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# Phytochemical Screening and Antibacterial Activity of *Ocimum Gratissimum* L. (Lamiaceae) Leaf Extract Against Pathogens Causing Ear Infections

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**Keywords**: *Ocimum gratissimum,* pathogens, ear infections, antibacterial activity, phytochemical screening.

**Abstract:** Medicinal plants have been used traditionally for curative purposes due to their diverse array of constituents. *Ocimum gratissimum* is acclaimed for its forkloric use in the management of headaches, diarrhea, pneumonia, infections etc. This investigation aimed to determine the phytochemical constituents and antimicrobial activity of the aqueous-ethanolic extract of *O. gratissimum*. Qualitative phytochemical analysis was done and the quantification carried out using Ultraviolet-visible (UV) spectrophotometry method. Qualitative analysis involved the detection of alkaloids, tannins, quinones, coumarins, flavonoids, cardiac glycosides, saponins, terpenoids and phenolic compounds in the extract, while the quantitative analysis involved quantification of both flavonoid and phenol using the UV-spectrophotometer. The antimicrobial activity of the extract was determined using agar disk diffusion method for activity against *Proteus mirabilis*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract were also done. Phytochemicals present include both hydrolyzed and condensed tannins, alkaloids, flavonoids, phenolics, saponins, cardiac glycosides, terpenoids and coumarins but not quinones. The total content of flavonoids and phenols present were 11.27 mg/g and 117.08 mg/g respectively. The extract presented activities against *P. mirabilis, S. pneumonia* and *P. aeruginosa* while a higher activity was observed against *S. aureus*. The extract contained a number of phytochemicals which singly or synergistically, could be responsible for most of the extract's pharmacological activities. The extract of *O. gratissimum* showed antimicrobial activity against common pathogens causing ear infections and this further affirms the

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ethnobotanical use of the plant for treating ear infection

## INTRODUCTION

Most medicinal and aromatic plants are sources of raw materials in drug production due to the presence of natural compounds present in these plants. These natural compounds serve as lead compounds which can be manipulated to enhance pharmacological effects (Thilagavathi *et al*., 2015). Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Saxena *et al*., 2013).

The discovery of antibiotics has helped in the control of infections, unfortunately there is an increased rise in resistance by most microbes causing infections. Therefore, natural plants are being screened for search of new drugs that could serve as alternatives in the treatmet of infections (Agholor *et al*., 2018).

*Ocimum gratissimum* L. (Lamiaceae) is widely distributed in tropical and temperate regions. The plant is commonly used in folk medicine to treat different diseases such as upper respiratory tract infections, diarrhoea, headache, ophthalmic infections, skin infection, pneumonia, cough and fever (Ugbogu *et al*., 2021). It has been recorded that this plant contains mostly phenols, particularly thymols and that these are probably responsible for its reported antimicrobial action (Oboh 2006).

Other phytoconstituents such as tannins, flavonoids and terpenoids have been reported to contribute to the antibacterial activity of *O. gratissimum* (Matias *et al*., 2011). Ear infection is responsible for up to 40 % of preventable hearing impairment; it is one of the reasons for frequent and unwise antibiotic usage, especially in the developing world. *Staphylococcus aureus, Proteus mirabilis, Proteus vulgaris, Streptococcus sp.,* and *Pseudomonas aeruginosa are* the leading cause of ear infections. The presence of high number of multidrug-resistant strains calls for the need for periodic and continuous follow-up on evaluation of antibiotic usage (Getaneh *et al*., 2021). This investigation aimed to determine the phytochemical constituents and antimicrobial activity of the aqueous-ethanol extract of *O. gratissimum*.

## MATERIALS AND METHODS

### Collection and Identification

Fresh leaves samples of *O. gratissimum* were collected from a cultivated garden located in Ibafo in Obafemi Owode local government area of Ogun state, Nigeria (6.747519N, 3.424827E). The fresh leaves plant samples were washed under runny water then oven dried at 45 $^{o}$C for 6 hours and it was milled well into a fine powder in a grinder and stored in the refrigerator. A sample of the fresh leaves was authenticated by Dr. Nodza George, the herbarium curator of the Department of Botany, University of Lagos, Akoka and specimen deposited (LUH 7129).

