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Phytochemical analysis and antibacterial activities of various leaf extracts of *Ocimum gratissimum* and *Newbouldia laevis*

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Abstract

Leaves of *Ocimum gratissimum* and *Newbouldia laevis* were subjected to cold maceration in ethanol, ethyl acetate, and water for 24 hours. The extracts which on phytochemical screening showed the presence of alkaloids, flavonoids, saponins, tannins, glycosides, and phenols, were tested for antimicrobial activities. Various concentrations of the plant extracts (200, 100, and 50 mg/ml) were assayed for antibacterial activity using the agar well diffusion method. Two Gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and four Gram-negative (*Escherichia coli, Klebsiella aerogenes, Salmonella typhi,* and *Pseudomonas aeruginosa*) bacteria were used for the study. The ethanol extract of *O. gratissimum* showed the highest zone of inhibition against *E. coli* with 18mm at 200mg/ml concentration, followed by ethyl acetate and aqueous extracts with 15 mm and 8 mm, respectively. Ethanol extract of *N. laevis* displayed the best activities against *E.coli* and *S. aureus* with both displaying inhibition zones of 10 mm, followed by 8.5mm against *S. typhi*. The aqueous extract had the best activity against *K. aerogenes* (9.5 mm), followed by *S. aureus* (5 mm). The zones of inhibition against the test organisms decreased with decreasing concentration of the plant extracts. Overall, the ethanol extracts displayed the best activity, while the aqueous extracts had the least activity. The results obtained showed that leaf extracts of the two plants have broad-spectrum activities, and could serve as potential sources for the development of useful natural antibacterial agents.

Keywords: Phytochemical; Antibacterial; *Ocimum gratissimum*; *Newbouldia laevis*

1. Introduction

Infectious diseases rank among the leading cause of death in developing and tropical countries [1]. The frequency of life-threatening infections caused by pathogenic microorganisms is increasing worldwide and has been worsened by the development of microbial resistance to available antimicrobial agents [2]. The development of antimicrobials despite being one of the greatest accomplishments of modern medicine is greatly threatened by the continuous development of drug resistance by pathogenic microorganisms. This has led scientists to investigate other natural sources for novel and more active agents

From ancient times, medicinal plants have served as good sources for drug discovery and the focus for the exploration of new lead compounds against infectious pathogens [3]. Herbal drug technology has become very important as they are considered to have very less or no adverse and toxic effects [4]. The medicinal values of some plants lie in some bioactive substances that produce definite physiological actions *in vitro*, some of which include alkaloids, tannins, flavonoids, and phenolic compounds [5]. Medicinal plant-based drugs owe the advantages of being simple, effective, and exhibiting broad-spectrum activity [3].

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According to WHO, 88% of all countries are estimated to use traditional medicine, such as herbal medicines as the basic need for human primary health care, with a greater percentage in developing countries [6]. In Nigeria and Southern Nigeria in particular, medicinal plants are considered by several researchers to form an important component of the natural wealth of the country [3]. Therefore, many indigenous plants are used in traditional medicine to cure diseases and heal infections and injuries. These ancient and indigenous medicinal practices were discovered, but then could not be proven by scientific theories, despite having beneficial and efficient results when applied. On the other hand, several medicinal plants have been evaluated for possible antimicrobial activity, some of which have been shown to possess antibacterial, antifungal, antiprotozoal, and antiamoebal activities among others [7].

The use of plant extracts or chemicals derived from plants has been described as the bases for the development of modern medicines a natural blueprint for the development of new drugs [8]. In this study, we investigate the phytochemical and antibacterial activities of the leaf extracts of the ethnobotanical plants *Newbouldia laevis,* and *Ocimum gratissimum.*

2. Material and methods

2.1. Sample Collection

Fresh and healthy leaves of *Newbouldia laevis* and *Ocimum gratissimum* (Figure 1) were collected from a domestic neighborhood at Ugbawka, Nkanu East L.G.A. Enugu State, Nigeria. The plant leaves were properly identified by a taxonomist and processed at Project Development Institute (PRODA) Enugu, Nigeria.

