ANTIOXIDANT ACTIVITY OF THE VOLATILE OIL OF *Cymbopogon citratus* AND ITS INHIBITION OF THE PARTIALLY PURIFIED AND CHARACTERIZED EXTRACELLULAR PROTEASE OF *Shigella sonnei*

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ABSTRACT

The low susceptibility of pathogenic organisms to antibiotics and other conventional drugs and the discovery that the oil of some plants contains therapeutic and antimicrobial properties encouraged research into the use of volatile oils as antibacterial agents. This study was carried out to evaluate the antimicrobial effect of the volatile oil of *Cymbopogon citratus* leaves against *Shigella sonnei* and to partially purify and characterize the extracellular protease of this organism. The percentage degree yield of the oil from this plant was 0.56. This oil showed a significant lower *($p<0.05$)* antioxidant effect (37.0 ± 6.9) and IC_{50} (3.83%v/v) against DPPH as compared to BHT (54.0 ± 3.0 and 4.25%v/v). This volatile oil inhibited the growth of *Shigella sonnei* with IC_{50} value of 1.58%v/v. Optimal activity of the extracellular protease of *Shigella sonnei* was observed at pH 7.5 and 40^{o}C. Chloride salts of Cu^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, $Mg^{2+}$and K^{+} appeared not to have any effect on the enzyme while Hg^{2+}, Co^{2+} and Ba^{2+} inhibited the activity of this extracellular protease. However, Mn^{2+} appeared to activate the enzyme. The volatile oil of *Cymbopogon citratus* as inhibitor of the extracellular protease showed a competitive inhibition with a of V_{max} = 800 µmol/min, K_m = 0.27mg/ml and K_m apparent of 0.53mg/ml. Highest fold of 3.73 was achieved with specific activity of 48.33 µmol/min/mg protein as compared to the crude extract. The gel filtration (G-100) produced two peaks for total protein and a peak for enzyme activity. The volat*ile* oil of *Cymbopogon citratus* will prove to be an effective nutraceuticals against *Shigella sonnei* infections, which are commonly characterized by immediate diarrhea, fever and stomach cramps.

{Adeola S. Adesegun, Folorunso O. Samuel, Raimi G. Olawale and Sowunmi A Funmilola. Antioxidant activity of the volatile oil of *Cymbopogon citratus* and its inhibition of the partially purified and characterized extracellular protease of *Shigella sonnei*. American Journal of Research Communication, 2013, 1(1): 31-45} www.usa-journals.com, ISSN: 2325-4076.

Keywords: *Shigella sonnei, antimicrobial agent, volatile oil, Cymbopogon citratus, extracellular protease*

INTRODUCTION

Medicinal plants are plants with proven chemical properties, which have been used for centuries as remedies for human diseases especially those caused by bacterial, fungal, viruses, protozoans and associated diseases because they contain chemical components of therapeutic values (Sumner, 2000; Tapsell et al, 2006). Some of these components include alkaloids, tannins, polyphenols, saponins, sugars, amino acids, vitamins, minerals, ashes and volatile oils (Parvathi & Kumar, 2002; Dónal & Walt, 2001). The essential (volatile) oils as one the phytocomponents of plants have been used in industries to produce fragrance, perfumes, confectionery, food additives, emulsion, grease, ornaments and many others. Medically, these volatile oils have not been maximally utilized, despite series of available information about the medicinal importance of the volatile oils.

Cymbopogon citratus (lemongrass/oil grass) is a perennial grass that grows spontaneously around the world, mainly in the tropical and savannah regions. Infusions of the leaves are used in traditional medicine as antimicrobial, anti-inflammatory and sedative (Figueirinha et al, 2010). Leite et al, (1986) have reported that this plant has no toxic effect in human. The essential oil of this plant is used in the food, perfumery, soap, cosmetic, pharmaceutical and insecticide industries. *Cymbopogon citratus* has been generally supported for its relieving effect against fever, flu, headaches, pain and oral thrush (Wright et al, 2009). Lemongrass has been used over many years to make caffeine free tea and natural herb drink and this confers its advantage over caffeinated tea products (Blanco et al, 2009). The volatile oil from *Cymbopogon citratus* contained monoterpene hydrocarbons, which accounted for 94.25% of the oil. The monoterpene fraction was characterized by a high percentage content of geranial (39.53%), neral (33.31%), myrecene (11.41%) and other sesquiterpene (0.78%) and medicinal values of these phytocomponents have been reported (Bastos et al, 2010; Wright et al, 2009).

