Phytochemical and Antibacterial Properties of *Moringa oleifera* leaf extracts on *Escherichia coli* and *Staphylococcus aureus*

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Abstract: This present study was aimed to investigate the phytochemical constituents and antimicrobial properties of ethanolic and aqueous extracts of *M. oleifera* leaf on *Staphylococcus aureus* and *Escherichia coli*. The phytochemical analysis was carried out using standard methods. The antibacterial activity of the plant extracts was determined using agar well diffusion method. The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts on the test isolates were determined using micro broth dilution method. Phytochemical analysis showed that terpenoids, phenols, flavonoids, glycosides, tannin, saponin, alkaloids, steroids and anthraquinolones were present in varying concentrations of the different extracts. The antibacterial activities of the ethanol and aqueous extracts of *M. oleifera* leaf at concentrations of 200, 100, 50, 25,12.5mg/ml showed the zone diameter of inhibition for *S. aureus* in response to the different extracts ranged between 9mm to 20mm while that of *E. coli* was between 7mm to 19mm. The MIC of aqueous and ethanol leaf extract on *S. aureus* is 25mg/ml and 12.5mg/ml respectively while the MIC of aqueous and ethanol leaf extract on *E. coli* is 12.5mg/ml and 6.25mg/ml respectively. The MBC of aqueous and ethanol leaf extract on *S. aureus* is 25mg/ml and 12.5mg/ml respectively while the MBC of aqueous and ethanol leaf extract on *E. coli* is 50mg/ml and 25mg/ml respectively. It can be concluded that some secondary metabolites present in *Moringa oleifera* leaf may be responsible for the inhibition of the bacteria observed in this study; and the ethanol extract of *M. oleifera* leaf possesses more antimicrobial activity (10 – 20mm) in a concentration dependent manner than the aqueous extract (9 - 15mm). This could justify its use as an antimicrobial agent. Therefore, *M. oleifera leaf* could be a promising natural antimicrobial agent with potential applications in pharmaceutical industries for controlling the pathogenic bacteria used in this work.
Key words: *Moringa oleifera,* Ethanol, Aqueous, *Staphylococcus aureus, Escherichia coli*

INTRODUCTION

Moringa (*Moringa oleifera* Lam.), one of the species of family *Moringaceae,* is a multipurpose tropical tree that can grow to a height of 10-12 m. It is mainly used for food and has numerous industrial, medicinal and agricultural uses, including animal feeding. The plant is referred to a number of names such as horseradish tree, drumstick tree, ben oil tree, miracle tree, and “Mothers best friend” (Julia-coppin, 2008). It has been dubbed the "miracle tree" or "tree of life" by the media (Kalibbala *et al.*, 2009; Iqbal *et al.,* 2006; Atawodi *et al*., 2010). It is also well known in all parts of Nigeria, in the North, the Hausas refer to it as Zogale or Bagaruwarmakka, in the south-west; the Yorubas call it Ewe Igbale or Idagbomonoye Moringa is mainly grown for its leaves in Africa, and much appreciated for its pods in and in the south-east, the Igbos call it Ikwaoyibo (Thilza *et al*., 2010).

Moringa originated from the southern hills of the Himalayas and was introduced in many tropical and subtropical areas, largely by migrant Asian populations (Kalibbala *et al*., 2009). Moringa is now naturalized in most African countries, in the Caribbean Islands and in Central America. Moringa is an important crop in India, Ethiopia, the Philippines and Sudan (Atawodi *et al*., 2010).

All parts of moringa are consumed as food. The plant produces leave during the dry season and during times of drought, and is an excellent source of green vegetable when little other food is available (Atawodi *et al*., 2010).

Asia (Iqbal *et al.,* 2006). Leaves, pods, roots and flowers can be cooked as vegetables.

The leaves are very nutritious and rich in protein, vitamins A, B and C, and minerals. They are highly recommended for pregnant and nursing mothers as well as young children (Kalibbala *et al*., 2009). They are generally cooked (boiled, pan-fried) and eaten like spinach or put in soups and sauces. Moringa leaves are also eaten as a salad or dried and ground to make a very nutritious leaf powder (Kalibbala *et al*., 2009). Moringa leaf powder is used to aid the restoration of infants suffering from malnutrition. Moringa flowers are used to make tea, added into sauces or made into a paste and fried (Kalibbala *et al*., 2009).

