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ANTIMICROBIAL POTENTIALS OF *OCIMUM GRATISSIMUM* LINN: LEAVE EXTRACTS AGAINST PATHOGENIC BACTERIA AND FUNGI.

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ABSTRACT

Curing of diseases caused by pathogenic organism is important in health care system and trial of alternative medicine is important in this for good health care delivery. In-vitro antimicrobial activity of extracts of Ocimum gratissimum Linn.leaves was conducted using ethanol (70%), Corn Steep Liquor (CSL), and water as solvents and tested against Staphylococcus aureus, Salmonella typhimurium, Candida albicans and Trichophyton rubrum using Agar well diffusion method. These organisms were collected on sterile agar slant and incubated at 37^0C for 24 hours. Minimum Inhibitory Concentration (MIC) was also determined. Four different concentrations of the extracts were employed including 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml. The findings indicated that the effect of the different concentrations of the extract of CSL, aqueous and ethanol were significantly different ($p < 0.05$) on the bacterial and fungal isolates tested. However, no significant difference ($p > 0.05$) between the zone of inhibition observed in aqueous extracts at 150 mg/ml and 200 mg/ml on Salmonella typhimurium, Candida albicans and Trichophyton rubrum. The least MIC value was found to be 6.25 ± 0.00 mg/ml and 5.21 ±1.04 mg/ml exerted by the ethanol extracts of Ocimum gratissimum at 200 mg/ml concentration against bacterial and fungal isolates respectively and these values were not significantly different ($p < 0.05$) with the value of the control (5.21 ± 1.04 mg/ml). Ethanol extracts of O. gratissimum L. leaves at 200

mg/ml concentration recorded highest tannin, alkaloid, flavonoid and phenol values of 12.53 ± 0.08 mg/ml, 0.910 ± 0.010 mg/ml, 0.765 ± 0.015 mg/ml and 0.805 ± 0.005 mg/ml respectively when compared with its aqueous and CSL extracts. The antimicrobial potency of Ocimum gratissimum leaves is determined so that the nutritional and medicinal properties could be exploited judiciously. The results confirm the effective use of this plant in medicine, food system and pharmacy.

Keywords: Alternative, Minimum Inhibitory Concentration, Extracts, Corn Steep Liquor (CSL)

INTRODUCTION

The increased usage of antibiotics has induced microorganisms to acquire resistance factors which have become a burning predicament (Abimbola *et al.,* 1993). As a result there is an urgent need to find the alternative of chemotherapeutic drugs in diseases treatment particularly those of plants origin which are easily available and have considerably less side effects (Khulbe & Sati, 2009). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. These plant products are known by their active substances, for example, the phenolic compounds which are part of the essential oils as well as in tannin. The use of higher plants and their extracts for treating the infectious diseases has long been practiced in many parts of the world (Sofowora, 1984). *Ocimum gratissimum* Linn. is an aromatic, perennial herb, 1-3 m tall; stem erect, round-quadrangular, much branched, glabrous or pubescent, woody at the base, often with epidermis peeling in strips. The whole plant and the essential oil have many applications in traditional medicine, especially in Africa and India. Preparations from the whole plant are used as stomachic and in treating sunstroke, headache and influenza. The seeds have laxative properties and are prescribed against gonorrhoea. The essential oil is applied against fever, inflammations of the throat, ears or eyes, stomach pain, diarrhoea and skin diseases. It is being

tested as an antibiotic (Orwa *et al.,* 2009). The emergence of multiple drug resistant bacteria (MDR) has become a major cause of failure of the treatment of infectious disease. Recently, assessed the antibacterial activity of *V. amygdalina and O. gratissimum* leaves extract on selected food borne pathogens. The high zone of inhibition at low concentration proved the plants to be medically useful. Tested the antibacterial activity of the extract of leaves of *O. gratissimum* on *Listeria monocytogens* (Singleton *et al.,* 1999).Their findings yielded great significance in health delivery system, since it could be used as an alternative treatment to orthodox antibiotics in the treatment of diseases caused by the bacterial isolates especially as they frequently develop resistance to known antibiotics and reduce the cost of obtaining health care as observed by (Gislene *et al.,* 2000).

