Journal of Advances in Biology & Biotechnology

22(2): 1-8, 2019; Article no.JABB.51236
ISSN: 2394-1081

Phytochemical Properties and Antibacterial Activity of Leaf Extract of *Ocimum gratissimum* on *Salmonella* Species

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SMJ, AAF and AMM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GDM, MKN, AG, SU and ASB managed the analyses of the study. Authors AM, AMJ and AMR managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2019/v22i230112
Editor(s):
(1) Dr. Maria Serrano, Department of Applied Biology, EPSO, University Miguel Hernández, Ctra Beniel km 3.2, E-03312 Orihuela, Alicante, Spain.
Reviewers:
(1) Ronald Bartzatt, University of Nebraska, USA.
(2) Senthil Kumar Raju, Swamy Vivekanandha College of Pharmacy, India.
Complete Peer review History: http://www.sdiarticle3.com/review-history/51236

Original Research Article

Received 22 June 2019
Accepted 04 September 2019
Published 13 September 2019

ABSTRACT

Aim: *Ocimum gratissimum* is commonly used as food and health purposes. This study is aimed at evaluating the bioactive compounds and antibacterial activity of leaf extract of *O. gratissimum* against *Salmonella* species.
Methodology: The Phytochemical screening of *O. gratissimum* was conducted using standard methods. Screening for antibacterial activity of the leaf extracts against *Salmonella* species was determined using agar well diffusion method. An *in-vivo* toxicity study was carried out with albino rats.
Results: The phytochemical screening revealed the presence of saponins, tannins, cardiac glycoside, flavonoid, glycosides, alkaloid, volatile oils and steroids. A zone of inhibition of 14mm

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was recorded against the organisms using ethanolic extract with a concentration of 100 mg/ml and the lowest was recorded against *Salmonella paratyphi* with the concentration of 25 mg/ml of the ethanolic extract. Zone of inhibition of 9.00 mm and 10.0mm was recorded against *S. typhi* and *S. paratyphi* on a concentration of 100 mg/ml of the aqueous extract. A minimum inhibitory concentration of 100 mg/ml and 25 mg/ml of the aqueous and ethanolic extract of the leaf was recorded. After the toxicity test, no death was recorded after 2 (two) weeks.

Conclusion: The leaf extract of *O. gratissimum* shows promising potentials in the treatment of infectious diseases associated with *Salmonlla typhi* and *Salmonella paratyphi*, due to its antimicrobial activity and low toxicity. However, further studies are needed to non-polar solvents to isolate other bioactive compounds as well as identify the active metabolites responsible for these activities.

Keywords: Ocimum gratissimum; antibacterial activity; Salmonella typhi; Salmonella paratyphi; phytochemical; toxicity.

1. INTRODUCTION

Medicinal plants are known to contain, in one or more of its organs, substances that can be used for therapeutic purposes or as precursors for the synthesis of useful drugs [1]. Many of such plants known to be used primitively to alleviate symptoms of illnesses have been screened to have medicinal importance, some of which include: *Vernonia amygdalina* (bitter leaf), *Ocimum gratissimum* (scent leaf), *Zingiber officinale* (ginger), *Azadirachta indica* (Dogonyaro), *Piper guineese* (Iyere), *Allium sativum* (garlic), Cotton leaf (*Gossypium* spp*)* etc. These plants have been reportedly used in the traditional treatment of ailments such as stomach disorder, fever symptoms and cough [2].

Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against cancer, as well as viral and microbial infections. The phytochemical evaluation of *Ocimum gratissimum* shows that it is rich in alkaloid, tannins, oxalate, flavonoids and essential oil [3]. In the coastal area of Nigeria, the plant *Ocimum gratissimum* is used in the treatment of epilepsy, high fever and diarrhoea [4]. *Ocimum gratissimum* (Scent leaf) is a perennial plant which is widely distributed in the tropics of Africa and Asia. It belongs to the family Labiatae and it as the most abundant of the genus *Ocimum.* In the southern part of Nigeria, it is called “Efirin nla” by the Yoruba speaking tribe. “Nichonwu” in Igbo while in the northern part of Nigeria, it is called “Daidoga” [5]. Leaf extract of *Ocimum gratissimum* and *Xylopia aethiopiea* were analyzed against five pathogenic organisms. *Staphylococcus aureaus, Escherichia coli, Streptococcus fecalis, Pseudomonas aeruginosa and Lactobacilli* [3]. The findings justify the application of *Ocimum gratussimum* in dermatological cream and indicate the effective doses could be achieved at very low concentration and also shows that the aqueous fractions of both plants have more potential as antimicrobial agents than their ethanolic fractions [3]. The findings of Silva et al. [6] showed the extracts of *Ocimum gratissimum* to be active against human pathogenic dermatophytes.