Figure 1: Picture of *O. gratissimum* plant (Ibafo, Obafemi-Owode, Ogun state)

### Extraction Process

The aqueous ethanolic extract of fine pulverized leaves were prepared by soaking 200 g of fine powdered leaves sample in proper volume of 10 % aqueous ethanol for 72 h. The extract was turned intermittently and then filtered using Whatman filter paper No. 1. The extract was allowed to dry using rotary evaporator at 40 $^{o}$C. Then the concentrated yield was stored in a refrigerator at 4 °C until further use (Salem *et al*., 2016).

### Method for Qualitative Analysis

Qualitative phytochemical investigations were carried out on the aqueous extract to identify the constituents using standard procedures (Salem *et al*., 2016). The class of phytoconstituents tested for are tannins, flavonoids, cardiac glycosides, phenols, saponins, terpenoids, alkaloids, reducing sugars, quinones and coumarins.

### Method for Quantitative Analysis
### Flavonoid

The content of flavonoids was determined by the reaction with aluminum chloride using the method described by Matias *et al*., (2011). To 2 ml of sample was added 2 ml of $AlCl_3$ 2 % solution in ethanol. After 1 hour the absorbance was measured

at 420 nm at room temperature, concentrations of 0.1 and 1 mg/mL were prepared in methanol and used, while the rutin concentrations of 0.01, 0.02, 0.04, 0.08 and 0.10 mg/ml were used to obtain the calibration of the curve. The data were calculated based on the calibration curve of rutin and expressed in mg equivalents (RE) per gram of crude extract (Matias *et al*., 2011).

**Total phenol**

Total phenols were determined by using the Folin Ciocalteu reagent (McDonald *et al*., 2001). A dilute extract of each plant extract (0.5 ml of 1:10 g/l) or gallic acid (the phenolic compound commonly used as the standard) was mixed with the Folin Ciocalteu reagent (5 ml of the reagent diluted tenfold with distilled water) and aqueous $Na_2CO_3$ (4 ml, 1 M), Concentration of 0.1mg/mL of plant extract were also prepared in methanol. The mixtures were allowed to stand for 15 min and total phenols were determined by colorimetry at 257 nm. The standard curve was prepared using 0, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL solution of gallic acid in methanol/water (50:50 v/v). The total phenol value was expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a commonly used reference value (Cheng, 2003).

**Antimicrobial Activity**
**Isolation and Identification of Bacteria**

The pure clinical isolates of some pathogenic ear bacteria (*P. mirabilis, S. pneumoniae, P. aeruginosa,* and *S. aureus*) were obtained from the Medical Microbiology laboratory, College of Medicine, University of Lagos. All the isolates were checked for purity and confirmed by Gram staining and by subculturing them on selective media to observe the colony characteristics and morphology of the cells (Agholor *et al*., 2018).

**Preparation of the Bacterial Suspension**

Bacterial suspension with a few well-isolated colonies from an overnight (18-24 hours at 35 ± 2°C) agar culture in sterile saline solution, adjusted to 0.5 MacFarland by comparison to the standard, was prepared. Not more than 15 minutes after preparing the inoculum suspension, cotton swab was dipped into the bacterial suspension and carefully streaked on the entire surface of Mueller-Hinton agar plate, three times, rotating 60 degrees each time. The surface was then allowed to dry completely before the next step. Similar procedure was repeated for each of the bacteria isolates (*P. mirabilis, S. pneumoniae, P. aeruginosa,* and *S. aureus*) used in this study.