Shigella sonnei is a pathogenic bacillus, non-motile, non-spore forming, facultative anaerobic gram-negative, lactose fermenter bacterium. Its non-motile characteristic means that this species does not have flagella to facilitate its movement like *Salmonella typhimurium* and *Campylobacter jejuni*, which are also human pathogenic enterobacteriaceae. It is one of the causative agents of shigellosis/dysentery (Yang & Yang, 2005) among other species, which are *dysenteriae, boydii, flexneri*. *Shigella sonnei* is extremely fragile in experimental settings because it survived mostly in a relatively low pH environment at 37^{o}C. This may probably suggest why this pathogen preferred human GIT to any other zone. In both developed and developing countries, the endemic enteric infectious disease called shigellosis is majorly caused by *Shigella sonnei* and other species. *Shigella sonnei* continues to be a major food-borne threat to public health in many developed countries where the issues of sanitation are closely monitored. This enterobacterium is generally transmitted by fecal-oral contamination, uncooked food, contaminated water, subcutaneous contact with inanimate objects and most rarely, sexual contact (Shiferaw et al, 1996-1999; Sureshbabu & Venugopalan, 2006). It uses both chromosomal and plasmid coded gene for its virulence (Niyogi, 2005). *Shigella sonnei* has plasmids that increase in the toxicity of the microbe to their host or other organisms around them. They produce a toxin called the “Shiga toxin”. It is a unique kind of toxin that works its toxicity in the

body in multiple ways that will bring potential harm to the neurons, cytoplasm of the cells and enteric epithelial cells (Niyogi, 2005). The use of antibiotics and antidiarrheal drugs are becoming compromising due to increasing drug resistant by this pathogen coupled with drug abuse and misappropriation (Brian et al, 1993; Sebhat et al, 2007). Extracellular proteases have been associated to the virulence nature of enteric pathogen because they play an important role either directly or indirectly regarding the pathogenesis of this organism. It has been found that majority of these proteases are responsible for breaking protein moieties of endothelial glycoprotein thereby creating access for pathogenic invasion and colonization (Crowther et al, 1987; Stewart et al, 1986). The inhibition of this enzyme regarding the pathogenicity of enteric bacteria by the volatile oil of *Cymbopogon citratus* is therefore hypothesized.

The fame gained by most extracellular proteases in the degree of pathogenicity of microorganism has led to the purification and characterization of these proteases in order to get drugs, which could effectively terminate the infections caused by such pathogens. The low susceptibility of the pathogenic organisms to antibiotics and other conventional drugs has led to the need for an alternative means of tackling the pathogenicity of these microbes. The aim of this study is to examine the antibacterial effect of the volatile oil of *Cymbopogon citratus* as well as its inhibitory effect against the extracellular protease of *Shigella sonnei*.

MATERIALS AND METHODS

Collection of Plant Materials

Cymbopogon citratus (lemongrass) was obtained from a small garden at Abesan Estate, Ipaja. Lagos State, Nigeria. The foliage sample was taken to the Botany Department, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria for proper identification and authentication. The sample was gotten as green foliage and air-dried for a week.

Extraction of Volatile Oil of *Cymbopogon citratus*

The volatile oil of *Cymbopogon citratus* was extracted by hydrodistillation method as described by Lawrence & Reynolds, (1993). Briefly, 350g of air-dried parallel-veinated leaves of *Cymbopogon citratus* were loaded into a 5L round bottom flask containing 2.5L of distilled water. This was fixed with Clevenger and placed into the heating mantle. The volatile oil was extracted at a steady temperature of 80^{o}C for 3 hours over 2.0ml *n*-hexane solvent. The oil was collected in corkscrew dark sample bottle and kept at 4^{o}C until it was tested.

Bacteria

Shigella sonnei was obtained from Nigeria Institute of Medical research (NIMR) Yaba Lagos State, Nigeria on a sub-cultured *Salmonella-Shigella* medium. After 24 hours of incubation at 37^{o}C, this organism was then sub-cultured in *Salmonella-Shigella* broth (SSB) and agar (SSA), while the broth was used to produce enzyme extract; agar was used to estimate the MIC and MBC of the volatile oil. All incubation was done at 37^{o}C under sterile and anaerobic environment.

Antioxidant Activity of the Volatile Oil of *Cymbopogon citratus*

The antioxidant activity of the volatile oil of *Cymbopogon citratus* was carried out using the process described by Koleva et al,

(2002). Briefly, 20µl of the volatile oil at 30%v/v concentrations in acetone was added to 80µl methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.2mg/ml) and mixed thoroughly and the absorbance was read at 517nm after 30 minutes at room temperature using microplate reader using Butylated hydroxyltoluene (BHT) standard positive control. The IC_{50} value denotes the concentration of the volatile oil, which is required to scavenge 50% of DPPH free radicals. Percentage radical scavenging activity of the volatile oil was estimated spectrophotometrically (using microplate reader) at 517nm using the formula:

$$\frac{A_o - A_1}{A_o} \times 100$$

where A_o represents the absorbance of DPPH solution in the absence of antioxidant A_1 represents the absorbance of DPPH solution in the presence of antioxidant.

Bacteria Growth inhibition and Determination of IC_{50} of the Volatile Oil

The volatile oil extracted from *Cymbopogon citratus* was tested for antimicrobial activity against *Shigella sonnei* and inhibitory concentration required to clear off 50% of the bacterial growth was estimated. This was done using microbroth dilution technique in *Salmonella-Shigella* broth as described by Akujobi and Njokwu (2010). Briefly, a colony of the organism was added to 200µl of susceptible test broth (prepared with 0.5%v/v Tween-80) containing two-fold serial dilutions of the pure volatile oil in the microtitre plate (21.5cm by 17cm). The plate was covered and incubated under anaerobic condition at 37°C for 24 hours. After 24 hours, each inoculum from the microwell was re-inoculated into a fresh SSB and growth inhibition of the bacteria was spectrophotometrically determined at 620nm using a microplate reader after 18 hours of anaerobic incubation at 37°C. The degree of percentage growth inhibition was estimated spectrophotometrically (using microplate reader) at 620nm using the formula:

$$\frac{A_o - A_1}{A_o} \times 100$$

where A_o represents the absorbance of the well in the absence of volatile oil and A_1 represents the absorbance of the well in the presence of volatile oil.