A B

Figure 1 Pictorial representations of A: *Ocimum gratissimum*, and B: *Newbouldia laevis*

2.2. Preparation and Extraction of Plant Material

Leaf samples were properly washed and allowed to dry at room temperature for 2 weeks. After which, they were pulverized into fine powder with the aid of a mechanical pulverizer. Measured quantities of the powdered sample were extracted separately in aqueous, 99% ethanol, and ethyl acetate for 72hrs followed by periodic stirring. The extracts were filtered using cheese-cloth and the filtrate re-filtered using Whatman No. 42 (125mm) filter paper. The filtrates collected were lyophilized using a freeze-dryer and stored in an airtight container for further analysis.

2.3. Qualitative Phytochemical Analysis

Using the protocols outlined by Osibemhe and Onoagbe, [9] preliminary phytochemical analysis was performed to detect the presence of the following bioactive components in the crude aqueous, ethanol, and ethyl acetate extracts: flavonoids, alkaloids, saponins, tannins, steroids, phenols, alkaloids, and glycosides. Alkaloids were identified using Mayer's, Wagner's, and hydrochloric acid, whereas saponins were detected using the froth and emulsion tests. The presence of flavonoids were found using 10% NaOH, while the Salkowski test was used for steroids. Tannins and phenols were detected using the ferric chloride reagent, whereas glycosides were detected using the Legal's test.

2.4. Quantitative Phytochemical Analysis

2.4.1. Determination of Alkaloid

This was estimated using the Harborne, [10] method. 10 g of the sample was weighed into a 250 ml beaker and 100 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. The solution was then filtered and the extract was concentrated on a water-bath to one-quarter of the original volume. Concentrated ammonium hydroxide (NH_4OH) was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with 20 ml of 1% NH_4OH and then filtered. The residue if present is the alkaloid which is dried and weighed.

$$\text{Percentage of alkaloid} = \frac{W_2 - W_1}{W_3} \; x \; \frac{100}{1}$$

W_1 = Weight of the empty filter papers
W_2 = Weight of the alkaloids + filter papers
W_3 = Weight of the samples used

2.4.2. Determination of Glycosides using 3,5,-Dintrosalicylic Acid Method

Glycosides were quantitatively measured using spectophotometric method. 5 g of each sample was soaked with 100ml of distilled water for 3 hours in a beaker. The soaked samples were filtered using filter papers. 1ml of the extracts were pipetted into clean test tubes, followed by the addition of 2ml of 3,5, dinitrosalicylic acid. The reaction mixtures were allowed to stand in boiling water for 5 minutes, after which the spectrophotometric absorbance were measured at 470nm and glycoside determined using the glycoside graph.

$$\text{Glycoside (mg/g)} = \frac{absorbance \; x \; gradient \; factor \; x \; dilution \; factor}{weight \; of \; sample}$$

2.4.3. Determination of Saponin

The method described by Obadoni and Ochuko, [11] was adopted for the estimation of saponin. The samples were ground and 10 g of each put into a conical flask followed by the addition of 100 ml of 20% ethanol. They were then heated over a hot water bath for 4 hours with continuous stirring at about 55 ℃. The mixture was filtered and the residue re – extracted with another 40 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90^0C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n – butanol was added. The combined n – butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight. Saponin content was calculated as percentage:

$$\text{Percentage of Saponin} = \frac{W_2 - W_1}{W_3} \; x \; \frac{100}{1}$$

W_1 = Weight of the empty beakers
W_2 = Weight of the beakers + sample after drying
W_3 = Weight of the samples used (10g)

2.4.4. Determination of Tannin

Estimation of tannin was carried out following the spectrophotometric method described by Van-Burden and Robinson [12]. 0.5 g of the samples was mixed with 50 ml of distilled water in a 250ml conical flask and agitated on a rotary shaker for 1 hour. Then 5ml of each filtrate was pipetted out into different 50ml volumetric flask. After which 5ml of 0.1% tannic acid was added into different 50ml volumetric flask. Blank was prepared using 5ml of distilled water in 50ml volumetric flask. The five flasks were incubated for $1_{1/2}$ hour at 30 ℃. The absorbance was measured at 760nm using UV-spectrophotometer. After which tannin was determined using the tannin graph.