Different studies from around the world has revealed some medicinal properties of moringa which includes its use in the treatment of edema, skin infections and sores; protection of the liver against damage caused by anti-tubercular drugs ,treatment of stomach disorders such as constipation, gastritis, and ulcerative colitis; and prevention of cancer (Caceres *et al*., 1992; Farooq *et al*., 2007; Raheela *et al*., 2008). It is also known in treating mood disorders, ulcers, diabetics, asthma, reducing high blood pressure (Rebecca *et al*., 2006; Abalaka *et al*., 2012). Its antioxidants properties might help prevent cardiac damage and has also been shown to maintain a healthy heart while its antibacterial, antifungal, and antimicrobial properties might help to combat infections caused by *Salmonella*, *Rhizopus*, and *E. coli* (Mahesh and Satish, 2008; Moyo *et al*., 2012).

The plant's usefulness as an antimicrobial agent has also been evaluated in a number of studies (Rani *et al*., 2018; Wang *et al*., 2016). While some have found that different preparations of the plant have broad-spectrum antimicrobial activity (Brilhante *et al*., 2017; Sayeed *et al*., 2012; Rani *et al*., 2018; Rahman *et al*., 2009), others report the plant as active only against Gram-positive organisms (Kheir *et al*., 2015), and some have suggested that its efficacy is mixed and species dependent (Marrufo *et al*., 2013). There are several potential explanations for such variation. Namely, the methods used for harvesting, drying, and preparing the plant have varied greatly from study to study (Kheir *et al.,* 2015; Sayeed *et al*., 2012).

In addition, it is important to bear in mind that the phytochemical contents present in leaves of *M. oleifera* depend on several factors such as geographical area where the plant is cultivated, type of soil, water and fertilizers, industrialization process, and storage conditions (Rani *et al.,* 2018). Taking these precedents into consideration, this study was aimed at investigating the phytochemical composition of the plant extract, examining antibacterial activity of the leaf extract (Moringa leaf) on the pathogenic organism (which are *Staphylococcus aureus and Escherichia coli*), and determining the appropriate concentration required to inhibit and kill the organisms in question through determination of minimum inhibiting concentration (MIC) and minimum bactericidal concentration (MBC) respectively.

MATERIALS AND METHODS

Sample collection

Fresh leaves of *M. oleifera* were purchased from Eke Agbani market in Enugu State, Nigeria and was authenticated by a Botanist at the Nnamdi Azikiwe University Herbarium with a voucher number of NAUH 35A**.**

The preparation was done at the Microbiology laboratory of Spiritan University Nneochi Abia State. The fresh leaves of *M. oleifera* were washed thoroughly with clean water, cut into bits and placed in a washed tray to air dry (Akinnibosun, 2009). With the help of a pestle and mortar, it was crushed to coarse powder. Different containers were used to label and store the powdered form of the leaves.

Test organisms

Bacterial cultures of *Escherichia coli and Staphylococcus aureus* obtained from the laboratory section of the Department of Microbiology, Nmamdi Azikiwe University, Anambra State, Nigeria; were used as antimicrobial test organisms. Their identity was confirmed using cultural, morphological and biochemical test as previously described by standard methods (Cheesebrough, 2002). The bacterial isolates were maintained on nutrient agar slants at 4°C for future use.

Biochemical Identification of the Test Organism

Escherichia coli

The *E. coli* was placed on Eosine Methylene Blue (EMB) agar for 24 hours. A positive result for *E. coli* was indicated with colonies with green metallic sheen. The distinct colonies with metallic green sheen on EMB agar were pick and confirmed by streaking onto Chromagar *E. coli* medium (Oxoid, Basingstoke, UK). Colonies with a blue/violet appearance were selected and analyzed further by gram staining and biochemical tests (Cheesebrough, 2002).