A thousand years ago an extensive use of plants as medicines has been reported and was initially taken in the form of crude drugs such as tinctures, elixirs, poultices, powders, and other herbal formulations [7]. However, the use of herbal products should be based on scientific origin; otherwise they would be useless and unsafe [7]. Furthermore, the irrational use of these herbal products may cause serious toxicity for humans. Unfortunately, many people underestimate the toxicity of natural products and do not realize that these agents could be as toxic as or more toxic than synthetic drugs [7]. A typical example of a toxic herbal product is the leaves of *Atropa Belladonna* and *Digitalis purpurea* [8], which show severe systemic toxicity if taken orally.

Toxicology is the important aspect of pharmacology that deals with the adverse effect of bioactive substance on living organisms prior to the use as drug or chemical in clinical use [9], As per the OECD 2001 guidelines, in order to establish the safety and efficiency of a new drug, toxicological studies are very essential in animals like mice, rat, guinea pig, dog, rabbit, monkey etc under various conditions of drug. Toxicological studies help to make decision whether a new drug should be adopted for clinical use or not. OECD does not allow the use of drug clinically

without its clinical trial as well as toxicity studies. The aim of this present work, therefore, was to carry out phytochemical screening of the leaf of *Ocimum gratissimum*, study the antibacterial effects of the leaf extracts of *Ocimum gratissimum* on selected Enterobacteriaceae (*Salmonella* species) and to estimate the toxic effects of aqueous and ethanolic extracts from *Ocimum gratissimum* in albino Rats.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Leaf Materials

Ocimum gratissimum (scent leaf) was obtained from Meat Market, Sokoto, Nigeria. The collected leaf was identified and authenticated at the Herbarium Section of the Department of Biological Sciences, Botany Unit of Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria. Voucher specimen numbers UDUH/ANS/101 was obtained.

2.2 Preparation and Extraction of Leaf Extracts

The fresh leaves were allowed to dry completely at room temperature before using them for this study. The leaf material was pulverized using mortar and pestle into a fine powder. Two different solvents were used for the extraction namely: Water and ethanol. A 100 g of powdered leaf was soaked in 1000 ml of each solvent in accordance with Udochukwu et al. [10]. Each solution was stirred intermittently and allowed to stand for 48 h, and then filtered by first, using a clean muslin cloth and then, No. 1 Whatman filter paper. Sterilization of the solutions was made using membrane filters. The sterile extract obtained was stored in sterile capped bottles and refrigerated [11].

2.3 Characterization and Identification of *Salmonella* Species

2.3.1 Source of test organism

The test organisms for this study (*Salmonella* species) are members of the family Enterobacteriaceae. The pure clinical isolates of *Samonella typhi* and *Samonella paratyphi* were obtained from the Department of Medical Microbiology and Parasitology, Specialist Hospital Sokoto, Nigeria. All the clinical isolates were checked for purity by sub-culturing the isolates onto Salmonella-Shigella Agar medium. After 24 hrs of incubation, there were growths of the isolates and they were maintained on nutrient agar slants at 4°C in the refrigerator until required for further use.

2.4 Biochemical Confirmation and Serotyping of *Salmonella*

The ISO-6579 [12], the standard recommendation was used for biochemical confirmation of *Salmonella*. The subculture of characteristic colonies from each Petri dish of Salmonella-Shigella agar medium was made. The triple sugar iron agar (TSI agar), Urea agar/broth, L-lysine decarboxylase, β-galactosidase (ONPG), Voges Proskauer and Indole tests were followed in this order.

In serotyping, the subculture of characteristic colonies from each Petri dish of Salmonella-Shigella agar was transferred onto nutrient agar slopes and incubated overnight at 37°C. Using a wire loop, 3 separate drops (each 0.02 ml) of saline solution were placed onto a clean microscope slide. Growth from the agar slope was added and emulsified to produce homogeneous suspension. A loopful of *Salmonella* polyvalent 'O' (PSO) anti-serum was mixed with the first drop of suspension and a loopful of *Salmonella* polyvalent 'H' (PSH) anti-serum with the second drop. It was rocked gently back and forth and examined for agglutination against a black background. Positive results were recorded if agglutination occurred within 20 min after shaking against dark background. In order to exclude any spontaneous agglutination (auto-agglutination), a negative control (using physiological saline solution and bacterial colony to be tested) was included in the test.