2.4.5. Determination of Flavonoid

Flavonoid was determined using the method described by Boham and Kocipai-Abyazan [13]. 10g of each sample was extracted repeatedly with 100 ml of 80% methanol 250ml conical flasks at room temperature. The whole solution was filtered through Whatman filter paper No 42 (125 mm). The filtrate was later transferred into clean weighed beakers and evaporated into dryness over a water bath and weighed to a constant weight. Flavonoid was calculated as difference in weight.

$$\text{Percentage of phenol} = \frac{W_2 - W_1}{W_3} \, x \, \frac{100}{1}$$

W_1 = Weight of the empty beakers
W_2 = Weight of the beakers + sample after drying
W_3 = Weight of the samples used (10g)

2.4.6. Modified Method for Determination of Total Phenol (Gravimtric Method)

10 g of each sample was soaked with 100ml of 2m hydrochloric (HCl) acid and incubated for 1 hour at 70 °C. After cooling, the filtrates were obtained followed by a wash with 30ml of diethyl ether. The mixtures were allowed to separate out into layers. The lower layers of each sample were removed into a beaker and the upper layers were poured into different weighed beakers. The washing process was repeated with 20ml of diethyl ether, and the filtrates heated to dryness. The phenol estimate was calculated as:

$$\text{Percentage of phenol} = \frac{W_2 - W_1}{W_3} \, x \, \frac{100}{1}$$

W_1 = Weight of the empty beakers
W_2 = Weight of the beakers + sample after drying
W_3 = Weight of the samples used (10g)

2.5. Antimicrobial Analysis

2.5.1. Test Organisms

Pure cultures of *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Klebsiella aerogenes*, *Salmonella typhi*, and *Pseudomonas aeruginosa* were used for the study. These organisms were obtained from the laboratory unit of the Department of Microbiology, University of Nigeria Nsukka. The organisms were further checked for purity before use, following Bergy's manual of bacteriology.

2.5.2. Antibacterial Susceptibility Assay

The plant extracts were tested for antibacterial activity against the test isolates using the Agar-well diffusion assay as described by Nwobodo *et al*. [14] By streaking with a sterilized swab stick, the McFarland's standardized (1.5 × 108 CFU/mL) isolates were inoculated on the surface of freshly gelled sterile Mueller Hinton Agar (MHA) plates. Using a sterile cork borer, 6mm diameter wells were aseptically bored and labeled on each agar plate. Different concentrations (200, 100 and 50 mg/ml) of the extracts were then added in fixed amounts (0.1 ml) into the corresponding wells of the plates. The plates were left on the bench for 40 minutes to allow for pre-diffusion of the extract, and then they were incubated for 24 hours at 37 °C. The resulting zone diameter of inhibition was measured with a millimeter-calibrated transparent ruler. The readings were interpreted to indicate the bacterial isolate in question's zone diameter of inhibition at that specific concentration. Ampicillin served as the positive control, while DMSO served as negative control.

2.6. Statistical Analysis

Statistical significance was determined by one-way variance analysis (ANOVA), with significant differences considered at $P<0.05$. Microsoft Excel (2016) software was used for analysis. All experiments were conducted in duplicate.

3. Results

3.1. Qualitative Phytochemical Analysis

The phytochemical analysis of *N. laevis* and *O. gratissimum* leaf extracts revealed the presence of alkaloids, flavonoids, saponin, tannins, glycosides, and phenol as shown in Table 1 below. Steroids were absent in all extracts of both plants studied, while Saponin was observed to be present only in the ethanol extract of *N. laevis* and ethyl acetate extract of *O. gratissimum*. Alkaloid, Tannin, and Phenol were present in all the extracts obtained.

Table 1 Qualitative Determination of active Phytochemicals in the Crude Extracts of *O. gratissimum* and *N. laevis* Leaves

Phytochemical	Water		Ethanol		Ethyl acetate	
	O. gratissimum	***N. laevis***	***O. gratissimum***	***N. laevis***	***O. gratissimum***	***N. laevis***
Saponins	_	_	_	+	+	_
Alkaloids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Flavonoids	+	+	_	_	_	_
Glycosides	+	+	+	+	_	_
Phenol	+	+	+	+	+	+
Steroids	_	_	_	_	_	_

Key: Present = +; Absent = -

3.2. Quantitative Phytochemical Analysis

The result of the qualitative phytochemical analysis is presented in Table 2. Flavonoid was observed to significantly ($p < 0.05$) have the height concentration among all phytochemicals identified with 5.56% and 4.90% in the extracts of *O. gratissimum* and *N. laevis* respectively. This was followed by saponins (1.42%) and alkaloids (1.42%) in *O. gratissimum* and *N. laevis* respectively. Glycoside (0.08%) was the lowest in *O. gratissimum*, while tannin (0.01%) was the least in *N. laevis*.