Staphylococcus aureus

Staphylococcus aureus which showed positive result for catalase test was subcultured on blood agar and incubated at 37°C for 24 hours. Then, the single colonies were placed on Mannitol Salt Agar (MSA) for 24 hours. A positive result was indicated by smooth circular colonies with yellow color (Cheesebrough, 2002).

Standardization of the Tests Organism

The test organisms (*E. coli* and *S. aureus*) were standardized by the use of 24 hours old broth cultures prepared by inoculating the test organism into 5 ml of nutrient broth and the culture was incubated for 2 hours. The growth of the organism was indicated by the turbid change in color of the nutrient broth which was adjusted to match the color of the 0.5 McFarland turbidity equivalent standards (Cheesebrough, 2002). .

Preparation of ethanolic and aqueous extracts

The extracts were prepared according to the method described by Akinnibosun (2009).

Preparation of Aqueous extract

Ten grams of dried grinded leaf powder was dissolved in 100 ml of distilled water for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate collected was evaporated to dryness using a water bath. The extract was collected in fresh sterile universal bottles and stored in the refrigerator at 4°C until when required for use (Akinnibosun, 2009).

Preparation of ethanol extract

Ten grams of dried grinded leaf powder was suspended in 100 ml of 95% ethanol for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate was placed into evaporator to drive-off the solvent and stored at 4°C (Akinnibosun, 2009).

Extract Dilution

After preparation of the extract as described, the ethanol and aqueous extract were reconstituted using sterile distilled H_20 to obtain concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.13 mg/ml (Akinnibosun, 2009) .

Sterility test of leaf extract

The leaf extracts (ethanol and aqueous) was tested for growth of contaminants according to the methods of (Cheesebrough, 2002). Standard leaf extract (1 ml) was inoculated aseptically onto Nutrient Agar and incubated at 37°C for 24hrs. The plates were observed for sign of growth. No growth on the plates signified sterility of the extracts.

Phytochemical screening of *Moringa Oleifera* extracts

The sample was screened for the following compounds, alkaloids, saponins, tannins, flavonoids, glycosides, phlobatannin and anthraquinones etc. This was done following standard methods of (Trease and Evans, 1989).

Test for Tannins

Two (2) gram of each extract was dissolved in 10ml of distilled water in separate test tubes and 3 drops of 10% ferric chloride ($FeCl_3$) was added to 2ml of the solution. The occurrence of blackish-blue, green or

blackish green coloration indicates the presence of tannins.

Test for phlobatannins

About 0.2g of each extract was boiled with an equal volume of 1% Hcl, the deposition of a red precipitate indicate the presence of phlobatannins.

Test for saponins

About 0.1g of each extract was dissolved in 5ml of distilled water and shaken vigorously. The formation of fronthing bubbles which lasted for 10 minutes indicate the presence of saponin.

Test for alkaloids

About 0.5g of each extract was dissolved in 3 drops of Dragendoffs reagent. An orange precipitate indicates the presence of alkaloid.

Test for flavonoids

About 0.2g of each extract was dissolved in 2ml of sodium hydroxide solution. The occurrence of a yellow solution which disappears on addition of Hcl acid indicates the presence of flavonoids.

Test for glycoside

Half (0.5)g of each extract was dissolved in 3ml of Fehling solution. A brick red precipitate indicates the presence of glycosides.

Test for steroids

Five (5) drops of concentrated H_2S0_4 was added to 0.1g of each extract in test tube, a reddish brown coloration indicates the presence of steroids.

Terpenoids

Four milligrams (4mg) of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated sulphuric acid solution was added slowly and red violet color was observed for terpenoid.

Anthraquinone

To ten milligrams (10mg) of the dissolved extract, magnesium acetate solution was added. Pink colour developed indicates the presence of anthraquinone and no colour change indicates negative.