**Antimicrobial Susceptibility Tests**

Agar well diffusion techniques were employed for the antimicrobial testing of the plant extracts. The 24 h old cultures were transferred into nutrient broth and incubated at 37 °C for 5 h and standardized to 0.5 MacFarland standard. Each of the test organisms (1 ml) from the broth cultures were placed into a sterile plate and 19 ml molten agar at 45 ºC poured and the plate shaken for even spread and proper mixing of the organisms and agar. This agar was allowed to solidify (Akoma *et al*., 2002, El-Mamood *et al*., 2008). All these were done under aseptic condition and were labelled accordingly. Wells of approximately 5 mm in diameter were made on the surface of the inoculated agar medium using a sterile cork borer and the wells labelled with a marker based on the concentration of the plant extract (100, 200 and 400 mg/ml) and the wells were filled with the different concentration of the extract. Distilled water and antibiotics discs were used as the negative and positive controls (Lino & Deogracious, 2006). The plates were incubated at 37°C and the susceptibility of the test organisms to the plant extract were recorded after 24 h by measuring the average diameter of the clear zone of inhibition around the wells in millimetres (mm).

**Preparation and Loading of Antibiotic Discs for Agar Disk Diffusion Method**

The disc diffusion test was done for each organism on Mueller-Hinton agar. For this, 19 ml of medium was poured into sterile Petri-dishes to a depth of 4 mm on a level surface to make the depth of the medium uniform. The antibiotics discs (Oxoid Holdings Ltd.) were applied by means of a sterile-forceps, not more than 15 minutes after swabbing, strictly under aseptic conditions. The discs were deposited onto the plate and tapped gently with the sterile forceps to make complete contact with the medium surface. The disks should not be moved once deposited. The lid of the plate was replaced, the plates inverted and placed in the incubator at 35°C. Plates were examined after 24 hours of incubation to measure the zones of inhibition.

**Inhibition Zone measurement**

The measuring tape was held on the back of the inverted plate over a black, non-reflecting background, and the more obvious margin was measured to determine the zone diameter. Growth within the apparent zone of inhibition was indicative of resistance.

**Minimum Inhibitory Concentration**

This is to determine the lowest concentration of the plant extract required to inhibit the growth of

known test organism. It was done by preparing sixteen working concentrations of the plant extract (0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 40, 80, 160 mg/ml) using the agar dilution method on Mueller Hinton agar plate.

**Agar Dilution Procedure**

The culture was standardized according to the Clinical Laboratory Standard Institute (CLSI) standards (CLSI., 2012). This was carried out by adjusting the bacteria isolates to the McFarland turbidity standard of 0.5. The Agar dilution method was used to determine the concentrations of the extracts under test, which can inhibit the growth of the test bacteria *in-vitro*. The MIC was then determined from the least concentration showing no growth after 24 hours of incubation.

**Minimum Bactericidal Concentration (MBC)**

The three lowest concentrations from the MIC test which resulted in an inhibition of the test organism were sub-cultured unto fresh Mueller Hinton Agar (MHA) plates by streaking. The MHA plates were incubated at 37 °C for 24 hours and were observed for growth. MHA plates that cannot show any growth indicates a 99.9 % bactericidal effect of the plant extract at that concentration (CLSI., 2014). The lowest concentration among these was the MBC.

**RESULTS**

**Qualitative Analysis Result**

The presence of tannins (hydrolysed and condensed), alkaloids, flavonoids, saponins, cardiac glycosides, phenolic nucleus, terpenoids and coumarin were confirmed, while quinone was absent in the *O. gratissimum* extract.

**Quantitative Analysis Result**

**Total flavonoid at 420 nm**

Using the equation of the graph to extrapolate the quantity of flavonoid present in the *O. gratissimum,* the equivalent concentration of total flavonoids in the extract was 117.08 mg/g.

**Total Phenol at 257 nm**

Using the equation of the graph to extrapolate the quantity of phenol present in *O. gratissimum,* the equivalent concentration of total phenols in the extract is 11.27 mg/g.