2.5 Standardization of Bacteria Cell Suspension

The nutrient broth cultures of the organisms for this study were taken and inoculated at 37°C on a fresh agar plate of nutrient agar for 24 hours. Sterile distilled water (2 ml) was poured on it and then mixed with the inoculums, 1 ml of each was taken and transferred into 9 ml of sterile distilled water and diluted to 0.5 Macfarland Standard giving a load of 10^5- 10^6 organisms/ml. One hundred microlitres of these were taken and poured onto the surface of the agar and then spread evenly with the use of a spreader on the plate to be used for the study [13].

2.6 Preparation of Extracts Concentration

The different extracts of the sample were reconstituted with sterile distilled water. The initial concentration of each plant extracts (1 g) was diluted using 10 ml of sterile water to obtain the stock culture. From this stock culture, different concentrations were gotten which were 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml for each of the extracts (water and ethanol).

2.7 Determination of Antibacterial Activities of Leaf Extracts

Agar-well diffusion Method was employed for the antibacterial testing [14]. The antibacterial screening of the extracts was done as described by Perex et al. [14]. One (1) gram of each crude extract (aqueous and ethanolic) was poured into 10 ml water. From this stock culture, different concentrations were gotten which were 100 mg/ml, 50mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml for each of the extracts (aqueous and ethanol). Nutrient agar was poured in sterile Petri dishes and was allowed to solidify. A loopful of the test culture of MacFarland standard was dropped on the solidified agar and the organism was spread all over the surface of the agar using a spreader (wire loop). The inoculated plates were allowed to dry after which wells of approximately 5 mm in diameter were made on the surface of the agar medium using a sterile cork borer. Then, 0.2 ml of different concentrations of the extract was separately introduced into the different wells that have been labelled accordingly. This procedure was repeated in triplicate and allowed to stay for 30mins on the bench after which they were incubated for 24 h at 37°C. At the end of incubation, observed zones of inhibition were measured and recorded to the nearest millimetre.

2.8 Determination of Minimum Inhibitory Concentration of the Extracts

This was carried out using the agar diffusion method following the recommendations of the Clinical and Laboratory Standard Institute [15]. Different concentrations 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml of the extracts were prepared and 1 ml from each of the concentrations of the extracts was added onto molten nutrient agar and was mixed thoroughly. Then, 1 µl of an overnight nutrient broth culture of the test isolates were added to each plate of the Mueller-Hinton agar containing the extracts and incubated at 37°C for 24 h. The experiment was conducted in triplicate for all the test isolate. Plates without visible growth of the organisms in each concentration were taken as the MIC [11].

2.9 Phytochemical Screening of Leaf of *Ocimum gratissimum*

The pulverized leaf obtained was subjected to phytochemical screening to determine the presence of bioactive compounds.

2.10 Test for Tannins

Five per cent (5%) ferric chloride was added drop by drop to 3ml of each extract and observed for brownish green or a blue-black colouration [16].

2.11 Test for Saponins

Two grams (2 g) of the powdered sample of each extract was boiled in 20 ml of distilled water in a water bath and filtered. Then, 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil, shaken vigorously and then observed for the formation of emulsion [17].

2.12 Test for Flavonoids

One millilitre (1 ml) of 10% NaOH solution was added to a portion of the aqueous filtrate of each plant extract, followed by addition of concentrated H_2SO_4. A yellow colouration observed in the extract indicated the presence of flavonoids [16].

2.13 Test for Cardiac Glycosides

Five millilitres (5 ml) of each extract was treated with 2 ml of glacial acetic acid containing I drop of ferric chloride solution (3.5%). The content was allowed to stand for one minute. One millilitre (1 ml) of concentrated H_2SO_4 was carefully poured down the wall of the tube. A reddish-brown ring of the interface indicated a deoxysugar characteristic of cardenolides [18].

2.14 Test for Alkaloid

Two milliliter (2 ml) of each extract was stirred with 2 ml of 10% dilute hydrochloric acid. Then, 1 ml was treated with a few drops of Wagner's reagent and second 1ml portion treated with Mayer's reagent. Deep brown precipitation indicated a positive test [18].

2.15 Test for Glycosides

The 2.5 ml of 50% H_2SO_4 was added to 5 ml of each of the extracts in test tubes. The mixture was heated in boiling water for 15 minutes. Cooled and neutralized with 10% NaOH, 5 ml of Fehling's solution was added and the mixture was boiled again. A brick-red precipitate was observed, which indicated the presence of glycosides [18].