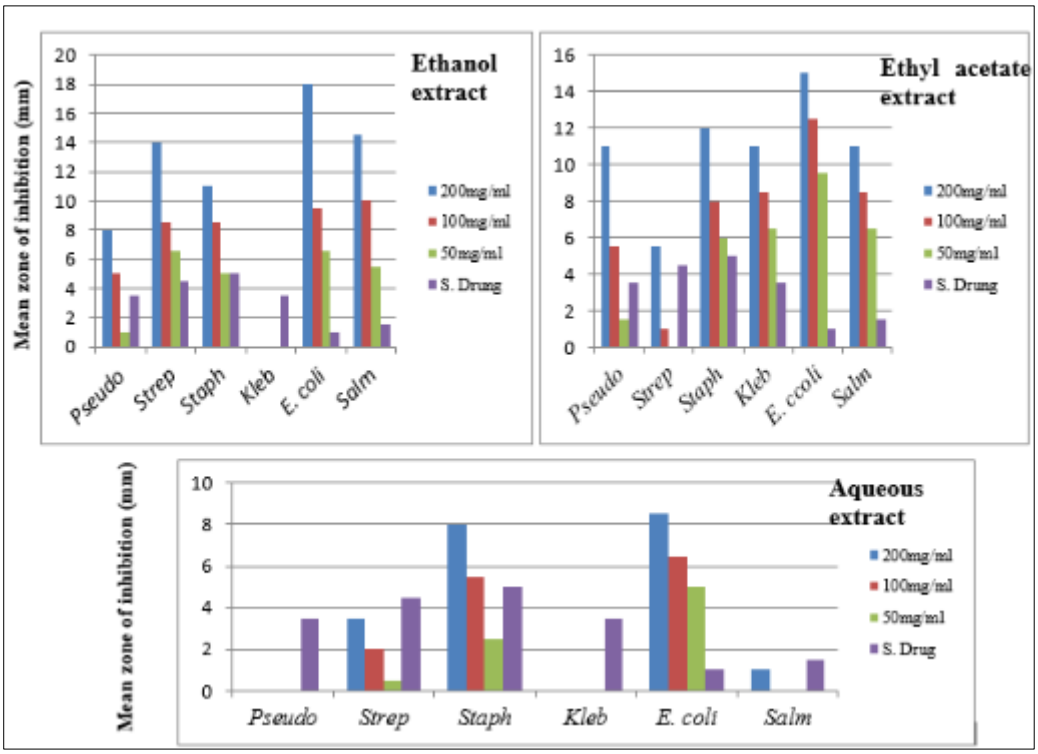
Table 2 Quantitative Analysis of Phytochemicals in the Crude Extracts of *O. gratissimum* and *N. laevis* Leaves

Parameter/ Sample	Alkaloid % w/w	Saponin % w/w	Flavonoid % w/w	Phenol % w/w	Glycoside mg/g	Tannin mg/g
O. gratissimum	1.00	1.42	5.56	0.81	0.08	0.11
N. laevis	1.42	0.98	4.90	0.56	0.12	0.01