Extraction of Crude Enzyme

As described by Makino et al, (1981) with little modification, a colony of *Shigella sonnei* was inoculated into the 5 ml susceptible *Salmonella-Shigella* broth. It was incubated for 24 hours at 37°C and then centrifuged (Kendros PicoBiofuge, Heraeus) at 9000 rpm for 10 minutes at room temperature. The supernatant, which contained the crude enzymes was decanted and stored in a sample bottle at 4°C until it was used.

Purification

The crude extract was saturated with 55% ammonium sulphate by adding 1.1g of NH_4SO_2 to 2ml of the crude extract supernatant. This mixture was shaken and allowed to stand for about 10min. The mixture was centrifuged at 3000g for 20min. The pellet was redisolved in small amount of Tris-HCl buffer pH 7.5 and dialyzed against 0.05M Tris-HCl buffer of pH 8.0 overnight with intermittent replacement of the buffer solution (using SIGMA Dialysis Tubing Cellulose Membrane, D9402). 1ml of the resulting supernatant obtained from centrifuging the dialysate was loaded on sephadex G-100 gel and allowed to percolate. Elution was done using 0.05M Tris-HCl buffer pH 8.0 with a flow rate of 0.3ml/min. 50 fractions of 3ml each were

collected. Protein and enzyme activity were determined at 280nm. Fractions showing protease activity were pooled together and concentrated.

Protein Determination

The protein concentration of the crude extract was determined using Lowry et al, (1951) method. This was done by adding 5.0ml of alkaline solution containing a mixture of 50ml of solution A (20g sodium trioxocarbonate IV and 4g sodium hydroxide in 1L) and 1ml of solution B (5g cupper II tetraoxosulphate VI pentahydrate and 10g sodium-potassium tartrate in 1L) to 0.1ml of crude extract and mixed thoroughly. The solution was allowed to stand for 10 minutes at room temperature and 0.5ml of freshly prepared Folin Ciocalteau's phenolic reagent (50%v/v) was added. The solution was mixed thoroughly and the absorbance was read at 750nm after 30 minutes. Bovine serum albumin (BSA) was used as standard.

Enzyme Assay

The extracellular protease of *Shigella sonnei* was assayed using Folin & Ciocalteau, (1927) method. Protease activity was determined by adding 5.0ml of casein solution (0.6%w/v in 0.05M Tris buffer at pH 8.0) to 0.1ml of the crude enzyme and the mixture was incubated for 10 minutes at 37^{o}C. The reaction mixture was stopped by adding 5.0ml of a solution containing 0.11M trichloroacetic acid, 0.22M NaCl and 0.33M acetic acid mixed in ratio 1:2:3. The turbid solution was filtered and 5.0ml of alkaline solution was added to 1.0ml of the filtrate followed by 0.5ml of freshly prepared Folin Ciocalteau's phenolic reagent after 10 minutes of thorough mixing. The absorbance was read at 750nm after 30 minutes. L-tyrosine solution (0.20mg/ml) was used as standard for the protease activity. *A unit of protease activity was defined as the amount of enzyme required to liberate 1.0µmol of tyrosine in 1 minute at 37^{o}C. The specific activity was expressed in units of enzyme µmol/min/mg protein.*

Determination of Optimum pH

This was carried out by adding as described by Mikano et al., (1981) with slight modification, 5.0ml of 0.6% w/v casein solution in 0.05M Tris buffer (pH range of 6.0-9.0), as substrate, to 0.1ml of the crude enzyme extract and the enzyme assay was carried out at 37^{o}C for 10 minutes as earlier discussed.

Determination of Optimum Temperature

5.0ml of 0.6% casein in 0.05M Tris buffer at pH 8.0 was mixed with 0.1ml of crude enzyme extract and the enzyme assay was carried out at temperature range of 30-60^{o}C for 10 minutes. The reaction was stopped and enzyme activity was carried out at each stage of temperature.

Inhibitory Assay

This was carried out by slightly modifying the method described by Makino et al, (1981). Briefly, 0.1ml of the crude enzyme extract and 0.1ml of 3.5%v/v of the volatile oil in 0.5%v/v Tween 80 solution were added concomitantly to different concentration of casein solution (0.2-1.0%w/v) in 0.05M Tris buffer at pH 8.0 and the reaction mixture was mixed and incubated at 37^{o}C for 10 minutes. The reaction was stopped by adding 5.0ml of the stop solution. Protease assay was carried out as earlier discussed. The procedure was repeated in the absence inhibitor.

