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Antimicrobial Assay for the Volatile Oils of *Chromolaena odorata* and its Inhibition against the Partially Purified and Characterized Extracellular Protease of *Pseudomonas aeruginosa*

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ABSTRACT

The recent development in the antimicrobial therapy to meet the challenges of resistant pathogenic bacteria has called for rapid growth in exploring natural products as alternative. The antimicrobial activity of the oils from the leaf and stem of *Chromolaena odorata* against nine enteric pathogenic bacteria was assessed and compared with commonly used antibiotics. Specifically, we examined the mode of inhibition of these oils on the extracellular protease of *Pseudomonas aeruginosa*. The inhibition zones of the oils range from 13.0±1.0-43.5±2.5 mm in *Salmonella paratyphimurium* and *Shigella dysenteriae*, respectively. Ceftriaxone has the highest inhibition of 26.0±2.0 mm against *Salmonella paratyphimurium*. There was a significant difference ($p<0.05$) between the average inhibition of the antibiotics, 5.0±0.82 mm and the volatile oils, 18.0±4.0 mm. Each of the microbes was sensitive to at least one of the oils. The extracellular protease of *Pseudomonas aeruginosa* had optimal activities at pH 7.5 and 35°C. The volatile oils competitively inhibited the extracellular protease of *Pseudomonas aeruginosa* with V_{max} = 0.91 μmol min^{-1} and K_m = 0.48 mg mL^{-1} (oil absent) but the K'_m increased to 0.93 and 1.25 mg mL^{-1} in the presence of the volatile oils of the leaf and stem of this plant, respectively. The highest purification fold of 2.35 (6.92 μmol min^{-1} mg^{-1} protein) was achieved from the crude enzyme with DEAE anion exchange chromatography. The chromatographic peaks may be an evidence of multiple subunits of this enzyme. Therefore, the oil of *Chromolaena odorata* possessed antimicrobial activity and its ability to inhibit extracellular protease of *Pseudomonas* aeruginosa will be a possible source of nutraceuticals for clinical purpose.

Key words: Volatile oil, *Chromolaena odorata*, antimicrobial, *Pseudomonas aeruginosa*, inhibition, extracellular protease

INTRODUCTION

Medicinal plants are plants with proven chemical properties which have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic values (Sumner, 2000). Chemically, essential/volatile oils, as one of the phytoconstituents of medicinal plants, are extremely complex mixtures containing compounds of every major functional group like ether, alkoxide, enol, polyaromaphenolic compounds and many others. Most of these phytoactive components have been reported to possess antibacterial, antifungal, antiviral and antiprotozoans (Bassole *et al*., 2011; Akin-Osanaiye *et al*., 2007; Martins *et al*., 2001). Asides these, the essential

oils from medicinal plants have displayed some credible effects as antidiarrheal, antihypertensive, antidiabetic, anti-inflammatory and also as immunomodulatory and antioxidant (Hamid *et al*., 2011; Fujiki, 2005). They are hydrophobic liquids containing volatile fragrant aroma (Wanda, 2001). The oils are isolated by steam distillation, hydrodistillation, solvent extraction or mechanical expression of the plant material and the plant parts often used are the roots, buds, leaves, stems and flower parts. *Chromolaena odorata* is herbaceous perennial shrubs belonging to the plant family *Asteraceae.* It occurs naturally in South America and Central America and was introduced to the tropical regions of Asia, Africa and the pacific as invasive weeds (Struhsaker *et al*., 2005). The volatile oil from its leaves has been shown to have antimicrobial and antiseptic properties (Akah, 1990), as well as enhancing homeostatic activity and stimulating the re-epithelialization process thus promoting wound healing (Pandith *et al*., 2013).

Pseudomonas aeruginosa is a gram-negative aerobic bacillus belonging to the bacterial family *Pseudomonadaceae.* Although, members of its genera are well-known plant pathogens, *Pseudomonas aeruginosa* have become increasingly recognized as an emerging opportunistic pathogen of clinical relevance (Pollack, 2000). *Pseudomonas aeruginosa* is one of the major causes of nosocomial pneumonia and spread mainly through hospital equipment and health care workers than from person-to-person (Amadi *et al*., 2009). Their frequent contamination of ventilators and hospital equipment is attributed to the fact that they are resistant to extreme temperature and drying. *Pseudomonas aeruginosa* is the fourth most commonly isolated nosocomial pathogen accounting for 10.1% of all hospital acquired infections. It is found on the skin of healthy persons and has been isolated from the throat and stool of 3-5% of non-hospitalized persons. *Pseudomonas aeruginosa* is involved in the aetiology of many diseases including endocarditis, meningitis, bronchopneumonia, burns and wound infections; wound infection is one of the major causes of limb amputations in Nigerian children (Amadi *et al*., 2009). One of the most worrisome characteristics of *Pseudomonas aeruginosa* is its high antibiotic resistance which is attributable to a concerted action of multidrug efflux pumps, chromosomally encoded antibiotic resistance genes and low permeability of the bacterial cellular envelopes (Baltch and Smith, 1994).