3.3. Antibacterial Activity

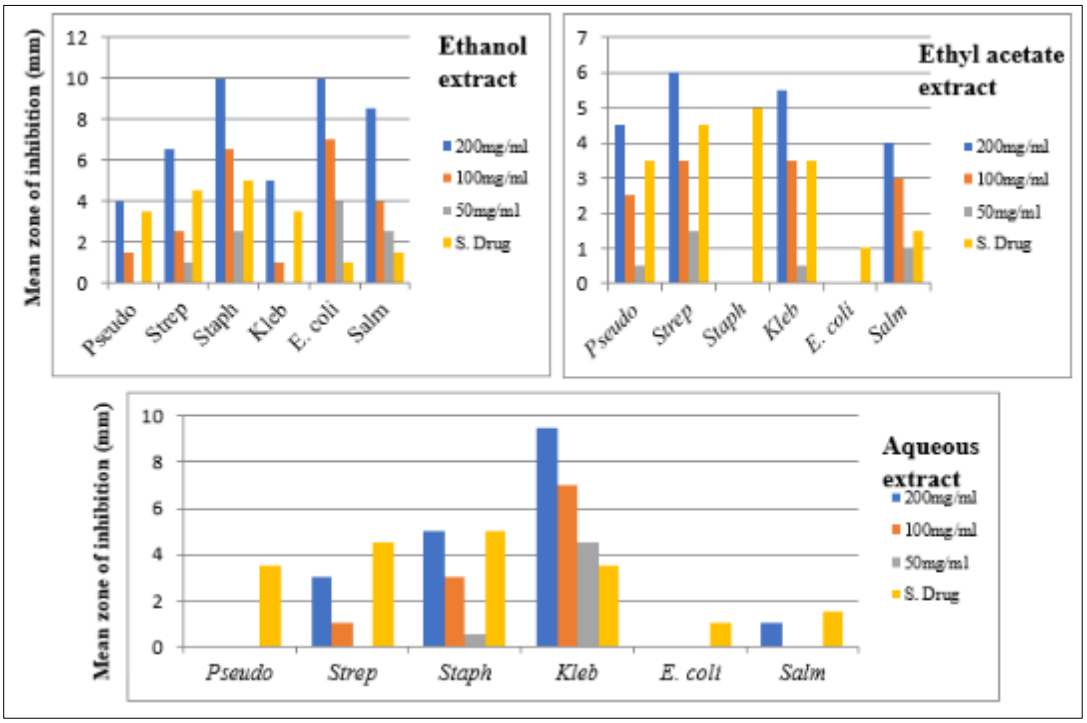
The antibacterial activities of the plant extracts against the tested organisms are represented by bar charts and presented in figures 2 and 3. The extracts were observed to display varying degrees of activity against the test isolates in a concentration-dependent manner. All extracts of *O. gratissimum* displayed the best activity against *E. coli*. At 200 mg/ml concentration, the ethanol extract displayed the highest inhibition with 18 mm, followed by ethyl acetate extract and aqueous with 15 mm and 8 mm, respectively. All crude extracts were observed to inhibit both Gram-positive and Gram-negative organisms. However, the aqueous extract had no effect against *P. aeruginosa* and *S. typhi*, while the ethanolic extract had no effect against *K. aerogenes*.

At 200 mg/ml, ethanol extract of *N. laevis* displayed the best activities against *E.coli* and *S. aureus* with both displaying inhibition zones of 10 mm, followed by 8.5mm against *S. typhi*. The aqueous extract had the best activity against *K. aerogenes* (9.5 mm), followed by *S. aureus* (5 mm). The ethyl acetate extract had the best inhibition against *S. pyogenes* (6mm), followed by *K. aerogenes* (5.5mm). Statistically, at $p < 0.05$ the plant extracts have a significant effect on the tested organisms when compared with the positive control.



Key: S. drug = Standard drug (Ampicillin antibiotic); *Pseudo = P. aeruginosa; Strep = S. pyogenes*; *Staph = S. aureus*; *Kleb = K. aerogenes*; *E.coli = E. coli*; *Salm = S. typhi.*

Figure 2 Mean antibacterial zones of inhibition of various extracts of *Ocimum gratissimum*



Key: S. drug = Standard drug (Ampicillin antibiotic); *Pseudo = P. aeruginosa; Strep = S. pyogenes*; *Staph = S. aureus*; *Kleb = K. aerogenes*; *E.coli = E. coli*; *Salm = S. typhi.*

Figure 3 Mean antibacterial zones of inhibition of various extracts of *Newbouldia laevis*

4. Discussion

The plant leaf extracts in this study were obtained using three different solvents (water, ethanol, and ethyl acetate) to take advantage of their differential extracting properties. The phytochemical analysis of *O. gratissimum* and *N. laevis* leaf extracts showed the presence of alkaloids, flavonoids, saponin, tannins, glycosides, and phenol (Table 1). This is supported by the studies of other researchers who reported the presence of the same phytochemicals in *O. gratissimum*, [15,16] and *N. laevis* [17]. According to Ungogo *et al.* [3] and Dahiru *et al.* [18], these classes of compounds show inhibitory activity against several bacteria, explaining their applications in traditional medicine. The antibacterial activity of plant extracts containing flavonoids has previously been documented long ago [19]. The results obtained in this study thus suggest that the identified phytochemical compounds may be the bioactive constituents responsible for the antibacterial efficacy of the plant leaves.

