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Antioxidant and Inhibitory Properties of Essential Oil of Ocimum Gratissimum Against Extracellular Protease of Escherichia Coli

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Abstract:*The oil of Ocimum gratissimum extracted by hydrodistillation was tested against the partally purified extracellular protease of E.coli The kinetics of the extracellular protease showed that the inhibition was competitive and that the extracellular protease has a k_m of 0.056 mg/ml in the presence of the inhibitor (oil of Ocimum gratissimum) and 0.08 mg/ml in its absence and with a V_{max} of 1.66 $vol^{-1}(\mu mol/min)^{-1}\times10^{-4}$. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were 0.53% and 0.72% respectively. The volatile oil of Ocimum gratissimum showed good antioxidant activity when used in scavenging DPPH radicals as compared to the BHT control. The oil has a mean activity value of 66.983 compared with 53.290 of BHT. The minimum percentage scavenging activity against DPPH is 50% for Ocimum gratissimum volatile oil as compared to 30% for BPH. The enzyme had an optimal pH of 6.5 and optimum temperature of 40^{o}C. Fe^{2+}, Ca^{2+}, Mn^{2+} and $Fe^{2+}$are metal activators of the extracellular protease of E.coli.*

I. Introduction

Medicinal plants have contributed immensely to health care in Nigeria. This is due in part to the recognition of the value of traditional medical systems, particularly in Asian origin, and the identification of medicinal plant from indigenous pharmacopoeias, which have significant healing power. (John *et al,* 2000). The medicinal properties of plants have been discovered many centuries ago before now, and had been the backbone of all therapies for several years until the intervention of synthetic drugs. Though the synthetic drugs have been effective in therapy, the world is now witnessing a return to herbal remedies due to the numerous and dangerous side effect accompanying the action of synthetic drugs. (Amar *et al,* 2004).The medicinal values of these plants lie in their component phytochemicals, which produce definite physiological actions on the human body. Some of these phytochemicals include alkaloids, tannins, flavonoids and phenolic compounds. Extracts from the leaves of *O. gratissimum* possesses good antioxidant potential presumably because of its phytochemical constituents (Hill, 2001). Among all families of the plant kingdom, members of the Lamiaceae have been used for centuries in folk medicine. *Ocimum gratissimum* L (Lamiaceae), commonly known as "alfavaca" is naturally used in the treatment of different diseases, for example upper respiratory tract infections, diarrhea, headache, fever, ophthalmic, skin disease and pneumonia (IIori *et al* ., 1996).

Several species and varieties of plants of the genus *Ocimum* have been reported to yield oils of diverse nature, commonly known as basilic oils. Craveiro *et al*., (1981). Janine de Aquino Lemos *et al*., (2005) reported some chemical components and active ingredients found in these plants such as; eugenol, linaol, methyl cinnamate, camphor and thymol. It has been demonstrated that the eugenol isolated from *O. gratissimum* presented antimcrobial (Janine de Aquino Lemos *et al*., 2005) insecticidal (Nikam 1982), antihelmintic (Pessoa *et al*., 2002), nematicidal and fungistatic properties (Reuveni *et al*., 1984). Volatile oils confer the characteristic fragrance of a plant on it. They are made up of secondary metabolites especially the neutral ones such as terpenes and autogenins. Apart from their uses in the production of many fragrances, they have also been used as antimicrobial agent. (Danuta 1998). The essential oil of *O. gratissimum* possesse insecticidal,, antibacterial, and antifungal properties. The essential oil of *O. gratissimum* and its main component eugenol were reportedly efficient in inhibiting eclosion of *Haemonchus contortus*, a gastrointestinal parasite of small ruminants. Essential oils contain the true essence of the plant . (Waung *et al*., 2000)Many bacteria, fungi and protozoan are human pathogens, which produce proteases both intracellularly and extracellularly with which they accomplish their physiological activities. In addition, some extracellular proteases produced by the pathogens are geared towards the effectiveness of their virulence (Boyd *et al*, 2000). Proteases play a crucial role in numerous pathologic processes. Arthritis, tumor invasion and metastasis, infections and a number of degenerative diseases have been linked with the involvement of one or more proteolytic enzymes. Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. The virulence of *E.coli* is