Sharma and Dietz, 2006 showed that extracts of *Zingiber officinale, Myristica fragrans, Ocimum gratissimum,* thyme, sage, rosemary, yarrow and guava showed antibacterial activity against antibiotic resistant bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus* sp. and *Shigella* sp. Koshy *et al.,* 2009 found ethanolic extracts and essential oil of *Zingiber officinale* and *Myristica fragrans* to be effective against the Enterobactericae. Also Braga *et al.,* 2005 tested the methanolic extract of *Z. officinale* to be effective against *Proteus* sp., *Bacillus* sp. *and Staphylococcus* sp. Okigbo and Ajale, 2005 found the ethanolic extract of *Z. officinale* and *M. fragrans* to be effective on *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. For these reasons, researchers are increasingly turning their attention to herbal products for new leads to develop much better drugs against MDR microbial strains (Nweze *et al.,* 2005).

MATERIALS AND METHODS

Collection and identification of plant sample : The leaves of *Ocimum gratissimum* Linn. was collected from the botanical garden at Moshood Abiola Polytechnic, Ojere, Abeokuta. It was identified

and authenticated at the Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria.

Extraction Procedure

Two hundred (200) grammes of the leaf sample was weighed, air-dried for two weeks and mashed into powder and sieved with mesh of size 0.50mm. The milled samples were labeled in a sterile conical flask. About 500ml of each solvent (i.e., ethanol, water and Corn steep liquor) were added to each of the milled sample vigorously shaken and allow standing for 24 hour using cold extraction method. The samples were filtered using filtration apparatus. After extraction, the solvent was evaporated off in a rotary evaporator (Stuart, Barloworld model RE 300) leaving the extract in the flask. After preparation of the crude extract as described by Junaid *et al.* (2006) the organic extracts were diluted with 50% Dimethylsulphoxide and distilled water (for aqueous extracts) to obtain concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml.

Quantitative Phytochemical Screening

Phytochemical screening was carried out on hot water and ethanolic extracts of *Ocimum gratissimum* Leaves using standard procedures to identify the constituents as described by Odebiyi and Sofowora (1990).

Test for Phenols

The quantity of phenols was determined using the spectrophotometer method. The plant sample was boiled with 50 ml of diethyl ether for 15min. 5 ml of the boiled sample was then taken into 50 ml flask, and 10 ml of distilled water was added. After the addition of distilled water, 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol is added to the mixture. The sample was made up to the mark and left for 30 min to react for colour development and measured at 505 nm wavelength using a spectrophotometer (Harbone, 1998).

Test for Alkaloids

Five (5) g of the plant sample were prepared in a beaker and 200 ml of 10% acetic acid in ethanol was added to the plant sample. The mixture was covered and allowed to stand for 4 hours. The mixture then filtered and the extract was allowed to become concentrated in a water bath till it reaches 1/4 of the original volume. Concentrated ammonium hydroxide was added until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is alkaloid, which was then dried and weighed (Harbone, 1998).

Test for Tannins

Quantity of tannins was determined by using the spectrophotometer method. 0.5 g of plant sample was weighed into a 50 ml plastic bottle. 50 ml of distilled was added and stirred for 1 hour. The sample was filtered into a 50 ml volumetric flask and made up to mark. 5 ml of the filtered sample was then pipette out into test tube and mixed with 2 ml of 0.1 M ferric chloride in 0.1 M hydrochloric acid and 0.008 M potassium ferrocynide. The absorbance of the sample was measured with a spectrophotometer at 395 nm wavelength within 10 minutes (Harbone, 1998).