**Result of antimicrobial activity**

The aqueous ethanol extract of *O. gratissimum* caused an incremental inhibition of *Proteus mirabilis* from 20 ± 0.1 mm to 39 ± 0.1 mm (as depicted in the inhibition zone diameter) as the concentration of the extract increases from 100 mg/ml to 400 mg/ml. *Staphylococcus aureus* and *Streptococcus pneumonia,* were not inhibition at 100 mg/ml concentration of the extract, but inhibition became dose dependent from 200 mg/ml upwards. *Pseudomonas aeruginosa* was only inhibited with a inhibition zone diameter of 22 ± 1 mm at 400 mg/ml which was the highest concentration used in this study (Table 1).

The standard antibiotic disks of ceftazidime, cefuroxime, gentamicin, ceftriaxone, erythromycin, cloxacillin, ofloxacin, Augmentin®, cefixime, nitrofurantoin and ciprofloxacin were used against the ear pathogens of interest in this study. *Staphylococcus aureus* was only susceptible to gentamicin (10 µg) and ofloxacin (5 µg) with inhibition zone diameters of 25 ± 0.58 and 31 ± 1.00 respectively. Similarly, *Streptococcus pneumoniae* was susceptible to gentamicin (10 µg) and ofloxacin (5 µg) with inhibition zone diameters of 8 ± 1.15 and 21± 2.31 respectively (Table 2).

*Pseudomonas aeruginosa* was not susceptible to any of these antibiotics at the respective disc load used, while *Proteus mirabilis* was susceptible to gentamicin, ofloxacin, Augmentin®, cefixime and ciprofloxacin.

The Minimum Inhibitory Concentration (Table 3) obtained for the respective pathogens were *Staphylococcus aureus* (40 mg/ml)*, Proteus mirabilis* (5.12 mg/ml)*, Pseudomonas aeruginosa* (40 mg/ml) and *Streptococcus pneumoniae* (10.24 mg/ml).

The Minimum Bactericidal Concentration (Table 4) obtained for the respective pathogens were *Staphylococcus aureus* (5.12 mg/ml)*, Proteus mirabilis* (10.24 mg/ml)*, Pseudomonas aeruginosa* (5.12 mg/ml) and *Streptococcus pneumoniae* (40 mg/ml).

**Table 1: Zones of inhibition against extract and negative control**

| Organism | Gram type | 400 mg/ml | 200 mg/ml | 100 mg/ml | Water |
|---|---|---|---|---|---|
| | | Zones of Inhibition (mm) | | | |
| *Proteus mirabilis* | Negative | 39± 1.0 | 29 ± 1.0 | 20 ± 1.0 | - |
| *Pseudomonas aeruginosa* | Negative | 22 ± 1 | - | - | - |
| *Staphylococcus aureus* | Positive | 45 ± 0.5 | 26±1.0 | - | - |
| *Streptococcus pneumonia* | Positive | 21 ± 1 | 12 ± 0.5 | - | - |

- No inhibition

**Table 2: Actions of the standard antibiotic disks on microorganisms with Inhibition Zones *(mm)***

| Organism | Antibiotics Disc Load (µg) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAZ (30) | CRX (30) | GEN (10) | CTR (50) | ERY (5) | CXC (5) | OFL (5) | AUG (30) | CXM (5) | NIT (300) | CPR (5) |
| *P. mirabilis* | - | - | 17±1.00 | - | - | - | 29± 0.58 | 11± 1.00 | 28± 2.31 | - | 38.33 ± 0.58 |
| *P. aeruginosa* | - | - | - | - | - | - | - | - | - | - | - |
| *S. aureus* | - | - | 25±0.58 | - | - | - | 31 ± 1.00 | - | - | - | - |
| *S. pneumoniae* | - | - | 8 ± 1.15 | - | - | - | 21± 2.31 | - | - | - | - |

Ceftazidime CAZ, Cefuroxime CRX, Gentamicin GEN, Ceftriaxone CTR, Erythromycin ERY, Cloxacillin CXC, Ofloxacin OFL, Augmentin AUG, Cefixime CXM, Nitrofurantoin NIT, Ciprofloxacin CPR.