Effect of Metal Ions

Following the method described by Jahan et al. (2007) with slight modification, the extracellular protease activity was carried

out in the presence of 1.0mM chloride salt solutions of Hg^{2+}, Ca^{2+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and K^{+}. 1.0ml of different metal concentration was added to 0.1 ml of purified enzyme. This mixture was then added to 2.0 ml of 0.5%. Other steps were as described for enzyme assay.

Statistical Analysis

Statistical analysis of antioxidant activity was evaluated using the *t-test* analysis. The mean was considered statistically significant at $p<0.05$

RESULTS

The analysis of the extraction of the volatile oil of *Cymbopogon citratus* is presented in Table 1. The volatile oil was extracted by hydrodistillation method. The percentage yield after 3 hours of extraction was 0.56 at steady temperature of 80^{o}C.

The summary of purification process and elution chromatogram of the crude enzyme extract of *Shigella sonnei* is shown in table 2. Highest purification fold of 3.73 and specific enzyme activity of 48.33 µmol/min/mg protein were obtained from the crude extracellular protease. Further analysis by gel filtration using Sephadex G-100 revealed a peak for the enzyme activity and two peaks for protein assay (Figure 1). This may be dimeric protein.

Therefore, the activity of the extracellular protease of *Shigella sonnei* has been investigated under the effect of pH, temperature, metallic ions and the volatile oil of *Cymbopogon citratus*, which was used as potential inhibitor against the catalytic activity of this enzyme. The oil exhibited a competitive inhibition (Figure 6) and this re-affirmed the potential antibacterial activity of this volatile oil especially against *Shigella sonnei*.

The antioxidant property of the volatile oil of Cymbopogon citratus as compared with BHT (positive control) is represented in Figure 2. The degree of decolourization was measures spectrophotometrically at room temperature. The volatile oil showed a statistically lower ($p<0.05$) percentage degree of antioxidant activity with an average mean value of 37.0 ± 6.9 as compared to BHT with average value of 54.0 ± 3.0. The volatile oil has an IC_{50} value of 3.83%v/v while BHT was 4.25%v/v. The slopes of the two lines (BHT and Volatile oil) differed significantly ($p<0.014$) with BHT having higher slope compared to the volatile oil (Figure 2).

Figure 3 shows the microbial inhibition of *Shigella sonnei* by the volatile oil of *Cymbopogon citratus*. The volatile oil showed an IC_{50} of 1.58%v/v.

The effect of pH on the protease activity of *Shigella sonnei* showed that the protease have highest activity at pH 7.5, thus indicating that the extracellular protease is slightly alkaline (Figure 4). The effect of pH on the activity of extracellular protease of *Shigella sonnei* shows that the protease have highest activity of 1.80 x 10^3 µmol/min at pH 7.5, thus the adaptive survival of this bacteria in a mild alkaline microenvironment.

The protease has highest activity of 2.15 x 10^3 µmol/min at 40°C (Figure 5**)**. This relatively affirmed the survival of this bacterial within the human body temperature even in feverish condition. The effect of the volatile oil on enzyme activity shows a competitive inhibition (Figure 6) with a V_{max} of 8.0 x 10^2 µmol/min and K_m of 0.27 mg/ml and $K_{mapparent}$ of 0.53 mg/ml. This

invariably reduced the catalytic affinity of this proteolytic enzyme to its true substrate (casein), probably due to both structural resemblance of a component (s) of the volatile oil and the substrate for the enzyme catalytic site.

The effect of different chloride salt solutions on the enzymatic activity of the extracellular protease *Shigella sonnei* is shown in figure 7. It was found that chloride salts of Cu^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and K^{+} showed no effect on enzyme activity as compared to the control but salts of Hg^{2+}, Co^{2+} and Ba^{2+} inhibited the activity of this enzyme (Figure 7). There was a positive modulation of this enzyme in the presence of Mn^{2+} chloride salt, which may suggest the extracellular protease to be a metalloproteinase.

Table 1: Extraction of volatile oil of *Cymbopogon citratus*

Weight of plant before extraction	350g
Volatile oil yield	2ml
Percentage yield	0.56
Volume of distilled water	2.5 L
Duration of extraction of volatile oil	3 hours
Steady temperature	80^{o}C