Antibacterial Assay

The agar-well diffusion technique was used to carry out the antibacterial assay of the plant extracts in comparison with standard antibiotic gentamicin (20 mg/ml) *in vitro* on the isolates according to the methods of NCCLS (2007). Pure culture of the bacteria was grown on nutrient agar. Three colonies of each organism were pick into the Mueller Hinton broth (Oxoid, England), incubated for 4 hours at 37°C and diluted with sterile saline to a density visually equivalent to McFarland Standard. Using a sterile 6 mm diameter cork borer, four wells were bored in the agar to which the two extracts of *M. oleifera* leaves were added, as well as the standard drug, gentamicin (GEN, 20 mg/ml) and sterile water separately, which served as the positive and negative controls respectively. The plates were placed on the bench for 30 minutes for pre-diffusion of the extract to occur. The plates were incubated at 37°C for 48 hours. The zones of inhibition were measured with the use of a metric rule.

Determination of Minimum Inhibitory Concentration (MIC)

The micro broth dilution method was employed in the determination of the Minimum inhibitory concentration (MIC) of *M. oleifera* extracts against the test organisms. To each 5ml of the various extracts in different test tubes was added 5ml of nutrient broth each and serially diluted out to various concentrations ranging from 200 to 3.13mg/ml. A loopful of each test organisms was inoculated into each of the test tubes and incubated at 37°C for 24 hours. The MIC was the lowest concentration of the leaf extracts that inhibited growth (Cheesebrough, 2002).

Determination of Minimum Bactericidal Concentration (MBC)

Briefly, 1ml bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and were sub-cultured onto nutrient agar and incubated at 37°C for 24hours.This was obtained by streaking out the samples from the MIC tubes that showed no visible growth on nutrient agar plates. The lowest concentration of the sample that showed no growth was noted and recorded as the minimum bactericidal concentration (Cheesebrough, 2002). .

RESULTS

Table 1 shows the Phytochemical components of ethanolic and aqueous extracts of *M. oleifera* leaf. Phenols, flavonoids and tanins were present in high amount in ethanol extracts and slight amount in aqueous extracts. Steroids, saponin and alkaloids were present in moderate amount on ethanol extracts and slight amount in aqueous amount. Lastly glycosides and anthraquinolones were present in slight amount in both ethanol and aqueos extracts while terpenoids were absent in both extracts.

Table 1: Phytochemical composition of *M. oleifera* leaf extracts

Phytochemical components	Ethanol extracts (EE)	Aqueous extracts (AE)
Phenols	+++	+
Flavonoids	+++	+
Steroids	++	+
Glycosides	+	+
Tanins	+++	+
Saponins	++	+
Alkaloids	++	+
Anthraquinolones	+	+
Phlobatanin	+	-
Terpenoids	+	+

KEY: - = Absence
+ = Slightly present
++ = Moderately present
+++ = Highly present

The antibacterial activities of ethanol and aqueous leaf extracts of *M. oleifera on S. aureus and E. coli* is found on table 2. The diameter zone of inhibition of *S. aureus* on aqueous extracts at concentrations of 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml are 17mm, 15mm, 12mm, 9mm, and 8mm respectively. While the diameter zone of inhibition of *S. aureus* on ethanol extracts at concentrations of 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml are 20mm, 18mm, 17mm, 13mm and 10mm respectively. The diameter zone of inhibition of E. *coli* on same concentrations of aqueous and ethanol extracts are found on table 2.

Table 2: Antibacterial activities of ethanol and aqueous leaf extract of *M. oleifera* leaf on *S. aureus* and *E. coli*

	Extract Concentrations (Zone diameter of inhibition in mm)							
Isolates	200	100	50	25	12.5	+C	-C	Extracts
S. aureus	17	15	12	9	8	18	0	AE
S. aureus	20	18	17	13	10	18	0	EE
E. coli	15	12	9	8	7	19	0	AE
E. coli	19	17	15	13	11	19	0	EE

Key: AE = Aqueous Extract
EE = Ethanolic Extract
+C = Positive control (gentamicin, 20 mg/ml)
-C = Negative control (sterile water)

The MIC and MBC of leaf extracts of *M. oleifera on S. aureus and E. coli* is found on table 3. The MIC and MBC of aqueous leaf extract on *S. aureus* are both 25mg/ml while the MIC and MBC of aqueous leaf extract on *E. coli* is 12.5 and 50mg/ml respectively. That of the ethanol extract on *S. aureus* and *E. coli* is found on table 3.