2.16 Test for Steroids

This was carried out according to the method of Harborne [18]. One (1) ml of each leaf extract was added in 2 ml of chloroform, and 2 ml of sulphuric acid (H_2SO_4) was added thereafter. A red colouration confirmed the presence of steroids.

2.17 Test for Volatile Oils

One millilitre (1 ml) of each of the extract fractions was mixed with 5 ml of dilute HCL. A white precipitate was formed, which indicated the presence of volatile oils [17].

2.18 Toxicity Study of the Leaf Extracts of *Ocimum gratissimum*

Acute oral toxicity test was carried out using the procedure of the Organization for Economic Cooperation and Development [19]. Ten (10) randomly selected Albino rats were used. The rats of both sexes weighing 160-200 g were used for the study. The animals were obtained from the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto. The animals were acclimatized for a period of seven days. All animals were housed, caged and allowed free access to food and water before they were used for the experiment. The animals' weights were taken and starved of food. Then 5000mg/kg bodyweight of the extract was administered in a single concentration. Concentrations were calculated according to the bodyweight of the animals. Oral administration of extracts was done using a graduated syringe and cannula. They were placed under observation for 48 hours for behavioural changes and daily for 14days for mortality [19], upon which the number of deaths and LD_{50} were determined.

3. RESULTS AND DISCUSSION

The results of phytochemical screening of *O. gratissimum* leaves revealed the presence of the following secondary metabolites; tannins, saponins, flavonoid, steroid, cardiac glycoside, glycosides, alkaloid, and volatile oil. This is similar to the findings of Nweze et al. [20] who reported the presence of alkaloids, tannins, glycoside, saponin, cardiac glycoside, steroid and flavonoids in *O. gratissimum* which is similar to the results obtained in this study.

The results revealed that the aqueous extract of *Ocimum gratissimum* had less inhibitory activity on the test organisms (Table 2), while the ethanolic extracts of *Ocimum gratissimum* (Table 3) had antibacterial activity against the isolates tested. At 100 mg/ml concentration, the ethanolic extracts showed greater antibacterial activity than the aqueous extracts as indicated by zones of inhibition. At 12.5 mg/ml – 3.125 mg/ml, the ethanolic extracts of *Ocimum gratissimum* (Table 3) was not effective on the isolates. While at 50 mg/ml – 3.125 mg/ml the aqueous extracts of *Ocimum gratissimum* (Table 2) was not effective on the isolates. This indicates that the antibacterial activity of this leaf extracts is concentration-dependent. Ethanolic extract showed high inhibitory zones than aqueous extracts and when compared to standard antibiotic such as Pemaclav drug had an appreciable zone of inhibition of the test organisms. The result of this work showed that the ethanolic extract showed high inhibitory zones than aqueous extracts. This observed difference between these plants extracts may be due to insolubility of active compounds in water or the presence of inhibitors to the antimicrobial components Okigbo and Ogbonnanya [21], Amadioha and Obi [22], Okigbo and Ajale [23]. They have attributed this observation to the high volatility of ethanol which tends to extract more active compound from the sample than water, hence, this study follows similar trends. The aqueous extract of *O. gratissimum* showed a decrease in the level of inhibition against isolates at the highest concentration compared to the positive control, inhibition zones ranging from 9.0 to 10.0 mm.

The minimum inhibitory concentrations (MIC) of aqueous and ethanolic leaf extracts on the test organisms ranged between 25 mg/ml –100 mg/ml. The minimum inhibitory concentrations of ethanolic extracts of *O. gratissimum* as 25 mg/ml while aqueous leaf extracts of *O. gratissimum* had their MIC as 100 mg/ml. Minimum inhibitory concentrations (MICs) of both aqueous and ethanolic extracts on test organisms using agar dilution method revealed low MIC, which is an

indication of high efficacy of the leaf extracts while high MIC may indicate low efficacy or possible development of resistance by the microorganisms to the antimicrobial [24]. The aqueous extract showed its MIC at high concentration of 100 mg/ml while ethanolic extract showed its MIC at 25 mg/ml.