Most pathogenic organisms secrete both intracellular and extracellular proteases with which they accomplish their pathogenic activities. These extracellular proteases also lead to the virulent nature of the pathogens (Kuehn and Kesty, 2005). *Pseudomonas aeruginosa* secretes quite a lot of extracellular proteases some of which include elastase, alkaline protease, exotoxin A and exoenzyme S as well as some soluble cytotoxic proteins. These extracellular proteases make it a very virulent organism with high level of antibiotic resistance thus indicating the need for a more novel approach towards fighting this microbe (Livermore, 2002). The prominence gained by most extracellularly produced proteases in the virulence of microorganisms has led to the purification and characterization of these proteases in order to design drugs which could effectively prevent the infections caused by such pathogen.

The *in vitro* activity of a range of essential oils against *Pseudomonas aeruginosa* has been examined with some medicinal plants (Zuridah *et al*., 2008; Jahan *et al*., 2007). There were promising results of antibacterial activities of both aqueous and organic solvents extraction of these medicinal plants (Sukanya *et al*., 2011). In this work, assessment of the antimicrobial property of the volatile oils from the leaf and stem of *Chromolaena odorata* comparing with the standard antibiotics was carried out. In addition, the inhibition of these oils on the partially purified and characterized extracellular protease of *Pseudomonas aeruginosa* was determined.

MATERIALS AND METHODS

Plants materials: *Chromolaena odorata* plants were obtained at Amuwo Odofin Local Government Area of Lagos State. The sample was gotten as green foliage and air-dried for four days. The green sample was taken to the Department of Botany, Faculty of Science, Lagos State University, Ojo Lagos for proper identification and authentication.

Microorganisms: The microorganisms used in this work were obtained from the Nigeria Institute of Medical Research (NIMR), Yaba, Lagos Nigeria and maintained on nutrient agar petri-dishes at 4°C. These microbes were *Staphylococcus aureus* (a gram-positive bacterium), while others were gram-negative bacteria: *Enterohaemorrhagic Escherichia coli* (EHEC), *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphimurium*, *Salmonella typhimurium*, *Shigella flexneri*, *Salmonella enteritis* and *Shigella dysenteriae*.

Susceptible antibiotic drugs: A susceptible antimicrobial sensitivity discs was purchased from a pharmaceutical store in Ojo Local Government of Lagos Nigeria. The antibiotic discs were coated with the following drugs: Ofloxacin (Travid) -5 µg, Erythromycin -10 µg, Clindamycin -5 µg, Ciprofloxacin -5 µg, Gentamicin -10 µg, Cephalexin -30 µg, Cotrimoxazole -50 µg, Ampicillin -30 µg, Ceftriaxone -30 µg and Augumentin -30 µg.

Extraction of volatile oils: The volatile/essential oil of *Chromolaena odorata* was extracted by the method described by Lawrence and Reynolds (1986). Briefly, the five-day-air-dried plant was separated into leaves and stems and each part was cut into pieces and packed into the 5 L 34/35 Quick fit round bottom flask containing 2.0 L distilled water with fixed Clevenger. The oil was extracted at a steady temperature of 80°C for 3 h, collected over 2 mL n-hexane and kept at 4°C.

Antimicrobial susceptibility tests of antibiotics and volatile oils: The volatile oil of *Chromolaena odorata* was tested for antimicrobial sensitivity against nine microorganisms using a diffusion technique method (Wannissorn *et al*., 2009) on different suitable agars. A 5 mm diameter paper disc paper was soaked into the volatile oil, picked with a sterilized tong and placed on the media which have been surface spread with each colony of the bacteria. The plates were inoculated in their appropriate media for 24 h at 37°C. The results were recorded by measuring the zones of inhibition surrounding the paper disc.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MIC and MBC of the volatile oil of *Chromolaena odorata* were carried out using micro broth dilution method (Harkenthal *et al*., 1999) with little modification. A colony of each organism 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 (21.5×17 cm^2). The plates were covered and incubated at 37°C for 24 h. Each of the microwells was inoculated on a freshly prepared Muller Hinton agar where MIC and MBC were determined.

