate and the concentrations of each of the prepared powdered extracts of leaves and stem bark of *Acacia nilotica* 20mg/ml, 40mg/ml, and 60mg/ml respectively was added to each well in the plates, and the plates were then incubated at 37˚C for 24hours. The diameter of the zone inhibition was measured in millimeters and the results were recorded.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bacteria Concentration (MBC) of the crude extracts

The Minimum inhibitory concentration and Minimum bacteriocidal concentration of the extracts on the test bacteria- *Staphylococus aureus* and *Escherichia coli* was determined according to the method proposed by Samie *et al*., (2005) and Omori *et al*., (2012).
Twelve sterile test tubes were used and 1ml of sterile nutrient broth was dispensed from test tube 2 to test tube 12, a stoke solution of each of the extract leave and stem bark of *Acacia nilotica* was prepared, 400mg of each crude extract was dissolved into 2ml of distilled water, 1ml of the stoke solution were dispensed aseptically into tube 1 and 1ml into tube 2 and from the contents of test tube 2 a serial dilution was performed using 1ml transfer to tube 10, leaving tubes 11 and 12.
1ml was taken out from tube 10 and discard, 1:100 (10^{-2}) broth culture of *Staphylococus aureus*, and *Escherichia coli* were prepared separately and 1ml of the prepared broth culture was dispensed into each test tubes with exception of test tube 11 and 1ml of sterile nutrient broth was added to test tube 11, and were then incubated at 37^oC for 24 hours.

After 24 hours, the test tubes were examined for turbidity in order to determine the MIC and MBC. The MIC was the concentration in the tube that fails to show evidence of growth (turbidity), just immediately after the last one that shows growth. MBC were the two test tubes before the MIC test tube, which were cultured on nutrient agar. The absent of growth after incubation indicate a positive result for MBC.

RESULTS AND DISCUSSION

Morphology and Biochemical Characteristics of Isolates

In order to ascertain the bacteria isolates, the bacteria were re-characterized based on biochemical morphological characteristics (Oyeleke and Manga, 2008). Distinct biochemical tests such as catalase, coagulase, motility, indole, urease, citrate, oxidase, methyl red and Voge's Proskaver used on bacteria isolates were carried out in order to differentiate and identify the *Staphylococcus aureus* from the *Escherichia coli*. The positive and negative indications on the respective morphological characteristic distinguish the bacteria type. The results show that the test criteria such as catalase, coagulase, methyl red are positive while motility indole, urease, citrate, oxidase and voge's proskauer are negative on *Staphylococcus aureus*. On the contrary, citrate, oxidase, and voge's proskauer are positive while catalase, coagulase, motility indole, urease and methyl red are negative on *E. coli*

In Vitro Antibacterial Effect (Ethanol Extract)

Ethanol crude extracts of leaves and stem bark from *Acacia nilotica* tree were introduced at different concentrations (mg/ml) at 10, 50, 100, 150 and 200 mg/ml on bacterial isolates of *Staphylococcus aureus* and *Escherichia coli* in order to determine the values of inhibition using Ciprofloxacin tablet (200mg/ml) as control drug (Table 1).

Table 1. Antibacterial activity of ethanol crude extracts of *Acacia nilotica* obtained against the test bacterial.

		Zone of Inhibition in (mm)/Bacterial Isolates	
	Conc Extracts (mg/ml)	***Staphylococcus aureus***	***Escherichia coli***
Leave	10	9	7
	50	10	9
	100	8	10
	150	9	7
	200	12	15
Stem Bark	10	7	0
	50	8	7
	100	10	8
	150	12	7
	200	10	8
Ciprofloxacin Tab (200mg/ml) control		13	12

The result shows that for leave extract at 200 mg/ml, the inhibition factor is 12.0 mm compared to 13.0 mm of Ciprofloxacin tablet control for *Staphelococcus aureus* while in the case of *Escherichia coli* the experimental inhibition factor is 15.0 mm at 200 mg/ml compared to 12.0 mm for Ciprofloxacin tablet (control) of same concentration value (Table 1). This implies that the leave extract is effective for the control of *Staphylococcus aureus* infection while the efficacy of the leave on *E. coli* is very effective and more efficient in the control of *E. coli* related diseases compared to Ciprofloxacin tablet used as control drug (Figure 1). The efficacy of the stem bark extract was tested on the isolated *Staphylococcus aureus* and *E. coli* at different concentration (Table 2). The result suggests that the application of the stem bark extract at 150 mg/ml is effective on *Staphylococcus aureus* at 12.0 mm inhibition factor compared to 13.0 mm for the control tablet (Figure 2).