multifactorial, but it is partly determined by exoproducts such as alkaline protease and elastase, which are responsible for the damage of tissues by degrading elastin, collagen and proteoglycans. These enzymes have been also shown to degrade proteins that function in host defense in vivo (Sakata et al. 1993).Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most E. coli strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine. (Hudault *et al*, 2001). Herbal medicine in developing countries is commonly used for the traditional treatment of health problems. In recent years, multiple drug resistance in human pathogenic microorganisms have developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on host including hyper-sensitivity, immune suppression and allergic reactions. Therefore there is a need to develop alternative antimicrobial drugs for the treatment of infections. The volatile oils of these medicinal plants with antimicrobial activity, can serve as alternative source for the treatment of infectious diseases.The study is therefore designed to investigate the antimicrobial and antioxidant activities of the volatile oil of Ocimum gratissimum and its potential inhibitory effect on the extracellular proteases of partially purified and characterized Escherichia coli.

II. Materials and methods

2.1. Plants Materials

Ocimum gratissimum whole plants were obtained at Ojo Local Government Area of Lagos State, Nigeria, as green foliage and were air-dried. The plant was identified and authenticated at the Department of Botany, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria.

2.2. Microorganisms

The *E. coli* used in this work was a gift from the Nigeria Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria and maintained on nutrient agar petri-dishes.

2.3. Extraction of Volatile Oil (Hydrodistillation)

The air-dried *Ocimum gratissimum* plant was separated into leaves, stems, and roots and each part was cut into pieces and packed into the 5 L 34/35 Quick fit round bottom flask containing 1.5 L distilled water with fixed Clevenger. The oil was extracted at a steady temperature of 80 ^{o}C for 3 hrs and the oil was collected over 2 ml *n*-hexane. The extracted oil was stored at 4 ^{o}C.

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of the volatile oil of *Ocimum gratissimum* were determined using microbroth dilution method (Janssen *et al.,* 1989). A colony of *E.coli* was added to 200 µl of susceptible test Muller Hinton broth containing two-fold serial dilution of the volatile oil using Tween 80 (0.5%v/v) as diluent in a microtitre plate (21.5 x 17 cm^2). The plates were covered with aluminium foil and incubated at 37^{o}C for ≈24 hrs. Each of the micro well on the plate was inoculated on a freshly prepared Muller Hinton agar where MIC and MBC were determined.

2.5. Extraction of crude enzyme (Makino *et al.,* 1981)

A colony of the *E.coli* picked and inoculated into the MH broth was incubated for ≈24hrs at 37^{0}C after which it was centrifuged at 9000rpm for 10mins at room temperature. The supernatant was decanted and stored at 4^{0}C. The supernatant and the sediment were re-inoculated on a fresh medium of MH agar and the results were recorded after 24hrs of incubation. This is to establish the fact that only extracellular protease were collected and that the cells were not lyzed.

2.6. Enzyme partial purification, activity, optimal pH, temperature and inhibition assays.

All these were carried out according to the methods of Makino *et al*., 1981, while the protein was determined (Lowry et al, 1951)

2.7. Enzyme (protease) purification:

2.7.1. Dialyses: (Makino, et al., 1981)

Ammonium sulphate ($(NH_4)_2$ $SO_{4)}$ was added was added to 1ml of the supernatant-1 to 55% (crude extract) and dialyzed for 48hrs against tris buffer. The dialysate was then centrifuged at 9000rpm for 10min. The sediment-2 collected was assayed for total protein and enzyme activity. The sediment was further added to 0.5g of the same salt, dissolved in 1ml of the buffer and dialyzed for another 48hrs and then centrifuged to get supernatant-3 and sediment-3. Both again were assayed for protein and enzyme activity.

2.7.2. Gel filtration: (Makino et al., 1981)

The dailysate from above was packed and separated on a sephadex G-100 at a flow rate of 3ml/50min. Fractions were collected and assayed for protein and enzyme activity.All data presented were processed using SPSS and Graphpad

III. Results

Summary of Results

This micro broth dilution method shows that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the oil of *Ocimum gratissimum* against *E.coli* were 0.53% and 0.72% respectively (fig.1)