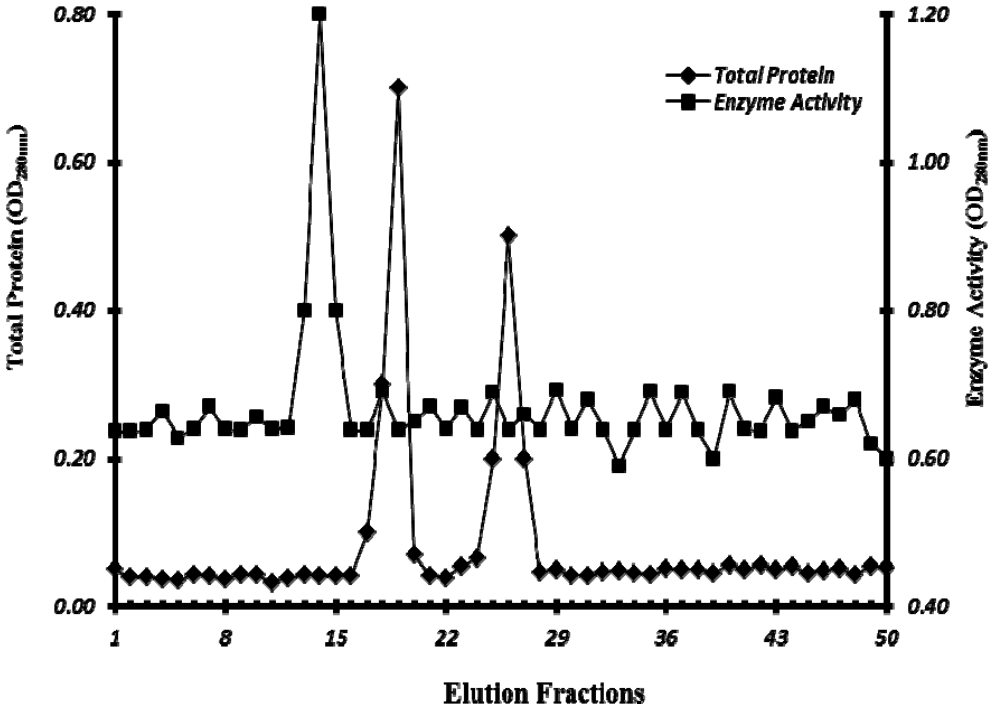

Figure 1: Elution profile of fractions obtained from Sephadex G-100 gel filtration

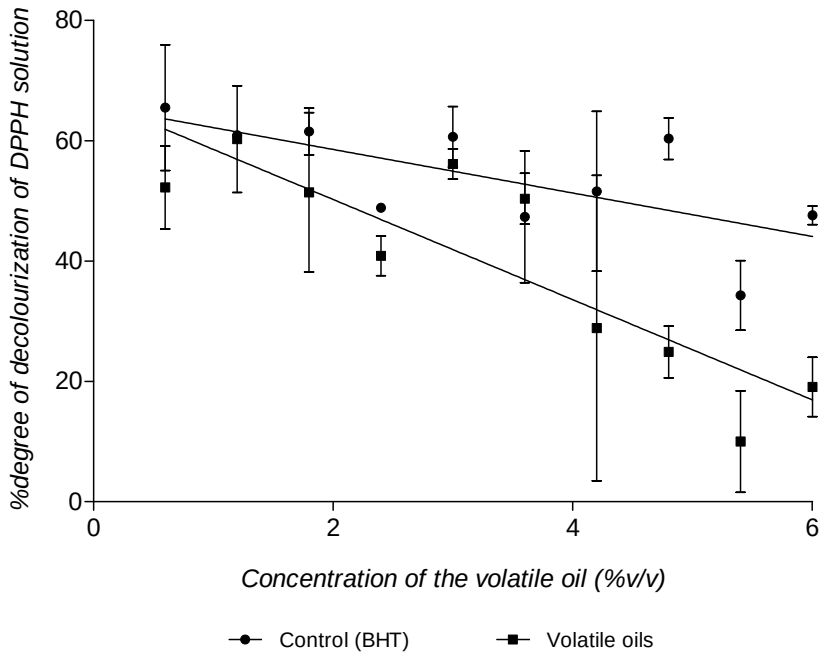

Figure 2: Antioxidant activity of the volatile oil of *Cymbopogon citratus* as compared with BHT

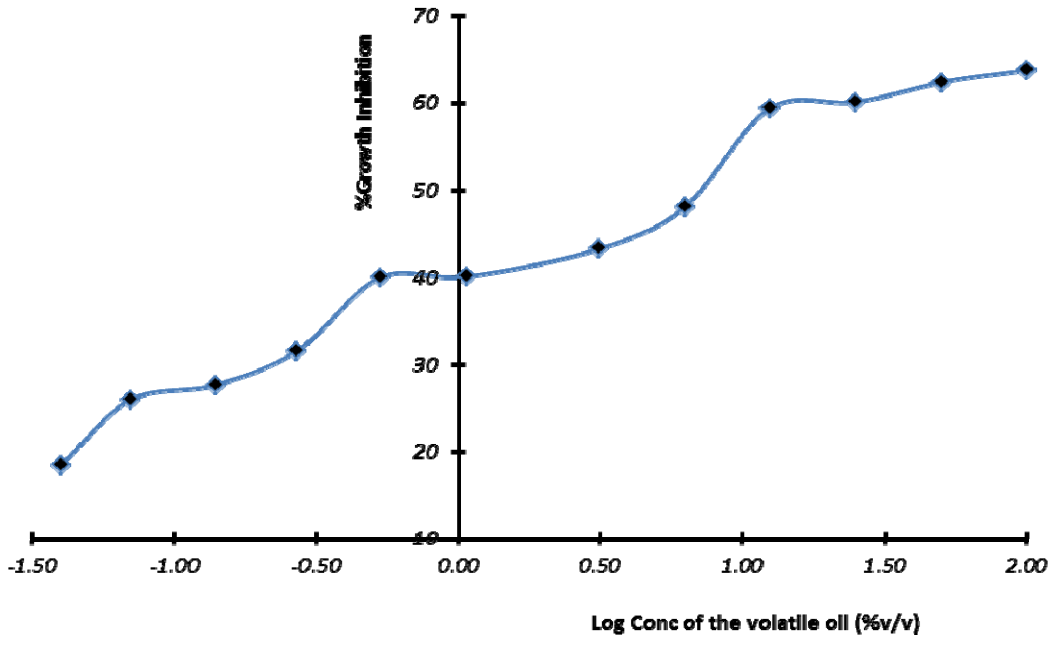

Figure 3: Microbial growth inhibition of *Shigella sonnei* by the volatile oil of *Cymbopogon citratus*