Extraction of crude enzyme: The extracellular protease of *Pseudomonas aeruginosa* was extracted by the method described by Bhaskar *et al.* (2007). A colony of the microbe was inoculated

into the Muller Hinton broth. It was incubated for 24 h at 37°C. The broth was centrifuged (Kendros PicoBiofuge, Heraeus) at 9000 rpm for 10 min at room temperature. The cell-free supernatant was decanted and stored in a sample bottle at 4°C until it was used.

Determination of total protein and protease activity: The total protein of the crude enzyme extract was determined using a method of Lowry *et al.* (1951), with casein as substrate. Total protein was determined by adding 5.0 mL of alkaline solution containing a mixture of 50 mL of solution A (20 g sodium trioxocarbonate IV and 4 g sodium hydroxide in 1 L) and 1 mL of solution B (5 g cupper II tetraoxosulphate VI pentahydrate and 10 g sodium-potassium tartrate in 1 L) to 0.1 mL of crude enzyme extract and mixed. The reaction solution was allowed to stand for 10 min at room temperature and 0.5 mL of freshly prepared Folin Ciocalteau's phenolic reagent (50% v/v) was added. The solution was mixed thoroughly and the absorbance was read at 750 nm (using Spectronic-21, Bausch and Lomb) after 30 min. Bovine Serum Albumin (BSA) was used as standard protein (0.20 mg mL^{-1}).

Protease activity was carried out by adding 5.0 mL of casein solution (0.6% w/v in 0.05 M Tris buffer at pH 8.0) to 0.1 mL of the crude enzyme extract and the mixture was incubated for 10 min at 37°C. The reaction mixture was stopped by adding 5.0 mL of a solution containing 0.11 M trichloroacetic acid, 0.22 M NaCl and 0.33 M acetic acid mixed in ratio 1:2:3. The turbid solution was filtered and 5.0 mL of alkaline solution was added to 1.0 mL of the filtrate followed by 0.5 mL of freshly prepared Folin Ciocalteau's phenolic reagent after 10 min of thorough mixing. The absorbance was read at 750 nm after 30 min. L-tyrosine solution (0.20 mg mL^{-1}) was used as standard for the protease activity. The one unit of protease activity was defined as the amount of enzyme required to liberate 1.0 μmol of tyrosine in 1.0 min at 37°C.

Determination of optimum pH: The method adopted was described by Chong *et al*. (2002) with little modification. Protease activity was assayed using 0.6% casein solution in 0.05 M Tris buffer solution (pH 6.0-9.0) at 37°C.

Determination of optimum temperature: As described by Bhaskar *et al*. (2007), protease activity was assayed under varying temperature conditions (30-70°C) using 0.6% casein solution in 0.05 M Tris buffer at pH 8.0.

Inhibitory assay: The method used was described by Chong *et al.* (2002) with a slight modification. Briefly, 0.1 mL of the crude protease extract and 0.1 mL of 3.5% v/v of the volatile oil (as inhibitor) in 0.5% v/v Tween 80 solutions were added concomitantly to different concentration of casein solution (0.2-1.0% w/v) in 0.05 M Tris buffer, pH 8.0. The reaction mixture was mixed and incubated at 37°C for 10 min. The reaction was stopped and the protease activity was assayed with the volatile oils of the two different parts of the plant. The procedure was repeated without an inhibitor.

Dialysis: Salting out technique was carried out on the crude enzyme extract. A 35% $(NH_4)_2SO_4$ saturated solution of the crude enzyme extract was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D9402) for 48 h and thereafter centrifuged at 5000 g for 5 min Then, 50%

$(NH_4)_2SO_4$ saturated solution of the sediment was dialyzed for 48 h. This was followed by the dialysis of 55 and 65% saturated solutions of the sediment for the same hours. In each case, both total protein and enzyme assay were carried out.

Gel filtration: We employed Bhaskar *et al*. (2007) method with little modification by soaking 3.0 g of Sephadex G-100 (BDH, pore sizes to exclude protein >100 kDa) in distilled water for 72 h. The gel was poured into the chromatographic column (28.0-1.5 cm column) and formed a bed length of 22 cm with a flow rate of 1.5 mL min^{-1} and this was used to separate 35% $(NH_4)_2SO_4$ dialysate. A total number of 50 elutions were collected. Each elution contained 3.0 mL of eluent and for each of the eluent; both total protein and enzyme assay were carried out.

Ion exchange chromatography: This was done by soaking 6 g of DEAE cellulose powder in 0.05 M Tris buffer of pH 8.0 for 48 h. It was packed in column of about 22 cm length. The flow rate was 0.6 mL min^{-1}. The column was prepared and equilibrated with Tris buffer (0.05 M, pH 8.0).