**Table 3: Result for minimum inhibitory concentration for *Ocimum gratissimum* at different concentrations.**

| Conc. (mg/ml) | *Staphylococcus aureus* | *Proteus mirabilis* | *Pseudomonas aeruginosa* | *Streptococcus pneumoniae* |
|---|---|---|---|---|
| 0.0025 | + | + | + | + |
| 0.005 | + | + | + | + |
| 0.01 | + | + | + | + |
| 0.02 | + | + | + | + |
| 0.04 | + | + | + | + |
| 0.08 | + | + | + | + |
| 0.16 | + | + | + | + |
| 0.32 | + | + | + | + |
| 0.64 | + | + | + | + |
| 1.28 | + | + | + | + |
| 2.56 | + | + | + | + |
| 5.12 | + | - | + | + |
| 10.24 | + | - | + | - |
| 40 | - | - | - | - |
| 80 | - | - | - | - |
| 160 | - | - | - | - |

+ Growth, – No growth

**Table 4: Result for minimum bactericidal concentration for *Ocimum gratissimum***

| Conc. (mg/ml) | *Staphylococcus aureus* | *Proteus mirabilis* | *Pseudomonas aeruginosa* | *Streptococcus pneumoniae* |
|---|---|---|---|---|
| 5.12 | - | + | - | + |
| 10.24 | - | - | - | + |
| 40 | - | - | - | - |
| 80 | - | - | - | - |
| 160 | - | - | - | - |

## DISCUSSION

Plants contain a wide array of phytochemicals, which have traditionally been utilized for centuries in folk medicines or ethnomedicines. The most phytochemical classification scheme is based on chemical structures such as phenolics, alkaloids, saponins, terpenoids, limonoids, polyacetylenes, secoiridoids e.t.c. (Patra, 2012). Phenolic compounds are a group of phytochemicals, which have a phenol structure, i.e. an aromatic benzene ring bearing at least one hydroxyl substituent (Robbins, 2003; Vermerris and Nicholson, 2006). Phenolic compounds are commonly found throughout the plant kingdom, where they protect the plants from microbial infections, ultraviolet radiation and chemical stressors. The commonly categorized subclasses of phenolic compounds are simple phenolics, phenolic acids and aldehydes, coumarins, flavonoids, chalcones, aurones, benzophenones, xanthones, stilbenes, benzoquinones, naphthaquinones, anthraquinones, betacyanins, lignans, and polyphenols (Vermerris and Nicholson, 2006; Handique and Baruah, 2002). Plants containing phenolics are becoming an important part of diets and phytomedicines due to their potential anti-oxidative and potent anti-microbial properties.

Alkaloids have been defined as N-heterocyclic basic metabolites, although the definition does not clearly separate from other N-containing compounds. Alkaloids have been classified in many ways depending upon biogenic precursors or carbon skeleton characteristics. Alkaloids are generally known according to their carbon skeleton structures. Pyridine (e.g. piperine), piperidine, quinoline, indole, pyrrolidine, quinazoline, isoquinoline, glyoxaline, lupinane, tropan, phenanthridine, imidazoline, alkaloidal amines and terpenoid types of alkaloids are commonly found in plants (Hegnauer, 1988). Alkaloid fractions isolated

from some plants had been tested for their antimicrobial properties against some pathogenic Gram-positive, Gram-negative and acid-fast bacteria and fungi. These fractions had shown considerable antimicrobial activity against both bacteria and fungi at the tested concentrations. Further, the growth of *Proteus vulgaris*, *S. aureus*, *Salmonella typhimurium*, *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Aspergillus niger* and *C. albicans* were significantly inhibited (Mallikharjuna and Seetharam, 2009).

Saponins are a group of high molecular-weight glycosides, in which saccharide chain units (1–8 residues) are linked to a triterpene (triterpene saponins) or steroidal (steroid saponins) aglycone moiety, i.e. sapogenin. Many plant extracts containing saponins from various plants and purified saponins show antimicrobial activities at different concentrations (Sen *et al*., 1998; Avato *et al*., 2006). However, the types of saponins exhibit different spectra of antimicrobial effects.