The plant extracts were observed to display varying degrees of activity against the test isolates in a concentration-dependent manner. Using the same plant, Mbata and Sanikia [20] reported a similar observation for *O. gratissimum* leaf extracts. They reported the highest zone of inhibition at 250mg/ml and the least at 50mg/ml. At 200 mg/ml concentration, the ethanol extract displayed the highest inhibition with 18 mm, followed by ethyl acetate extract and aqueous with 15 mm and 8 mm, respectively. In this study, all extracts of *O. gratissimum* displayed the best activity against *E. coli* (Figure 2). The antibacterial activities of the plant extracts of *O. gratissimum* in this study are in agreement with the reports of Unegbu *et al*. [21] and Alo *et al*. [22]. *O. gratissimum* is known for its various uses in traditional medicine, and is reported for its action against gastrointestinal infections (diarrhoea, dysentery), infections of the skin (dermatitis, eczema, scabies), infections of the upper respiratory tract, associated with cough, asthma and bronchitis, wounds and sores, insect bites, nosebleeds, stroke, anaemia [23].

All crude extracts were observed to inhibit both Gram-positive and Gram-negative organisms, a similar observation by Ugbogu *et al*. [16] and Unegbu *et al*. [21], who both reported the inhibitory effects of *O. gratissimum* leaf extracts against both Gram-positive and Gram-negative bacteria. However, the aqueous extract did not inhibit *P. aeruginosa* and *S. typhi*, while the ethanolic extract did not affect *K. aerogenes*. Generally, the aqueous extract of *O. gratissimum* in this study displayed the least antibacterial activity, when compared with other extraction solvents used. A similar observation has been reported by Nwinyi *et al*., [24] whose findings showed that the aqueous extracts of *O. gratissimum* had minimal antibacterial activity. The observed differences in the activities among the plant extracts may be due to the insolubility of active compounds in water or the presence of inhibitors of the antimicrobial components [25].

At 200 mg/ml, it was observed that the ethanol extract of *N. laevis* displayed the best activities against *E.coli* and *S. aureus*, followed by *S. typhi*. Obum-Nnadi *et al*., [26] most recently published agreeing data of the very good inhibitory effect of ethanol extract of *N. laevis* against *E.coli* and *S. aureus*. The aqueous extract had the best activity against *K. aerogenes*, followed by *S. aureus*. The ethyl acetate extract had the best inhibition against *S. pyogenes*, followed by *K. aerogenes*. Statistically, at $p < 0.05$ the plant extracts have a significant effect on the tested organisms when compared with the positive control. In time past, several other researchers had reported that *N. laevis* possess broad-spectrum antimicrobial properties toward Gram-positive and Gram-negative bacteria [27-29].

All antibacterial activity results obtained in this study were found to be encouraging when compared to that of standard antibiotics (Ampicillin). All the extracts showed a higher zone of inhibition than the standard antibiotic. Similarly, Ekhaise *et al*. [30] obtained a low zone of inhibition when Ampicillin was tested against the bacterial isolates used in this study (*P. aeruginosa, S. aureus,* and *E. coli*). The pattern remained unaffected in the presence of DMSO, indicating that the vehicles at the concentration used, did not have any microbial growth inhibitory effect on all the tested organisms, which was also reported by Anas [31] and Nwobodo *et al*. [32]. We strongly believe that further purification of the crude extracts of *O. gratissimum* and *N. laevis* could lead to an advancement in the search for novel natural antibacterial agents.

5. Conclusion

The results revealed the presence of important phytochemical constituents in the plant leaves studied. The phytochemical compounds identified in this study have earlier been proven to be bioactive components, which could be responsible for the antibacterial activities. The leaf extracts of the plants in this study displayed broad-spectrum potentials against the selected isolates, and could therefore be seen as a potential source for the development of useful antibacterial drugs.

Compliance with ethical standards

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Disclosure of conflict of interest

Authours have declared that no conflict of interest exists.

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