However, the effect of the stem bark on *E. coli* is very poor with highest inhibition factor of 8.0 mm at both 100 and 200 mg/ml compared to 12.0 mm obtained for control drug. It is suggested that the application of the stem bark at 150 mg/ml should be limited only for the treatment of *Staphylococcus aureus* related diseases.

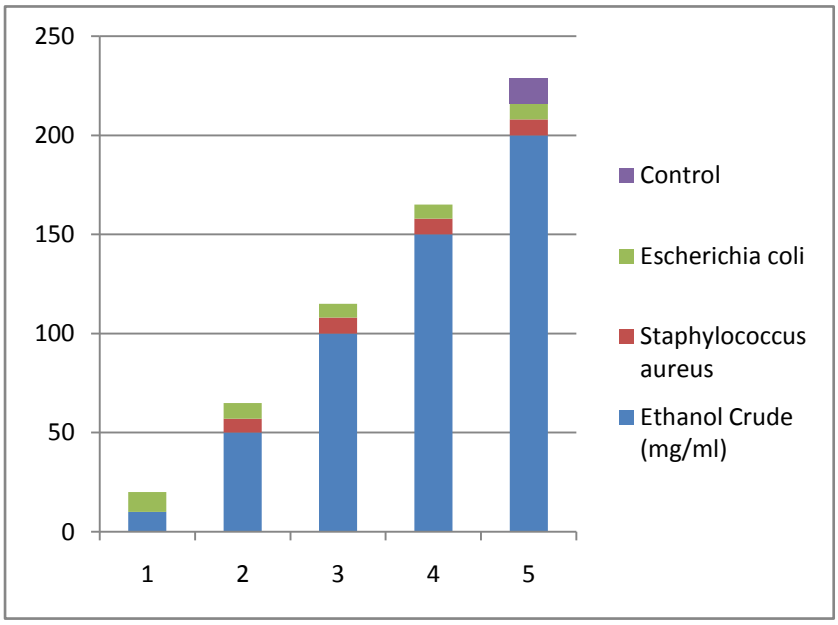

Figure 1. Comparative effect of *Acacia nilotica* leave extract on *Staphylococcus aures* and *Escherichia coli* of different concentration to Ciprofloxacin Tablet (200mg/ml) as control.

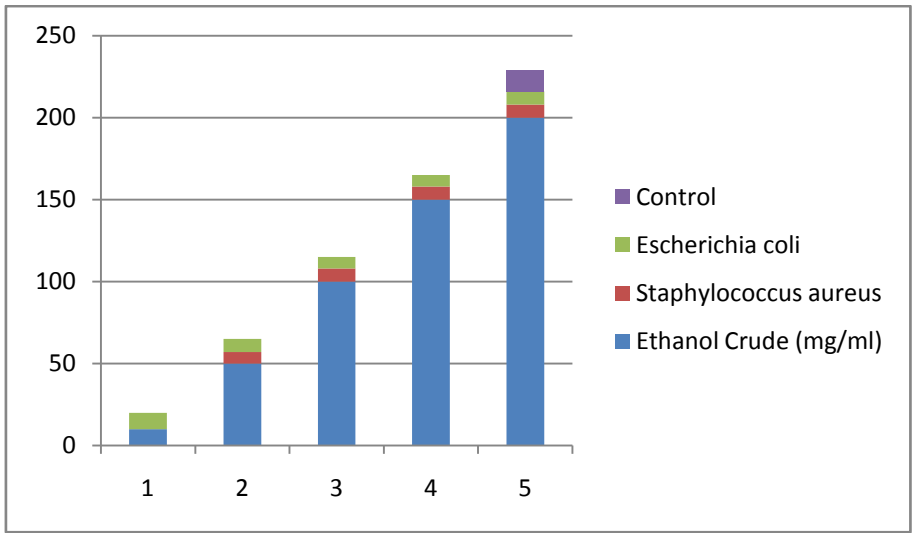

Figure 2. Comparative effect of *Acacia nilotica* stem bark extract on *Stephylococcus aureus* and *Escherichia coli* of different concentration to Ciprofloxacin Tablet (200mg/ml) as control.

In general, the results clearly indicate that the application of *Acacia nilotica* extracts is more effective against Gram-positive bacteria like *Staphylococcus aureus* in comparison to Gram-negative bacteria like *E. coli*.

In Vitro Antibacterial Effect (N-Hexane Extract)

Another form of extraction medium used is N-Hexane crude as an extract on the same Acacia nilotica leaves and stem bark on isolated bacteria such as *Staphylococcus aureus* and *Escherichia coli*. The details of the inhibition values on the bacteria isolates are presented in Table 2 below.