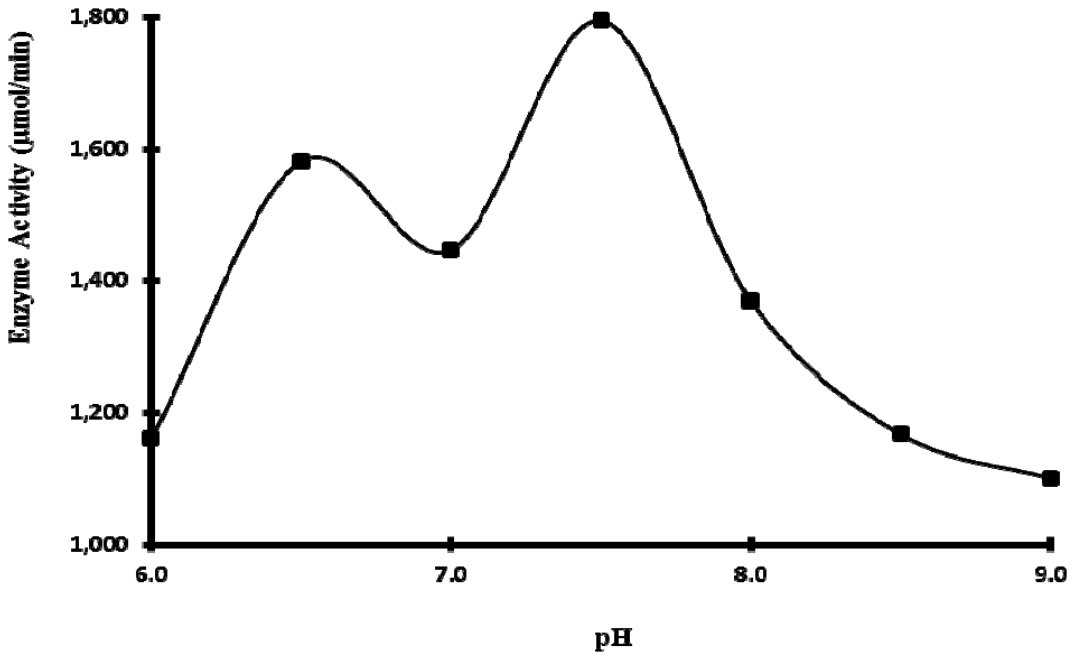

Figure 4: The effect of pH on the protease activity of *Shigella sonnei*

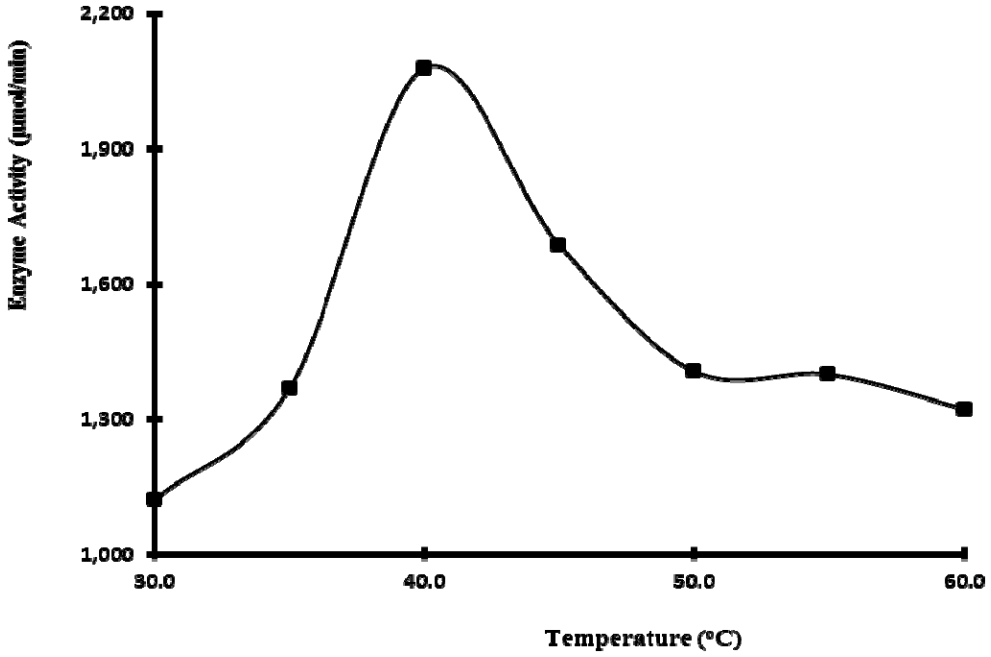

Figure 5: The effect of temperature on the activity of extracellular protease of *Shigella sonnei*