Statistical analysis: Comparison of the antimicrobial activities of the volatile oils and antibiotics was carried out using t-test analysis and the mean difference was considered significant at $p<0.05$.

RESULTS

The volatile oils obtained from the stem and leaves of *Chromolaena odorata* plants were tested for antimicrobial activity against nine infections bacteria using disc diffusion method and its inhibition on the extracellular protease of *Pseudomonas aeruginosa* was carried out. This protease was partially purified by ammonium sulphate precipitation, gel filtration and ion exchange chromatography.

Figure 1 shows the antimicrobial sensitivity of the volatile oils of the leaves and stems of *Chromolaena odorata* against nine infections bacteria. The zones of inhibition obtained ranged from 13.0±1.0-43.5±2.5 mm with *Salmonella paratyphimurium* having the lowest zone of inhibition and *Shigella dysenteriae* having the highest. The inhibition zones for *Pseudomonas aeruginosa* were 32.0±1.0 and 37.5±2.5 mm for the stems and leaves, respectively. The volatile oil from the stem of *Chromolaena odorata* showed higher inhibitions on seven out of nine organisms used while the volatile oil from the leaf inhibited five out of the nine microorganisms tested. The highest inhibition zone of 26.0±2.0 mm was observed in ceftriaxone against *Salmonella paratyphimurium*. Similarly, all antibiotics used had inhibition zones ≥10.0 mm except augumentin which has no effect. There was a significant difference ($p<0.05$) between the total average inhibition of the antibiotics, 5.0±0.82 mm and the volatile oils, 18.0±4.0 mm. Each of the microbes was either sensitive to both types of the oils or at least one the oils. The sensitivity test of *Chromolaena odorata* volatile oils from the stems and leaves on these nine different microorganisms showed that the oil from the stem of *Chromolaena odorata* is highly potential than the leaves because of its inhibitory effect on almost all the microorganisms tested.

Table 1 shows the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) obtained by micro-serial dilution. Most of the organisms were sensitive to

relatively low concentrations of the oils except *Staphylococcus aureus*. *Enterohaemorrhagic Escherichia coli* (EHEC) and *Escherichia coli* have the same lowest possible MIC and MBC values of 0.09 and ≥0.18% v/v of the oils.