Table 2. Antibacterial activity of N-Hexane crude extracts of *Acacia nilotica* obtained against the test bacterial.

		Zone of Inhibition in (mm)/Bacterial Isolates	
	Conc Extracts (mg/ml)	***Staphylococcus aureus***	***Escherichia coli***
Leave	10	0	10
	50	7	8
	100	8	7
	150	8	7
	200	8	8
Stem Bark	10	0	6
	50	0	6
	100	7	7
	150	7	7
	200	8	6
Ciprofloxacin Tab (200mg/ml) control		11	12

The N-Hexane extract on leaves of *Acacia nilotica* on bacteria isolates reveals zone of inhibition (mm) for *Staphylococcus aureus* to range from 0.0-8.0 mm for 10.0 and 200 mg/ml respectively (Table 2). A comparison with *Escherichia coli* shows that the inhibition value (mm) ranges from 7.0 mm (for 100 and 150 mg/ml) to 10.0 mm at 10.0 mg/ml (Figure4).

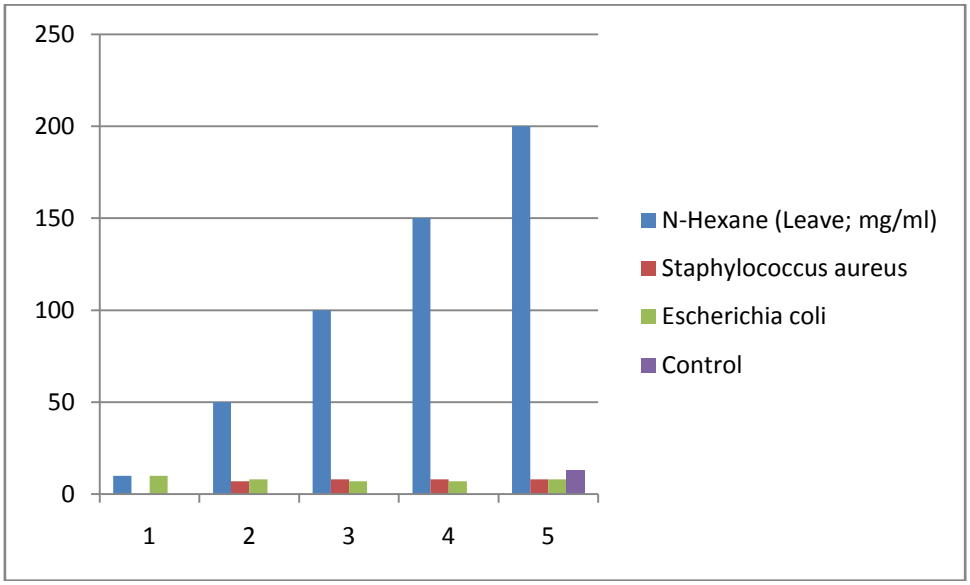

Figure 3. Comparative effect of Acacia nilotica leave extract using N-Hexane on *Staphylococcus aureus* and *Escherichia coli* of different concentration to Ciprofloxacin Tablet (200mg/ml) as control.

The values obtained for the bacteria are very low compared with the values obtained on Ciprofloxacin tablet (control) with 11.0 and 12.0 mm inhibition factors for *Staphylococcus eureus* and *Escherichia coli* respectively. It is observed that the inhibition value (mm) is not high enough; this suggests that N-Hexane leave extract is not effective enough for the control of both *Staphylococcus aureus* and *Escherichia coli* form of diseases.

Stem bark extract using N-Hexane shows values ranging from 0.0-8.0 mm for *Staphylococcus aureus*, while they vary from 6.0-7.0 mm for *Escherichia coli* (Table 3). This is graphically presented in Figure 5.