Test for Saponins

The plant samples were ground and 20 g of each plant sample was put into a conical flask and 100 ml of 20% ethanol was added to the plant sample. The sample was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% ethyl alcohol. The combined extracts are reduced to 40 ml over a water bath at about 90°C. The concentrated was then transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added to the extract and vigorously shaken. The aqueous layer was recovered while the diethyl ether layer was discarded and the purification process was repeated. 60 ml of n-butyl alcohol was

added and the combined n-butyl alcohol extracts was washed twice with 10 ml of 5% sodium chloride. The remaining solution was then heated in a water bath and after evaporation; the samples are dried in the oven to a constant weight (Harbone, 1998).

Test for Flavonoids

Ten (10) g of plant sample is repeatedly extracted10 g of plant sample is repeatedly extracted with 100 ml of 80% aqueous methanol at room temperature. The whole solution was then filtered through filter paper and the filtrate was later on transferred into a water bath and solution was evaporated into dryness. The sample was then weighed until a constant weight (Harbone, 1998).

Test for Steroids

One (1) ml of Methanolic extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hex cyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70 ± 20°C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank (Harbone, 1998).

Terpenoids

About 2 g of the plant powder was weighed and soaked in 50 ml of 95% ethanol for 24 hours. The extract was filtered and the filterate extracted with petroleum ether (60^0C) and concentrated to dryness. The dried extract was treated as total terpenoids (Harbone, 1998).

Anthocyanins

This was done gravimetrically by the method of Harborne (1998). Five (5) g of each test sample was hydrolyzed by boiling in 100ml or 2M Hydrogen Chloride solution for 30 minutes. The hydrolysate was filtered using Whatman No 42 filter paper. The filtrate was

transferred into a separation funnel and equal volume of ethyl acetate was added to it, mixed well and allowed to separate into two layers. The ethyl acetate layer (extract) was collected while the aqueous layer was discarded. The extract was separated to dryness in the crucible over a steam-bath. The dried extract was then treated with concentrated amyl alcohol to extract the anthocyanins. After filtration, the alcohol extract and the filtrate was transferred to a weighed evaporating dish and evaporated to dryness. It was then dried in the oven at 300^0C (Harbone, 1998).

Anthraquinones

Borntrager's reaction was used to detect anthraquinone aglycones in the extract. About 2 ml of 2M hydrochloric acid was added to 8 ml of the sample, and the mixture was heated on a hot water bath for 15 minutes, then cooled and filtered. The filterate was then extracted with chloroform. The chloroform layer was separated and shaken with 10% potassium hydroxide solution. The total anthraquinone content was analysed by UV spectrophometer at 515 nm (Harbone, 1998).

Collection and Maintenance of Test Organisms

The test organisms that were used for this study were all clinical isolates (two Bacteria and two Fungi) collected from the Department of Medical Microbiology and Parasitology, Sacred Heart Hospital, Lantoro, Abeokuta, Ogun State. These isolates include *Staphylococcus aureus, Salmonella typhimurium, Candida albicans and Trichophyton rubrum.* The organisms were collected on sterile agar slant and incubated at 37^0C for 24 hours. Biochemical analysis was carried out on each test organism (bacteria), the tests include: sugar fermentation, citrate utilization, oxidase reaction, vogue proskauer, methyl red, capsule staining, spore staining, motility, indole test, urease test, hydrogen sulphide test, gelatin liquidification and gram staining reaction. *Candida albicans* was identified by Gram staining, germ tube test, sugar fermentation and assimilation tests. *Trichophyton rubrum* was identified

macroscopically and microscopically using lactophenol cotton blue stain. These were then kept as stock cultures on slants in the refrigerator set at 4°C.