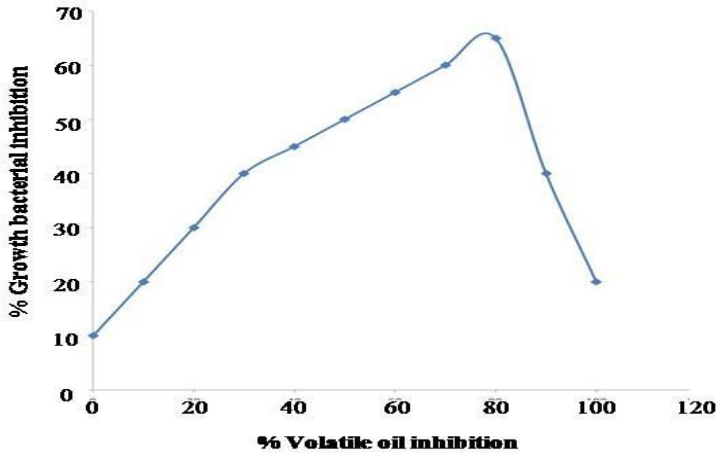

Figure 1**:** Plot showing the minimum inhibitory and minimum bacteriocidal concentration of the essential oil of *Ocimum gratissimum.*

The optimum pH of the extracellular protease of *E.coli* was 6.5 (fig.2), with their temperature optimum 40^{o}C (fig.3).

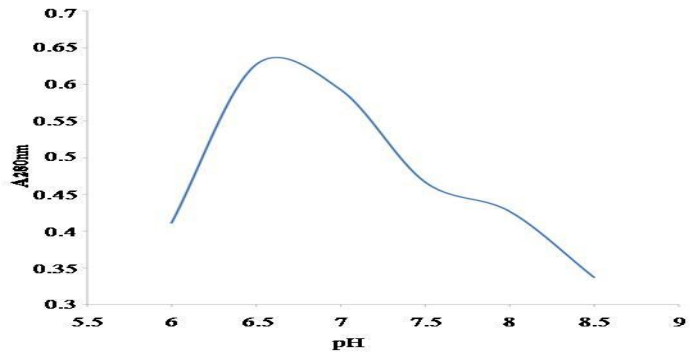

Figure.2**:** Plot showing the effect of pH on the extracellular protease of *E.coli.*

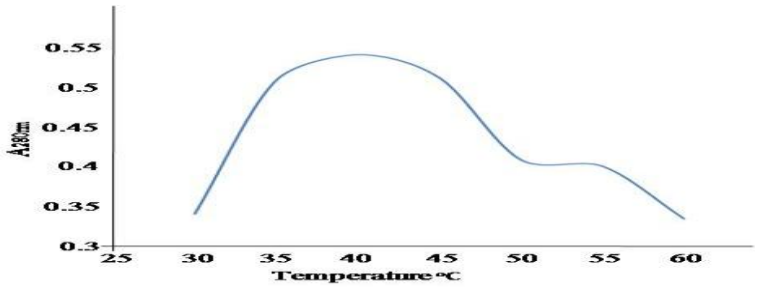

Figure.3: Plot showing the effect of temperature on the extracellular protease of *E.coli.*

The graph shows that the enzyme exhibited its optimum temperature at 40°C The double reciprocal plot shows that inhibition is competitive having the same Vmax but different Km value. This suggests that both substrate and inhibitor could have the same structural resemblance by competing with each other for the enzyme catalytic site. The K'_m value is 0.056mg/ml in the presence of the inhibitor and a K_m of 0.080mg/ml in the absence of inhibitor. It also has a V_{max} of 1.66µmol/min (fig.4).

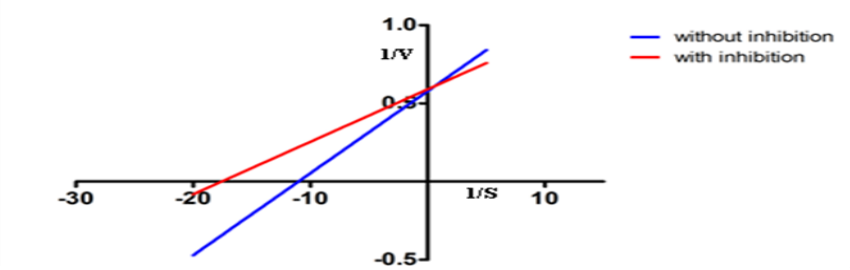

Figure 4**:**Double reciprocal Lineweaver Burke plot of the enzyme activity of the extracellular protease of *E.coli* using oil of *Ocimum gratissimum* as inhibitor.

Of all the metallic salts used on the protease of *E.coli*, Ca^{2+}, Fe^{2+}, K^{+}, and Mn^{2+} were observed to be activators of the protease (fig.5).