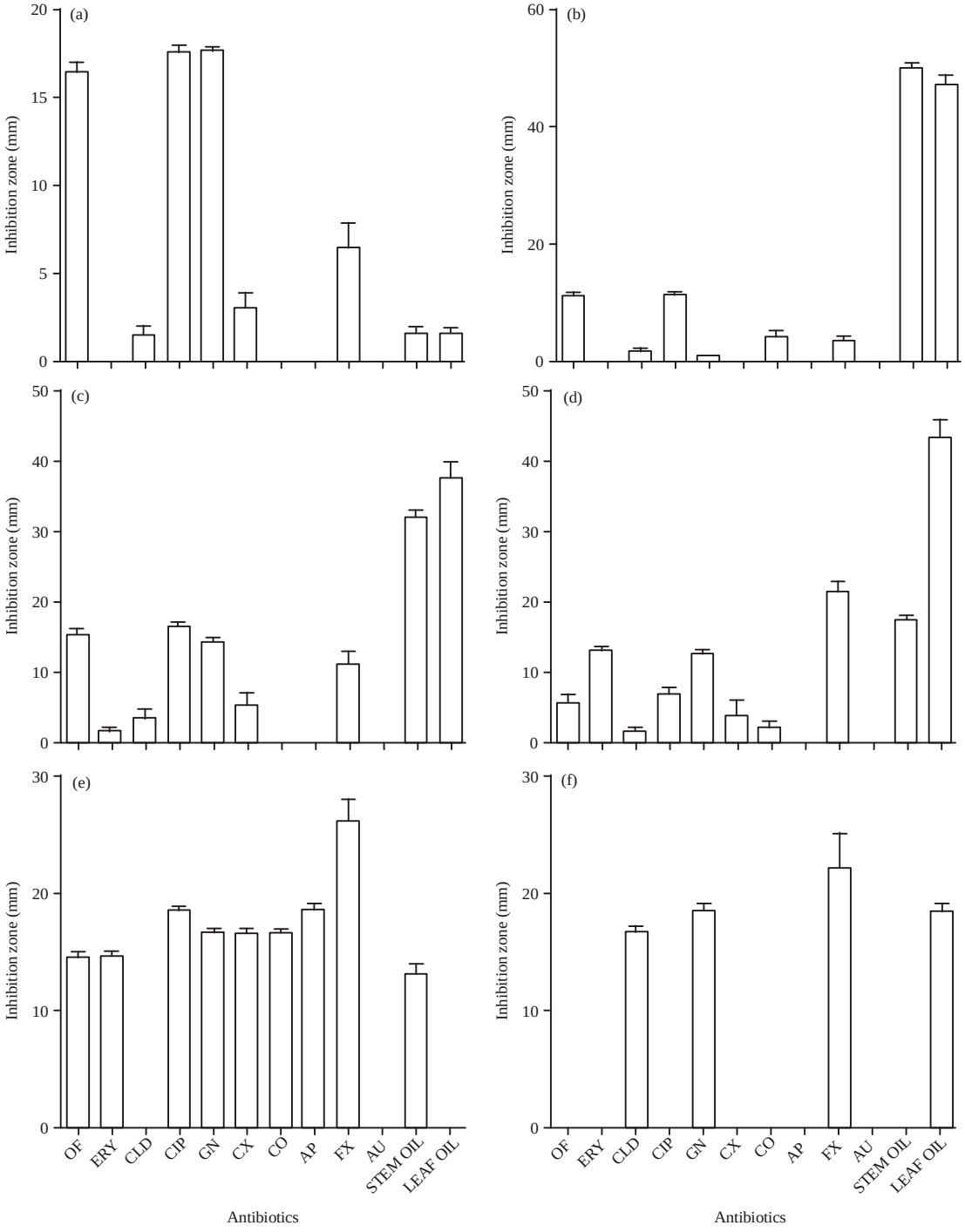

Fig. 1(a-i): Continue

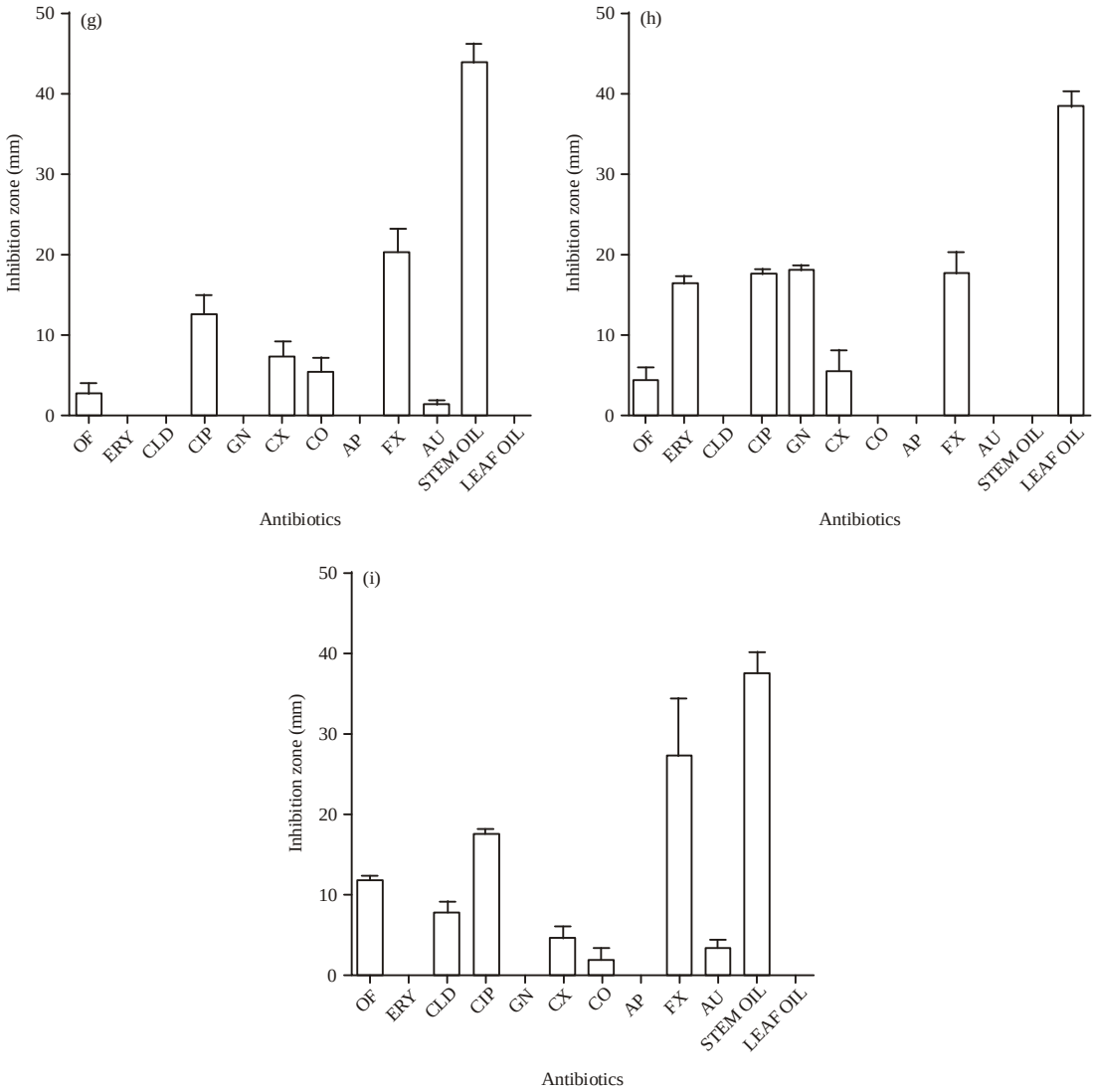

Fig. 1(a-i): Antimicrobial screening of the volatile oils of *Chromolaena odorata* as compared with the clinical antibiotics OF: Ofloxacin, ERY: Erythromycin, CLD: Clindamycin, CIP: Ciprofloxacin, GN: Gentamicin, CO: Cotrimoxazole, AP: Ampicillin, FX: Ceftriaxone, AU: Augumentin, (a) *Staphylococcus aureus*, (b) *Escherichia coli*, (c) *Pseudomonas aeruginosa*, (d) *Salmonella typhimurium*, (e) *Salmonella paratyphimurium*, (f) *Salmonella enteritidis*, (g) *Shigella dysenteriae*, (h) *Shigella flexneri* and (i) *Enterohaemorrhagic Escherichia coli*

Figure 2 and 3 show the effect of pH and temperature on the extracellular protease activity of *Pseudomonas aeruginosa*. The protease had highest activity at 7.5 and 35°C.

Figure 4 shows the enzyme kinetics of the extracellular protease of *Pseudomonas aeruginosa* under the influence of volatile oils of *Chromolaena odorata* as potent inhibitor. The double reciprocal plot shows that inhibition is competitive in both COLVO-*Chromolaena odorata* leaf volatile oil and COSVO-*Chromolaena odorata* stem volatile oil, because they both have the same V_{max} (0.91 μmol min^{-1}) but different K_m values (K_m in the absence of inhibitor = 0.48 mg mL^{-1}).