Preparation of Inoculi

Inoculum was prepared using Mueller Hinton broth prepared according to the Manufacturer's instruction. Five (5) ml of broth medium was dispensed into each screw capped test-tube and sterilized by autoclaving at 121^0C for 15 minutes. The test-tubes were cooled and kept in an incubator for 24 hours at 37^0C to check sterility. The isolates were inoculated into the sterilized test-tubes containing the medium, and placed in an incubator overnight at 37^0C. The presence of turbidity in broth culture was compared to 0.5 McFarland standards to obtain standardized suspension by adding sterile normal saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard or against a white background with contrasting black line. The McFarland 0.5 standard provides turbidity comparable to bacterial suspension containing 1.5 x 10^8 cfu/ml according to National Committee for Clinical Laboratory Standards (NCCLS, 1993). The inoculi should give semi confluent growth of colonies after overnight incubation. Denser inoculi will result in reduced zone of inhibition and lighter inoculi will have opposite effect.

Determination of antimicrobial activity (Agar well diffusion method).

Antimicrobial activities of extracts were evaluated by the well plate agar diffusion method using modified (Aida *et al.,* 2001).

Minimum Inhibitory Concentration (MIC)

The MIC for both bacterial and fungal isolates was determined according to Ochei and Kolhatkar (2008) using, microtubes dilution method described by National Committee for Clinical Laboratory standards (NCCLS, 2000).

RESULTS

Agar diffusion test for *Ocimum gratissimum* (Leaves)

The effect of the different concentrations of the extract of CSL, aqueous and ethanol were observed to be significantly different ($p < 0.05$) on the bacterial and fungal isolates tested (Table 1). There was no significant difference ($p > 0.05$) between the zone of inhibition observed in CSL extract at 200 mg/ml and the control on the fungal isolates. However, the control (Cloxacillin for Bacteria and Fluconazole for Fungi) showed a significantly higher inhibitory effect on all isolates than the different extract concentrations of CSL. No significant difference ($p > 0.05$) between the zone of inhibition observed in aqueous extracts concentration of the plant extract on the microbes challenged with. The control for both bacterial and fungal isolates was observed to exert significantly higher ($p < 0.05$) inhibitory effect than the different concentration of aqueous extract of *Ocimum gratissimum* (Table 1).

The rate of inhibition of extracts on the microbial isolates followed the trend 200 mg/ml > 150 mg/ml > 100 mg/ml > 50 mg/ml. However, no significant difference ($p > 0.05$) was observed in the inhibition zones of *Ocimum gratissimum* ethanol extract at 200 mg/ml and control on *Staphylococcus aureus* and *Trichophyton rubrum* for all the microbial isolates used. Comparing the inhibitory effect of the different extracts of *Ocimum gratissimum* (CSL, aqueous and ethanol), ethanol extracts were observed to have the highest inhibitory effect at 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml on all the microbial isolates tested (Table 1).

Minimum Inhibitory Concentration (MIC) of *Ocimum gratissimum*

The least MIC value was found to be 6.25 ± 0.00 mg/ml and 5.21 ±1.04 mg/ml exerted by the ethanol extracts of *Ocimum gratissimum* against bacterial and fungal isolates respectively and these values were not significantly different ($p < 0.05$) with the value of the control (5.21 ± 1.04 mg/ml).

DISCUSSION

The antimicrobial activity of crude extract of *Ocimum grattisimum* were evaluated using three different extracts including aqueous, ethanol and corn steep liquor. The entire test organisms were susceptible to all the extracts though to varying degrees. The ethanol extract (at 200 mg/ml concentration) showed the highest level of inhibition on all the test organisms. Though there was no significant difference ($p > 0.05$) between ethanol extract and the control at 200 mg/ml concentration. The result for minimum inhibitory concentration revealed that the ethanol extract exhibited a high degree of effectiveness against all the test organisms. This attests to the findings of Udochukwu *et al.* (2015) on phytochemical analysis of *Ocimum gratissimum* and *Vernonia amygdalina* extracts on some drug resistant bacteria. He reported that ethanol extract of *Ocimum gratissimum* was more effective against *Staphylococcus aureus* than ethanol extract of *Vernonia amygdalina*. Tannin was the highest phytochemical observed in *Ocimum gratissimum* leaf. Anthocyanin was the lowest secondary metabolite observed in *Ocimum gratissimum* ethanol, aqueous and corn steep liquor extracts respectively. High tannin, saponin, flavonoid and alkaloid content in *Ocimum gratissimum* could make it useful in the treatment of diabetes. This was supported by Ojewumi and Kadiri (2014) who worked on the physiological evaluation of the anti-diabetic properties of *Morinda lucida* on rats.