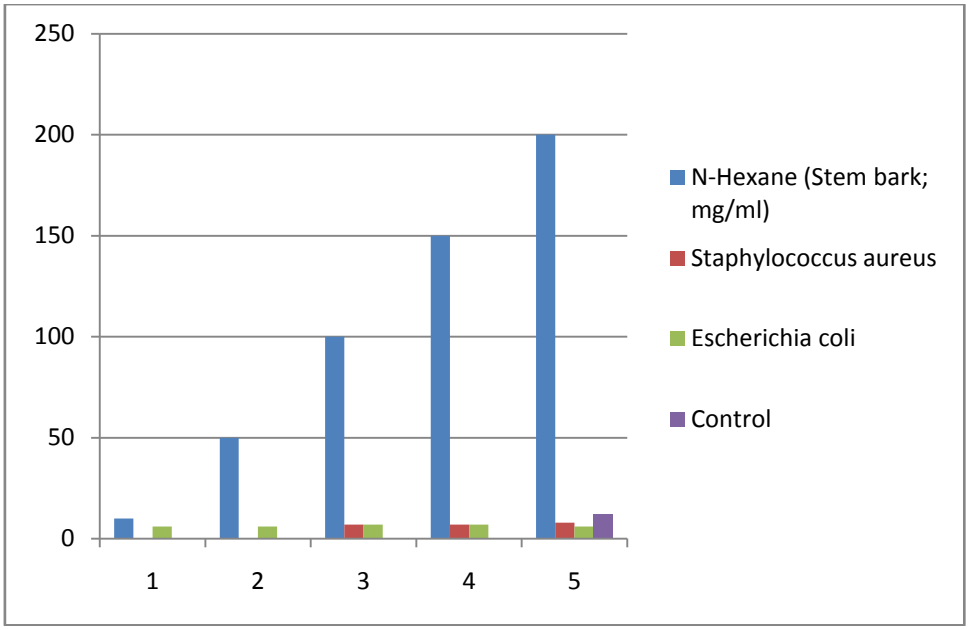

Figure 4. Comparative effect of Acacia nilotica stem bark extract using N-Hexane on *Staphylococcus eureus* and *Escherichia coli* of different concentration to Ciprofloxacin Tablet (200mg/ml) as control.

The contrast in inhibition values when compared with Ciprofloxacin tablet (control) values of 11.0 and 12.0 mm for *Staphylococcus aureus* and *Escherichia coli* suggests that the extract do not have effective positive impact on the control of *Staphylococcus aureus* and *Escherichia coli* related infections (Figure 5).

Generally speaking, the study compares the two extraction methods and evaluates the application of extracts through the use of ethanol crude extractor impact on leaves and stem bark to be more effective in the control of *Staphylococcus aureus* and *Escherichia coli* than extracts obtained from N-Hexane which has proven ineffective This assertion is compared with the work of Kalaivi and Mathew (2009) which suggests that the ethanol extracts are rich in phonolic and flavonoid contents which have potent antioxidant activity and were significant in comparison with all the positive controls used in this study. The possible antioxidant mechanism of the ethanol extract may be due to its hydrogen or electron donating and direct free radicals scavenging properties (Kalaivi and Mathew, 2009).

Determination of MIC and MBC (Ethanol Extract)

In this study the determination of the MIC and MBC is to serve two purposes: (1) to complement the earlier study on the zone of inhibition exhibited by the *Staphylococcus aureus* and *Escherichia coli* (2) to corroborate the results obtained and suggestions made on the potency of the leaves and stem bark extract of *Acacia nilotica* at various concentrations compared with the Ciprofloxacin tablet which serves as control drug. Therefore, the determination of the MIC and MBC test was carried out on *Staphylococcus aureus and E. coli* based on two different extractors – ethanol crude and N-Hexane crude on the leaves and stem bark of *Acacia nilotica*.

The minimum bactericidal concentration (MBC) is the lowest concentration of antibacterial agent required to kill a particular bacterium (Amyes *et al.,* 1996). This was determined through the method proposed by Samie *et al.,* (2005) and Omori *et al.,* (2012). The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculums by ≥ 99 .9 %. The MBC is complementary to the MIC, when the lowest level of antibacterial agent that resulted in microbial death. However, antibacterial agents are usually regarded as bactericidal if the MBC is not more than four times the MIC (French, 2006).

The results of the tests on both MIC and MBC based on the extracts of leaves and stem bark of *Acacia nilotica* using ethanol and N-Hexane against *Staphylococcus aureus* and *E. coli* are presented in Tables 4 and 5. All the extracts showed varying degree of antibacterial activity against the organisms (Tables 4 and 5) with some plant extracts from the leaves extracts of the ethanol crude showing strong antibacterial activity against *Staphylococcus aureus* and *E. coli*. The ethanol leave extract showed strong MIC value of 12.5 mg/ml and MBC value of 100 mg/ml for *Staphylococcus aureus*; whereas, the MIC for *E. coli* is 6.25 mg/ml and MBC of 50 mg/ml (Table 4). The MIC of stem bark extract for *Staphylococcus aureus* and *E. coli* is the same with value of 12.5 mg/ml and same value for MBC at 100 mg/ml.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) concentrations (mg/ml) produced by Ethanol crude extracts of *Acacia nilotica* against selected bacteria test cultures.