Table 1: Minimum inhibitory concentration and minimum bacterial concentration of the volatile oils of *Chromolaena odorata against* nine pathogenic bacteria

	Stem volatile oil (% v/v)		Leaf volatile oil (% v/v)	
Micro-organisms	MIC	MBC	MIC	MBC
Staphylococcus aureus†	100	≥100	100	≥100
*Escherichia coli**	0.09	≥0.18	0.09	≥0.18
Pseudomonas aeruginosa	0.18	≥0.39	0.18	≥0.39
Salmonella typhi	6.25	≥12.5	0.18	≥0.39
Salmonella paratyphimurium	0.18	≥0.39	100	≥100
Salmonella enteritidis	100	≥100	6.25	≥12.5
Shigella dysenteriae	0.18	≥0.39	100	≥100
Shigella flexneri	100	≥100	0.18	≥0.39
*Enterohaemorrhagic Escherichia coli**	0.09	≥0.18	0.09	≥0.18

† Gram positive bacteria and most insensitive microorganism, *Most sensitive organism to the volatile oils, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

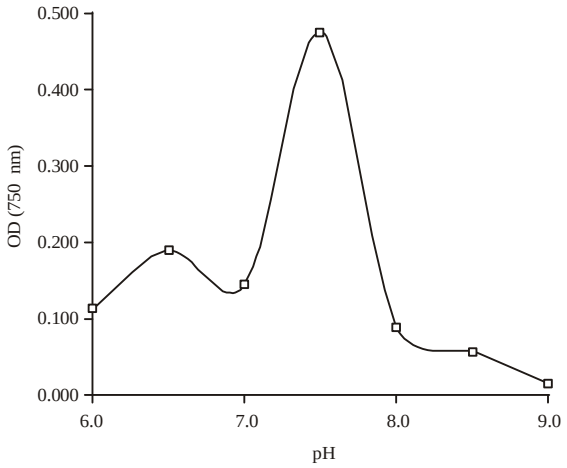

Fig. 2: Effect of pH on the enzymatic activity of extracellular protease of *Pseudomonas aeruginosa*. The enzyme exhibited highest activity at pH 7.5