Oral administration of a single dose of ethanol and aqueous extracts of *O. gratissimum* of 5000 mg/kg bodyweight of the test animals produced no mortality in them. The general signs and symptoms of toxicity were observed for a period of 14 days after administration of the extracts. However, the following observations were made during the exposure period; slow movement, scratching of hair and mouth, tremor, raised hair coat and weakness. Thus, the median dose (LD_{50}) of the leaf extracts was estimated to be greater than 5000 mg/kg because 5000 mg/kg is the highest dose according OECD [19]. From the experiment performed as per the OECD Guidelines 2001, the results reveal that both aqueous and ethanolic extract of *Ocimum gratissimum* has been found nontoxic at 5000 mg/kg body weight of experimental animals as in the first 1 hour of observation, no morbidity was observed but weakness, slow movement, scratching of mouth, fur and body, tremor was observed and in the next 48 hours of observation mortality were not found and all that parameters used for evaluation of toxicity were found to be normal. No significant changes were observed in body weight. In the last 2 weeks of observation, no death rate recorded. As per observations and calculations from Acute Oral Toxicity (OECD Guidelines 2001), the LD_{50} value of aqueous and ethanolic Extract of *Ocimum gratissimum* was found to be more than 5000 mg/kg body weight of the rats.

Table 1. Phytochemical constituents of the leaf of *O. gratissimum* leaf extract

Phytochemical	*O. gratissimum*
Tannins	+
Saponins	+
Flavonoid	+
Cardiac glycoside	+
Alkaloid	+
Glycosides	+
Steroid	+
Volatile oil	+

Key:- = Not detected, + = Detected

Table 2. The antibacterial activities of aqueous leaf extracts of *O. gratissimum*

Test organisms	Zone of inhibition(mm)						
	O. gratissimum						
Concentration (mg/ml	100	50	25	12.5	6.25	3.125	+ve control
Salmonella typhi	9.0	x	x	x	x	X	20
Salmonella paratyphi	10.0	x	x	x	x	x	20

Key: Values are mean of three replicates (n=3); × = No zone of inhibition; +ve control = Pemaclav drug (10 mg/ml)

Table 3. The antibacterial activities of ethanolic leaf extracts of *O. gratissimum*

Test organism	Zone of inhibition (mm)						
	O. gratissimum						
Concentration (mg/ml)	100	50	25	12.5	6.25	3.125	+ve control
Salmonella typhi	14.0	13.0	12.0	x	x	x	20
Salmonella paratyphi	14.0	11.0	7.0	x	x	x	20

Key: Values are mean of three replicates (n=3); × = No zone of inhibition; +ve control = Pemaclav drug (10 mg/ml)

Table 4. Minimum inhibitory concentration of the aqueous and ethanol leaf extracts of *O. gratissimum* against *Salmonella* spp

Bacterial isolates	Aqueous extract MIC (mg/ml)	Ethanol extract MIC (mg/ml)	Pemaclav drug (Amoxicillin combination) MIC (mg/ml)
Salmonella typhi	100	25	12
Salmonella paratyphi	100	25	12

Values are mean of three replicates (n=3)

Table 5. Acute toxicity results on twenty randomly selected albino rats

Dose (mg/kg)	Time duration	No. of animals	No of deaths	Observation signs
5000	0-30 minutes	5	0	Weakness, slow movement immediately after administration.
	1 hour			Continuously scratching of mouthpart, fur and body, tremor.
	24 hours			Ruffled fur, scratching of their nostril.
	48 hours			Normal movement and less scratching of the body part.
	2 weeks			No death rate recorded.
5000	0-30 minutes	5	0	Increased breathing
	1 hour			Scratching of mouth and body parts
	24 hours			Ruffled fur
	48 hours			No scratching of the body part
	2 weeks			No death rate recorded

Key: a) The first 5 rats were given aqueous leaf extracts of O. gratissimum
b) The last two 5 rats were given ethanolic leaf extracts of O. gratissimum

4. CONCLUSION

From this study, it was observed that ethanol extracts exhibited high inhibitory activity on the test organisms. This can be deduced to the ability of ethanol to extract more of the essential oils and secondary plant metabolites which are believed to exert antibacterial activity on the test organisms. This suggests the possibility of using the ethanol extracts of *O. gratissimum* in treating the diseases caused by the test organisms. Aqueous and ethanolic extracts of *Ocimum gratissimum* exhibit no toxic effects when given orally at concentration of 5000 mg/kg body weight. However, the normalcy and insignificant changes in toxicity parameters and body weights reveal the safety of aqueous and ethanolic extract at a dose of 5000 mg/kg body weight. This study, however, can justify the use of the leaf in traditional medicine practice as a therapeutic agent and can explain the long historical use of these plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/51236