Extract (Mg/ml)	TB	200	100	50	25	12.5	6.26	3.125	1.5625	0.78125	0.390625	MIC	MBC
Leave	SA	-	-	-	-	-	+	+	+	+	+	12.5	100
	EC	-	-	-	-	-	-	+	+	+	+	6.25	50
Stem bark	SA	-	-	-	-	-	+	+	+	+	+	12.5	100
	EC	-	-	-	-	-	+	+	+	+	+	12.5	100

Key: TB**=** Test Bacteria, MIC= Minimum inhibitory concentration, MBC= Minimum bacteriocidal concentration, SA= *Staphylococcus aureus*, EC= *Escherichia coli*, Mg/ml= Milligram/ml**,** - = Absent, += Trace

Determination of MIC and MBC (N-Hexane)

In comparison, the MIC and MBC obtained for leaves and stem bark extracts from *Acacia nilotica* using N-Hexane extractor is presented in Table 5. For the N-Hexane leave extract, the MIC and MBC are 25 and 200 mg/ml respectively; for stem bark extract, the MIC and MBC concentration values for *Staphylococcus aureus* is 100 mg/l but not showing any effectiveness at 200 mg/ml, while for *E.coli* the values are 25 and 200 mg/ml respectively. For N-Hexane extracts the most potent extract is the leave which has concentration value of 12.5 mg/ml and 100 mg/ml respectively for E. coli.

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) concentrations (mg/ml) produced by N-Hexane crude extracts of Acacia nilotica against selected bacteria test cultures.

Extract (Mg/ml)	TB	200	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625	MIC	MBC
Leave	SA	-	-	-	-	+	+	+	+	+	+	25	200
	EC	-	-	-	-	-	+	+	+	+	+	12.5	100
Stem bark	SA	-	-	+	+	+	+	+	+	+	+	100	-
	EC	-	-	-	-	+	+	+	+	+	+	25	200

Key: TB**=** Test Bacteria**,** MIC= Minimum inhibitory concentration**,** MBC= Minimum bacteriocidal concentration, SA= *Staphylococcus aureus*, EC= *Escherichia coli*, Mg/ml= Milligram/ml**,** - = Absent**,** + = Trace.

The leave extract based on ethanol crude shows high potency on *E. coli* with MIC and MBC values of 6.25 and 50 mg/ml respectively. However, MIC of 12.5 mg/ml is commonly exhibited for the two extracts in which MBC of 100 mg/ml is also common for both *Staphylococcus aureus* and *E. coli* in this study.

Comparatively, The MIC and MBC values for ethanol leaves and stem bark extracts vary from 6.25 to 12.5 mg/ml (MIC) and 50 to 100 mg/ml for MBC test. Whereas N-Hexane leaves and stem bark extracts values for both *Staph. aureus* and *E. coli* varies from 12.5 to 200 mg/ml for MIC and MBC with concentration values range from 100 to ≥ 200 mg/ml.

The result obtained on MIC and MBC corroborate the assertion that ethanol extract on leaves and stem bark of *Acacia nilotical* show strong microbial activity on *Staph. aureus* and *E. coli* than extracts obtained by N-Hexane. The conclusions drawn are similar to the work of Marita *et al.,* (2011) and Banso, (2008). *Acacia nilotica* was found to give potent antimicrobial extract. It has been reported as antimicrobial, antihyperglysemic and antiplasmodial properties (Satohy *et al.,* 1995; El-Tahir *et al.,* 1996).

CONCLUSION

The plant extracts exhibited antimicrobial activity against the test microorganisms such as *Staphylococcus aureus* and *Escherichia coli*. The minimum inhibitory concentration for ethanol extract of 200 mg/ml on *Staphylococcus aureus* and *E. coli* are 12.0 and 15.0 mg/ml respectively. Acacia nilotica ethanol extracts are more potent than N-Hexane extracts whereby ethanol leave extract shows strong antimicrobial activity The result obtained on MIC and MBC corroborate the assertion that ethanol extract on leaves and stem bark of *Acacia nilotical* show strong microbial activity on *Staph. aureus* and *E. coli* than extracts obtained by N-Hexane.

ACKNOWLEDGEMENTS

The author is grateful to Dr Ola-Buraimo for his support in carrying out this study. Gratitude is also extended to Waziru Umaru Federal Polytechnic Birnin Kebbi and Department of Microbiology, Kebbi State University of Science and Technology, Aliero, for their kind gesture in permitting me to use their laboratory facilities. The author is also grateful to the authority of Federal Medical Center, Birnin Kebbi, Nigeria for collection of bacterial cultures of *Staphylococcus aureus* and *Escherichia coli.*

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