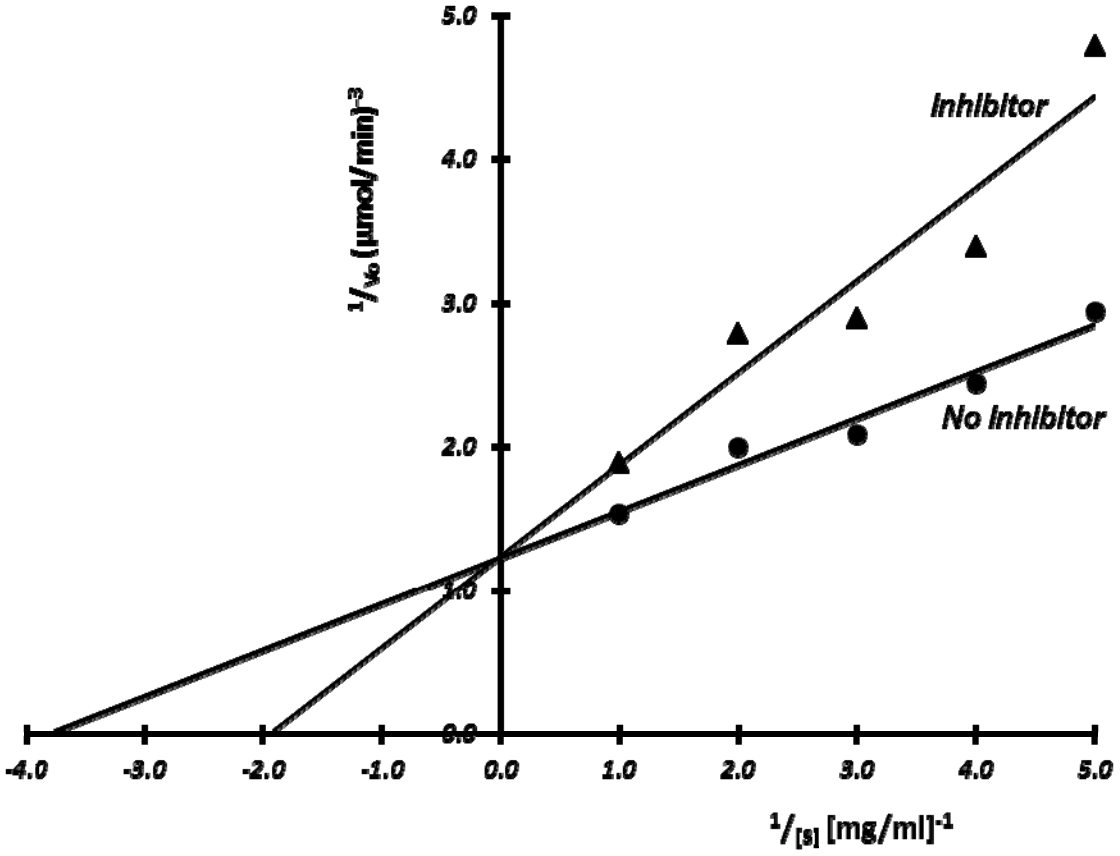

Figure 6: Double reciprocal plot for the activity of the extracellular protease of *Shigella sonnei* in the presence of volatile oil of *Cymbopogon citratus* as inhibitor