Table 3: Minimum Inhibitory and Bactericidal Concentration (MIC and MBC) of leaf extracts of *M. oleifera on S. aureus and E. coli*

	Concentration of Extracts (mg/ml)		
Isolates	MIC (mg/ml)	MBC (mg/ml)	Extracts
S. aureus	25	25	AE
S. aureus	12.5	12.5	EE
E. coli	12.5	50	AE
E. coli	6.25	25	EE

Key: AE = Aqueous Extract
EE = Ethanol Extract

DISCUSSION

Results from the phytochemical analysis confirms the presence of rich secondary metabolites such as steroids, alkaloids, terpenoids, glycosides, anthraquinolones, phenols, saponins and flavonoids in varying amounts in both aqueous and ethanol extract of *M. oleifera*. This was corroborated by the works of Daniyan *et al.* (2011) and Thilza *et al*. (2010), who also found similar compounds in *M. oleifera seeds and leaves respectively*. Antimicrobial effects of plant extracts have been attributed to the presence of these secondary metabolites (Oboh and Masodje, 2009). The presence of these metabolites in the investigated plant part account for its usefulness as an antimicrobial agent.

The absence of some phytochemicals might be due to differences in the polarity of the solvents, as the types of solvent used determined the kind of biologically active compounds that can be extracted from plant (Tiwari *et al.,* 2011).

It has been reported that different solvents have different extraction capabilities (Ashok *et al*., 2014; Srinivasan *et al*., 2001). Ashok *et al.* (2014) reported that the best way to extract broad spectrum antimicrobial compound from plant is by the use of ethanol solvents. The differences observed between antibacterial activities of the extracts could be explained by their abilitiy to dissolve in different solvent (Ashok *et al.*, 2014).

It was also shown that the ethanol extracts of *M. oleifera* inhibited the growth of both the *E coli and S. aureus* than the aqueous extract in a concentration dependent manner. The variation in the antibacterial activities is presumed to be due to difference in the quantity of compounds present in those plant extracts (Ezeifeka *et al*., 2004). Similar result was discovered in the work of (Abalaka *et al.*, 2012).

Result from this work showed a greater zone of inhibition produced by the ethanolic extracts (10 – 20mm) of *M. oleifera* at all concentrations used compared to that produced by the aqueous extract (9 - 15mm). This indicates the possibility that *M. oleifera* could serve as a better and alternative drug to treat infections caused by *S. aureus* and *E. coli* compared to most conventional antibiotics used presently in the world. Similar result was discovered in the work of (Njume *et al*., 2011).

The MIC obtained shows that different concentrations were effective against the two test organisms. The generally low MIC and MBC values of ethanol extracts against *S. aureus* and *E. coli* is an indication of their antibacterial potential (Elzein *et al.*, 2018). The different values obtained from the MBC of leaf extract on *E. coli* and *S. aureus* confirmed that both extracts have varying phytochemical properties, and hence exhibit different inhibitory effects and bactericidal effects on the test organisms but less when compared to standard used (Abubakar and Usman, 2016). This could be further explained by the differences in the chemical composition of these extracts (Bukar et al., 2010).

Similar results were obtained from the works of Thilza *et al.*, (2010) and Abalaka *et al.* (2012), but contrasted with the work of Bukar *et al.* (2010), who posited that *M. oleifera leaf* extracts exhibited bactericidal effects very well when compared to standard antibiotics.

CONCLUSIONS

From this study, it can be concluded that some secondary metabolites such as phenols, flavonoids, glycosides, tannin, saponin, alkaloids and anthraquinolones present in *Moringa oleifera leaf* may be responsible for the inhibition of the bacteria observed in this present study and could also justify its use as an antimicrobial agent.

It can also be concluded that the Ethanol extract of *M. oleifera* leaf possesses more antimicrobial activity (10 – 20mm) in a concentration dependent manner than the aqueous extract (9 - 15mm).

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