They found these phytochemicals to be present and high in *Morinda lucida* which had a hypo-glycemic effect on rats. In vitro studies show that flavonoids also have anti-allergic, anti-inflammatory, anti-microbial (Cushnie, 2005), anti-cancer and anti-diarrheal activities (Shueir *et al.*, 2005). In vitro, flavonoids have antiviral activity against several viruses; among them is poliovirus (Gonzaiel *et al.,* 1990). At very high concentration, flavonoids chelate metals such as iron and zinc and reduce the absorption of these nutrients. They also inhibit digestive enzymes and may also precipitate proteins. In one experiment, flavonoids were found to be strong topoisomerase

inhibitors and induce DNA mutations in the MLL gene, which are common findings in neonatal acute leukemia (Strick *et al.,* 2000). High level of alkaloids exerts toxicity and adverse effects to humans, especially in physiological and neurological activities. For instance, consumption of tropane alkaloids will cause rapid heartbeat, least amount of alkaloid was observed in aqueous extract , this is supported by the fact that lower dose of alkaloids mediate important pharmacological activities, such as analgesic, reducing blood pressure, killing tumour cells, stimulating circulation and respiration . Saponins are effective in maintaining liver function, lowering blood cholesterol, preventing peptic ulcer, osteoporosis as well as platelet agglutination (Kao *et al.,* 2008). The beneficial effects of saponins have been applied commercially in drugs and medicines, emulsifiers, adjuvants, taste modifiers, sweeteners and precursors of hormone synthesis. Tannins have shown potential antiviral, antibacterial and antiparasitic effects (Akinyama *et al.,* 2001). It was also reported that certain tannin are able to inhibit HIV replication selectivity and is also used as diuretic. Plant tannin has been recognized for their pharmacological properties and is known to make trees and shrubs a difficult meal for many caterpillars (Haslem, 1989). Phenol was highest in ethanol extract of *Ocimum gratissimum* leaf. Phenols are reported antitumour agents and exhibit antiviral and antimicrobial activities, hypotensive effects and antioxidant properties (Egbuna *et al.,* 2015).

Appreciable steroidal content of ethanol extracts of *Ocimum gratissimum* leaf (0.325 mg/ml) explains its usefulness in the treatment of some skin infections like ringworm. Steroids have been reported by Raquel (2007) to have antibacterial properties and they are very important compounds especially due to their relationship with compounds such as sex hormones too (Okwu, 2001). Terpenoid which was found to be minimal in ethanol extract of *Ocimum gratissimum* leaf have also shown antimicrobial activities (Islam *et al.*, 2003). This is important due to the increase in antibiotic

resistant bacteria, which is occurring globally and at an alarming rate.

Table 1: Agar Diffusion Test for *Ocimum Gratissimum* (Leaves)