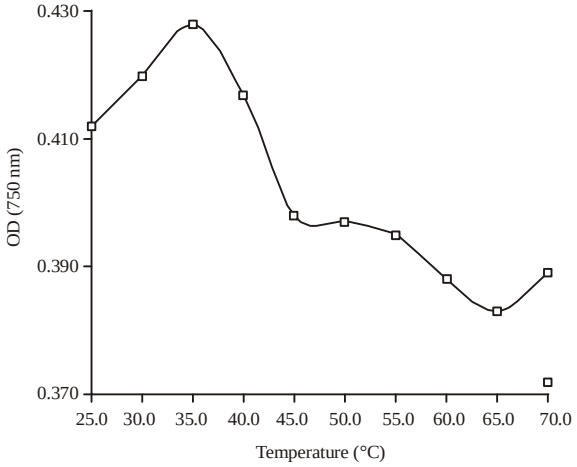

Fig. 3: Effect of temperature on the enzymatic activity of extracellular protease of *Pseudomonas aeruginosa*. The enzyme exhibited highest activity at 35°C

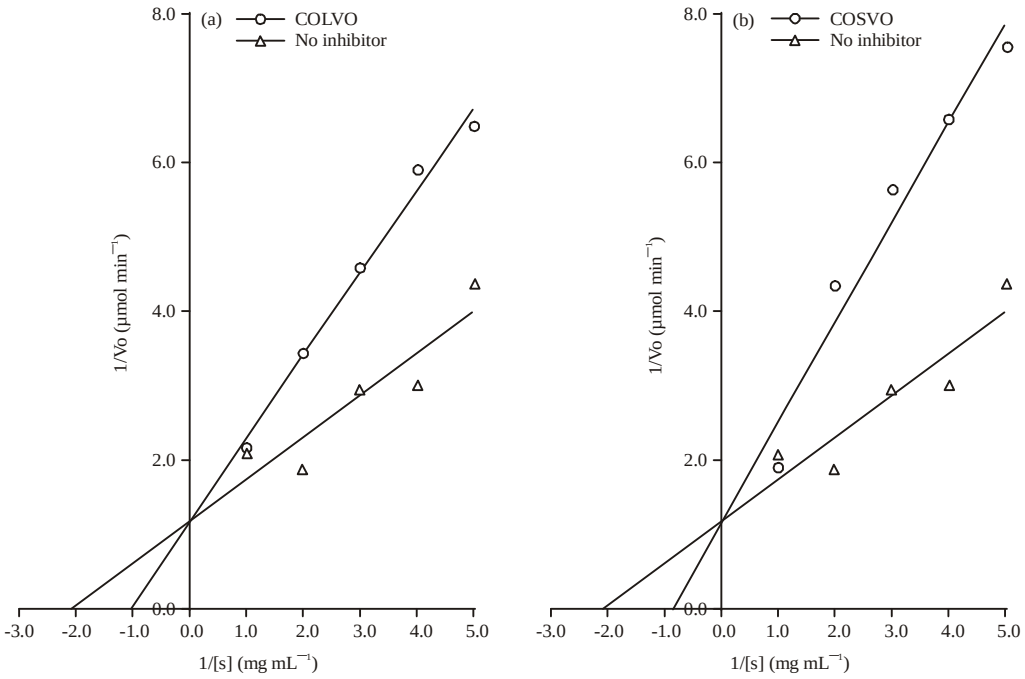

Fig. 4(a-b): Line weaver burke plot of enzyme activity. The double reciprocal plot shows that inhibition is competitive in both (a) COLVO-*Chromolaena odorata* leaf volatile oil and (b) COSVO-*Chromolaena odorata* stem volatile oil have the same V_{max} (0.91 µmol min^{-1}) but different K_m values (K_m in the absence of inhibitor = 0.48 mg mL^{-1}, K_{mCOLVO} = 0.93 and K_{mCOSVO} = 1.25 mg mL^{-1}). This suggests that substrate and one or more component(s) of the oils, as inhibitor, have the same structural/functional similarities and this made them to compete for the active site of the enzyme

Table 2: Purification table of the extracellular protease of *Pseudomonas aeruginosa*. Highest purification of 2.35 was achieved with DEAE cellulose ion exchange chromatography