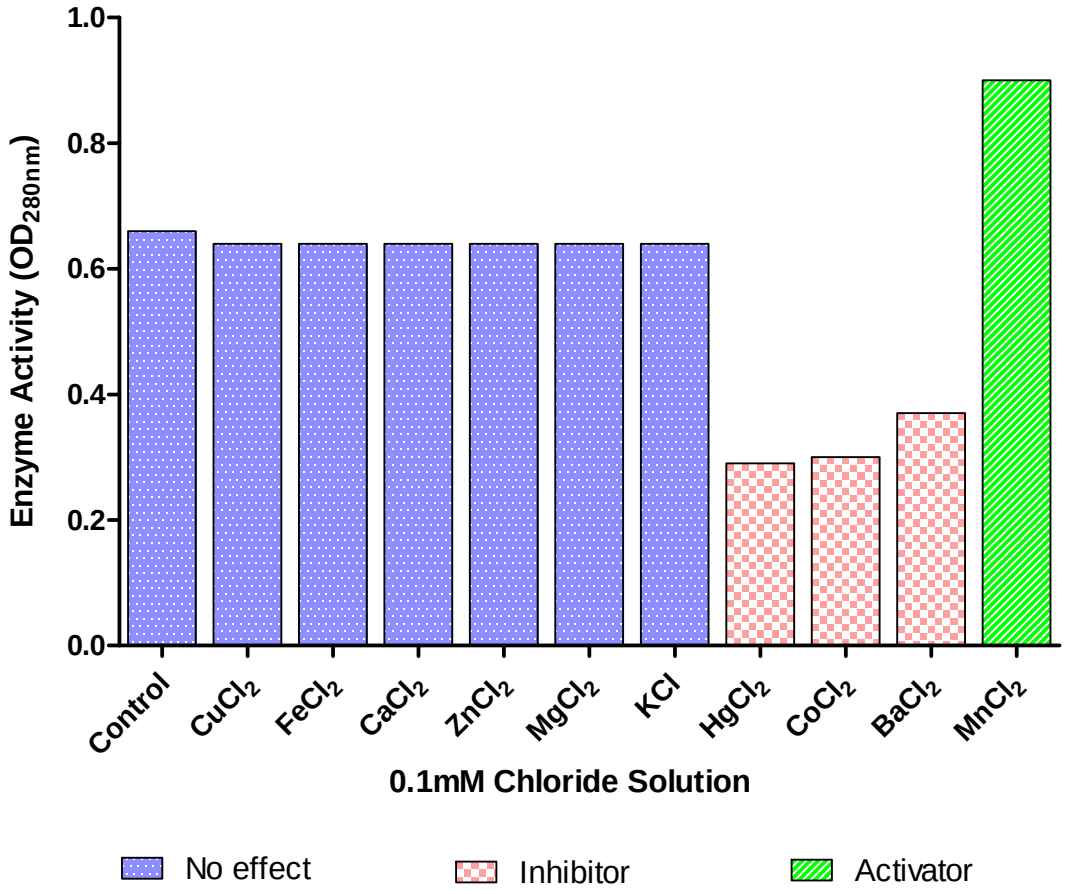

Figure 7: Effect of metallic chloride ions on the catalytic activity of the extracellular protease of *Shigella sonnei*

Table 2: Summary of purification profile

Purification Steps	***Total Protein (mg)***	***Total Activity (µmol/min)***	***Specific Activity (µmol/min/mg protein)***	***Percentage Yield***	***Purification Fold***
Crude enzyme	170	2200.0	12.94	100	1.0
55% $(NH_2)SO_4$ Precipitation	46	1233.3	26.81	56.06	2.07
Sephadex G-100	10	483.3	48.33	21.97	3.73

DISCUSSION

The volatile oil from the leaves of *Cymbopogon citratus* showed an antioxidant property against DPPH. Though this activity was not as strong as Butylated hydroxyltoluene (BHT), which was used as positive control but this oil has demonstrated a competitive value. The result shows presence of Geranial, neral and myrecene in this volatile oil (Gbenou et al., 2012). This oil has been shown to inhibit the growth of S*higella sonnei* with IC_{50} of 1.58%v/v. From the work of Akin-Osanaiye, lemon grass oil has been shown to inhibit the growth of fungal like *Candida albicans, Aspergillus fumigatus* and *Aspergillus niger* (Selvi et al., 2011; Sunita & Mahendra, 2008), *Salmonella typhi, Staphylococcus aureus* and *Escherichia coli* (Akin-Osanaiye et al., 2007). There was a report of the antimicrobial activity of two species of lemon grass oils of series of pathogenic bacteria excluding *Shigella sonnei* (Bassolé et al., 2011) but this work has exclusively displayed the antimicrobial activity of the volatile oil of *Cymbopogon citratus* on *Shigella sonnei.*

The enzyme has been shown to exhibit optimal activities at pH 7.5 and 40^{o}C. There was a sudden fall in the activities of this enzyme because of increase in pH and temperature beyond the optima values, this may be confirming the fragility of this enzyme and subsequently its susceptibility to high pH and temperature. Generally, the optimum activity of extracellular proteases of enteric pathogens is usually found between 35 -50^{o}C (Louboudy et al., 2007).
The Lineweaver Burke plot of enzyme activity in the presence the volatile oil of *Cymbopogon citratus*, which serve as an inhibitor, displayed a competitive inhibition (Figure 6). The competitive nature of this inhibition is likely to create a platform at which an antibiotic can be directed against the survival of this pathogen even in the GIT.

Metallic ions are prosthetic groups of holoenzymes that serve as either inhibitors or activators. Among the tested chloride salts on the activity of this extracellular protease of *Shigella sonnei*, Mn^{2+} exhibited positive modulatory effect on the activity of this enzyme and this has revealed the metalloproteolytic nature of this enzyme. Chloride ions like Hg^{2+}, Co^{2+} and Ba^{2+} on the other hand were inhibitors of the extracellular protease of *Shigella sonnei* (Figure 7).

Highest purification fold of 3.73 and enzyme activity of 48.33 µmol/min/mg protein were obtained as compared to the crude extract. The two peaks revealed by Sephadex G-100 elution chromatography may have indicated a dimeric nature of this protein.

CONCLUSION

The volatile oil of the leaves of *Cymbopogon citratus* possessed both antioxidant, antibacterial and inhibitory effects against the activity of the extracellular protease of *Shigella sonnei*. This is of great benefit to the development of drugs directed towards this pathogen because the pathogenesis of this microbe whose virulence is aided by the secretion of some extracellular protease has been shown to be sensitive to the antimicrobial and inhibitory effects of the volatile oil of *Cymbopogon citratus.*

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