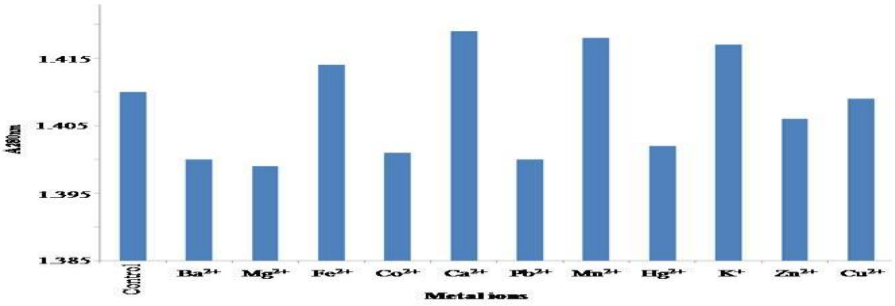

Figure.5**:** Plot showing the effect of different metal ions on the extracellular protease from *E.coli.*

The study also shows that the volatile oil of Ocimum gratissimum has antimicrobial activity and high antioxidant activity by scavenging DPPH more than BHT, with its minimum inhibition percentage was 50% (fig 6). The graph shows that in every volume, O.gratissimum volatile oil possesses more % radical scavenging activity compared to the BHT, which indicated that the oil has more antioxidant activity as compared to BHT.

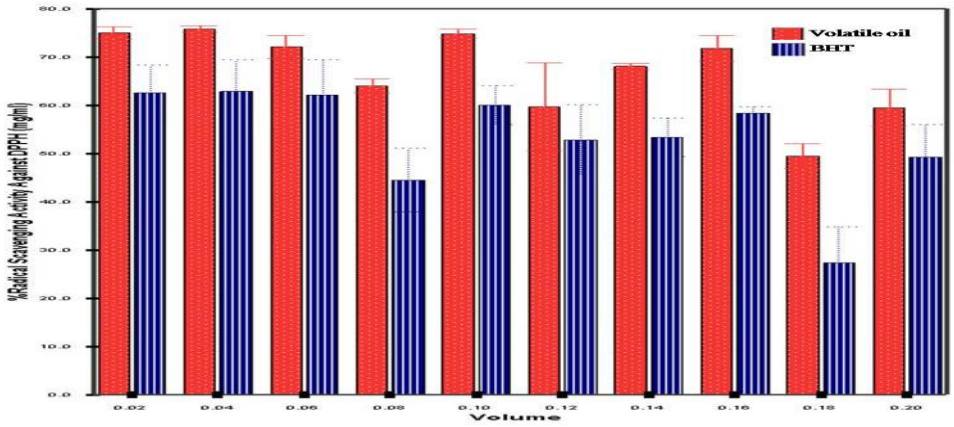

Figure .6**:** Plot showing the antioxidant activity of the volatile oil of *O.gratissimum* and BHT (control) against DPPH **.**

The elution profile of the extracted and partially purified protease from *E.coli* shows two peaks as presented in fig 7. The elution profile of the protease of *E.coli* shows a peak for the protease activity and two distinct peaks for total protein

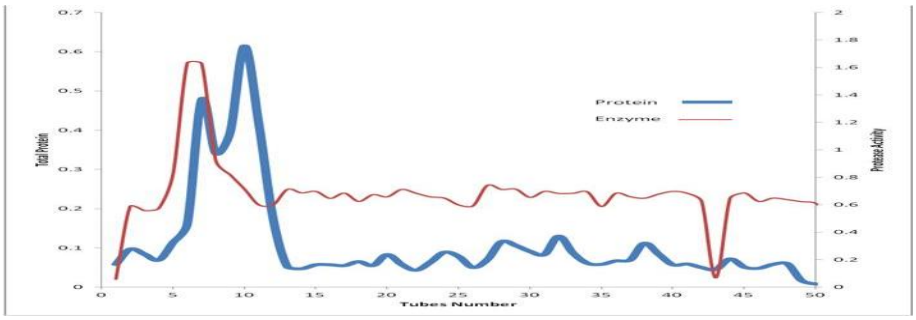

Figure.7: Elution profile of fractions eluted from Sephadex G-100 column.