Terpenoid compounds derive from a basic structure of C5 isoprene units. They are classified according to the number of isoprene unit involved for their synthesis, i.e. monoterpenoid (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25) and triterpenoids (C30). They can be acyclic (myrcene and geraniol), monocyclic (cymene and carvacrol), bicyclic (pinene) and tricyclic with different groups (alcohol, phenol, and aldehyde). A number of essential oil are known for their strong anti-microbial activities against many pathogenic and non-pathogenic bacteria and fungi (Singh *et al*., 2002, Mahady *et al*., 2003, De *et al*., 2009, Park *et al*., 2008).

The extract of *O. gratissimum* was observed to contain the following phytochemicals: hydrolysed

tannins and condensed tannins, alkaloids, flavonoids, phenolics, saponins, cardiac glycosides, terpenoid and coumarin while quinone is absent. The absence of quinone in aqueous-
ethanol extract of *O. gratissimum* is in contrast to the findings of Jumare (Jumare, 2018).
The presence of constituents like flavonoids, alkaloids and tannins gives more authentication to the findings of Afolabi *et al.,* 2007, where they were also detected in aqueous extract of *O. gratissimum* (Afolabi *et al.,* 2007). Also, it is observed that this water-ethanol extract has a better extractable activity of phytochemicals compared to the methanol and chloroform extract investigated by Umar *et. al.,* (2019). Several studies have proven that these metabolites have varied pharmacological actions in man and animals, the presence of these metabolites suggest great potentials of the plants as a source of useful phytomedicines. The phytochemicals are naturally occurring chemicals in plants which serve as medicine for the protection of human disease; the phytochemicals are also non-nutritive plant chemicals that have protection or disease preventive properties (Cheng, 2003; Venuprasad *et al*., 2014; Alexander, 2016; Justina and Solomon, 2017)..
According to the result obtained it can be seen that *O. gratissimum* has abundant quantity of tannins, alkaloid, flavonoid, phenolic ring, saponins, cardiac glycoside and terpenoid. These phytochemicals are most likely responsible for the various pharmacological actions of *O. gratissimum* on man.
The total flavonoid content in the extract of *O. gratissimum* leaves was obtained from the regression equation of the calibration curve of rutin equivalent. The flavonoid content of the plant extract is 117.08 mg/g. The total phenol content in the extract of *O. gratissimum* leaves was obtained from the regression equation of the calibration curve of gallic acid equivalent (GAE). The phenolic content of the plant extract is 11.27 mg/g.
The antimicrobial activity was determined using the agar well-diffusion method, agar disk diffusion method and the Agar dilution method for the MIC. The activity was quantitatively assessed based on the inhibition zone, and their activity index was also calculated alongside with the Minimum inhibitory concentration.
Plants and many other natural sources can provide a huge range of complex and structurally diverse compounds. Based on recent research, essential oils, pure secondary metabolites and synthesized molecules can act as potential antimicrobial agents (Runyoro *et al*., 2006).
Minimum inhibitory concentration is defined as the lowest concentration that is able to inhibit any visible bacterial growth on the culture plates. Determination of MIC is important in diagnostic laboratories, as it helps to confirm the resistance of the micro-organism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The aqueous-ethanolic extract of *O. gratissimum* showed the least MIC value of 5.12 mg/ml against *P. mirabilis* while the highest MIC value is observed at 40 mg/ml against *S. aureus* and *P. aeruginosa.* The results showed that the four organisms are susceptible to the aqueous-ethanolic extract of *O. gratissimum.* The MBC obtained were 5.12 mg/ml for *S. aureus* and *P. aeruginosa*, while 10.24 mg/ml and 40 mg/ml were obtained for *P. mirabilis* and *S. pneumoniae* respectively.

## CONCLUSION

The results revealed the presence of a number of phytochemical constituents in *O. gratissimum*. The extract had moderate activity against known pathogens responsible for ear infections such as *S. pneumonia* and *P. aeruginosa* and a higher activity against *S. aureus and P. mirabilis*

### Conflict of Interest

There is no conflict of interest

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