Extracts	Conc. (mg/ml)	*Staphylococcus aureus*	*Salmonella typhimurium*	*Candida albicans*	*Trichophyton rubrum*
CSL	50	11.33±1.20^{d}	12.67±0.88^{c}	11.33±0.88^{c}	10.00±0.58^{d}
	100	14.33±0.88^{cd}	14.33±0.33^{bc}	13.67±0.88^{bc}	13.00±0.58^{cd}
	150	16.00±0.58^{bc}	15.33±0.33^{bc}	15.33±0.88^{b}	14.33±1.20^{bc}
	200	19.33±0.88^{b}	17.33±0.88^{b}	16.67±0.88^{ab}	17.33±0.88^{ab}
	Control	24.33±0.88^{a}	22.00±0.58^{a}	19.67±0.67^{a}	19.33±0.88^{a}
Aqueous	50	9.00±0.58^{d}	9.33±0.33^{c}	9.67±0.33^{d}	8.67±0.33^{c}
	100	11.33±0.33^{cd}	10.67±0.67^{c}	11.67±0.33^{c}	12.00±1.15^{bc}
	150	12.67±0.88^{bc}	14.00±0.58^{b}	13.67±0.33^{b}	13.67±1.20^{b}
	200	15.00±0.58^{b}	15.33±0.67^{b}	14.67±0.33^{b}	15.00±0.58^{b}
	Control	24.33±0.88^{a}	22.00±0.58^{a}	19.67±0.67^{a}	19.33±0.88^{a}
Ethanol	50	13.00±0.58^{c}	14.33±0.88^{d}	11.67±0.88^{d}	11.00±0.58^{c}
	100	15.33±0.88^{bc}	15.33±0.33^{cd}	14.00±0.58^{cd}	14.00±0.58^{bc}
	150	17.67±0.88^{b}	18.00±0.58^{bc}	17.00±0.58^{bc}	16.00±0.58^{b}
	200	22.00±1.15^{a}	21.00±1.15^{ab}	21.33±1.45^{ab}	19.33±0.88^{a}
	Control	24.33±0.88^{a}	22.00±0.58^{a}	19.67±0.67^{a}	19.33±0.88^{a}

^{abc}Mean values (±Standard error) of each of the extracts in the same column having the same superscript are not significantly different at $p > 0.05$. CSL = Corn Steep Liquor;

Table 2: Minimum Inhibitory Concentration (MIC) of *Ocimum Gratissimum*

Plants	Extracts	*Staphylococcus aureus*	*Salmonella typhimurium*	*Candida albicans*	*Trichophyton rubrum*
Ocimum gratissimum	**CSL**	**8.33±2.08^{b}**	**10.42±2.08^{ab}**	**10.42±2.08^{ab}**	**10.42±2.08^{a}**
	Aqueous	20.83±4.17^{a}	12.50±0.00^{a}	16.67±4.17^{a}	16.67±4.17^{a}
	Ethanol	6.25±0.00^{b}	6.25±0.00^{b}	5.21±1.04^{b}	6.25±0.00^{a}
	Control	5.21±1.04^{b}	5.21±1.04^{b}	6.25±0.00^{ab}	6.25±0.00^{a}

[abc]Mean values (±Standard error) of each of the extracts in the same column having the same superscript are not significantly different at p > 0.05. CSL = Corn Steep Liquor.

Table 3: Quantitative Phytochemical Screening of *Ocimum Gratissimum* Leaves

Plant Extracts	Steroid	Alkaloids	Phenols	Tannins	Flavonoid	Saponin	Terpenoid	Anthocyanin	Anthraquinone
***Ocimum gratissimum* CSL**	0.200 ±0.010^{cd}	0.845 ±0.005^{b}	0.210 ±0.010^{b}	11.705 ±0.145^{c}	0.450 ±0.010^{c}	1.220 ±0.020^{e}	0.140 ±0.010^{c}	0.025 ±0.005^{b}	0.300 ±0.010^{ab}
***Ocimum gratissimum* Aqeous**	0.155 ±0.005^{d}	0.225 ±0.005^{e}	0.145 ±0.005^{c}	10.295 ±0.045^{d}	0.330 ±0.010^{d}	1.070 ±0.010^{f}	0.125 ±0.005^{c}	0.015 ±0.005^{b}	0.270 ±0.010^{b}
***Ocimum gratissimum* Ethanol**	0.325 ±0.015^{b}	0.910 ±0.010^{a}	0.805 ±0.005^{a}	12.525 ±0.075^{b}	0.765 ±0.015^{b}	2.325 ±0.025^{b}	0.200 ±0.010^{b}	0.050 ±0.010^{b}	0.315 ±0.005^{a}

[abc]Mean values (±Standard error) of each of the phytochemicals in the same column having the same superscript are not significantly different at p > 0.05. CSL = Corn Steep Liquor.

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