.Table 1. Purification table

Purification Steps	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg protein)	Percentage Yield	Purification Fold
Crude Cellular Extract	0.012	0.016	1.333	100	1
55% $(NH_2)SO4$ prep	0.532	0.883	1.468	67	3
Sephadex G-100	0.089	0.149	1.674	8.2	7

IV. Discussion

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by micro serial dilution and showed that the volatile oil from the leaves of Ocimum gratissimum inhibited E.coli by 0.53% and 0.72% inhibition respectively. This suggests that some strains of E.coli are susceptible to growth inhibition by volatile oils. This data corroborates the findings of Kareni et al, 2005, that reported MIC and MBC of the volatile oil of Ocimum gratissimum on E.coli as 0.58% and 0.77% respectively.
The extracellular protease from E.coli was partially purified using ammonium sulphate precipitation and gel filtration.. Optimum pH and temperature was determined to be 6.5 suggesting that the protease was slightly acidic, and the enzymes had highest activity at 40°C after which they were rapidly inactivated. This is close to the findings of Sakata et al, 2007 who reported a pH of 6.5 and optimal temperature of 37°C for an isolated protease of E.coli. This findings shows that E.coli carry out their pathogenic functions at normal body temperature and its acidic nature shows they can cause diseases at the urinary tract and the stomach which are acidic environment.The enzyme kinetics using 0.6% Casein as substrate showed that the volatile oil of Ocimum gratissimum competitively inhibit the protease indicating that the volatile oil of Ocimum gratissimum is potentially capable of reducing the catalytic activity of the extracellular protease of E.coli. The extracellular protease had a k_m of 0.056 mg/ml in the presence of the inhibitor (oil of Ocimum gratissimum) and a k_m of 0.08 mg/ml in the absence of inhibitor. It also had a V_{max} of 1.66 $vol^{-1}(\mu mol/min)^{-1}\times10^{-4}$. The competitive nature of the inhibition suggests that the volatile oils exhibited antimicrobial properties on the protease of E.coli by binding to the active site of the enzyme thereby preventing the real substrate from being bound. This result correlate with the findings of Adebolu et al, (2005) that uses three different extracts of Ocimum gratissimum oil, cold water extract (CWE), hot water extract (HWE), and steam distillation extract (SDE), on four different pathogenic microorganism, Staphylococcus aureus, Escherichia coli, Salmonella typhi and Salmonella enteritidis, and with only the steam distillation extract showed antimicrobial activity.Of all the metals tested (fig.5), Ca^{2+}, Fe^{2+}, K^{+}, and Mn^{2+} had enhanced enzyme activity that surpassed that of the control, suggesting activator potentials for extracellular protease of E.coli. This corresponds with the findings of Hoshman et al, 2009 that reported that Ca^{2+}, and Fe^{2+}, activated the extracellular protease of E.coli. The Sephadex G-100 gel filtration which is capable of separating proteins with molecular weight of 75 – 200kdal gave two peaks. The two peaks revealed by G-100 suggests the presence of more than one protease or a single protease made up of subunits (suggesting a polymeric nature) This finding agrees with the report of Harrison (1998), that the purified protease of E.coli had two subunit using gel precipitation methods.The antioxidant activity potentials of Ocimum gratissimum oil was determined by comparing it with that of BPH which was used as standard. In

fig.6, it is observed that Ocimum gratissimum oil possesses more antioxidant activitycapabilities. The oil has a mean activity value of 66.983 to mean value of 53.290 value of that of BHT and minimum percentage scavenging activity against DPPH as 50% for Ocimum gratissimum volatile oil as compared to BPH which is 30%. This corroborates the findings of Owolabi et al, 2009, that Ocimum oil have high scavenging activity than BPH. This indicates that the oil of Ocimum gratissimum can serve as a good antioxidant , capable of scavenging free radicals, which are implicated as oxidative stress in diseases, like Alzheimer's disease etc. This corresponds with the reports of Johnson et al, 2007, that the oil of Ocimum gratissimum has a large antioxidant activity by reducing free radicals when used on mice with Alzheimer disease for 3 months.

V. Conclusion

The volatile oil of Ocimum gratissimum possesses antimicrobial activity against E.coli which could present as a pathogenic microorganism whose virulence is enhanced by the secretion of some extracellular proteases. E.coli is known to infect the urinary and pulmonary tracts, it is therefore conceivable that this extract could be used to treat cases of disease caused by these pathogenic organisms in infected individuals. The high antioxidant activity of Ocimum gratissimum oil makes its volatile oil a very good anti-free radical agent to reduce oxidative stress associated with some diseases like dementia. Further studies and clinical trials may be carried out to evaluate the potential of this oil as an antimicrobial agent against a wide range of microorganisms as well as the isolation and purification of the active component in the oil

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