Purification steps	Total protein (mg)	Total activity (µmol min^{-1})	Specific activity (µmol min^{-1} mg^{-1} protein)	Percentage yield	Purification fold
Crude enzyme extract	3.163	9.310	2.94	1.00	1.00
65% $(NH_4)_2SO_4$ precipitation	0.033	0.104	3.15	1.12	1.07
55% $(NH_4)_2SO_4$ precipitation	0.028	0.098	3.50	1.05	1.19
50% $(NH_4)_2SO_4$ precipitation	0.021	0.094	4.47	1.00	1.52
35% $(NH_4)_2SO_4$ precipitation	0.020	0.102	5.08	1.10	1.73
Sephadex G-100	0.017	0.093	5.47	1.00	1.86
Sephadex G-75	0.016	0.092	5.75	0.99	1.95
DEAE cellulose ion exchange	0.013	0.090	6.92	0.97	2.35

The K_m in the presence of the volatile oil from the leaf (K_{mCOLVO}) increased to 0.93 mg mL^{-1} while stem (K_{mCOSVO}) increased to 1.25 mg mL^{-1}. This suggests that substrate and one or more component(s) of the oils, as inhibitor, have the same structural/functional similarities and this made them to compete for the active site of the enzyme.

Table 2 shows a summary of the purification profile obtained for the extracellular protease of *Pseudomonas aeruginosa*. Purification by 65% ammonium sulphate precipitation gave a purification

fold of 1.07 and percentage yield of 1.12 while, 35% ammonium sulphate precipitation gave a purification fold of 1.73 and percentage yield of 1.10. The highest purification recovery of 2.35, as compared to the crude extract, was achieved from DEAE cellulose ion exchange chromatography.

Figure 5-7 show the elution fractions obtained from Sephadex G-100, G-75 and DEAE cellulose ion exchange chromatography, respectively. The formation of multiple peaks in these orders shows the order of purifications and that the extracellular protease of *Pseudomonas aeruginosa* is likely to be a protein of multiple subunits but this needs some evidence-based procedures to be confirmed.

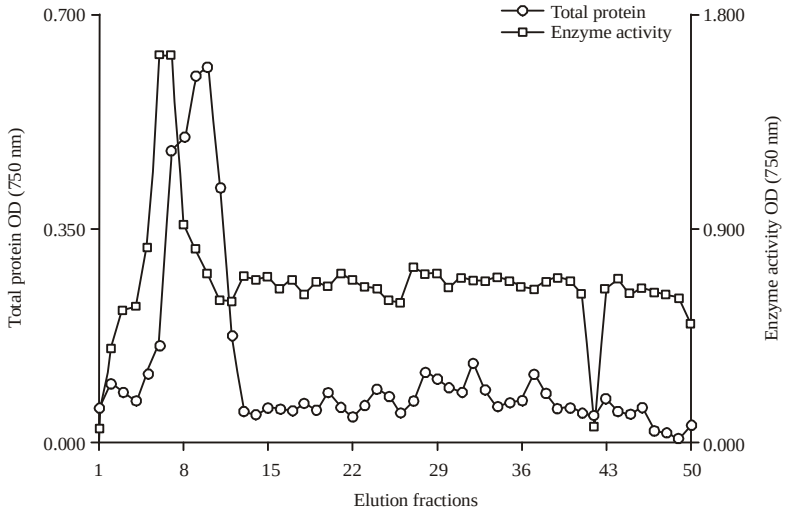

Fig. 5: Elution fractions obtained from Sephadex G-100 gel filtration

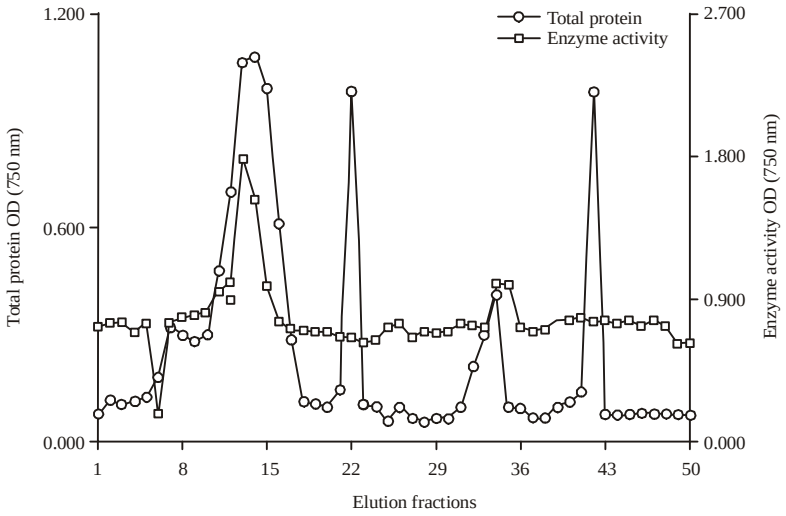

Fig. 6: Elution fractions obtained from Sephadex G-75 gel filtration

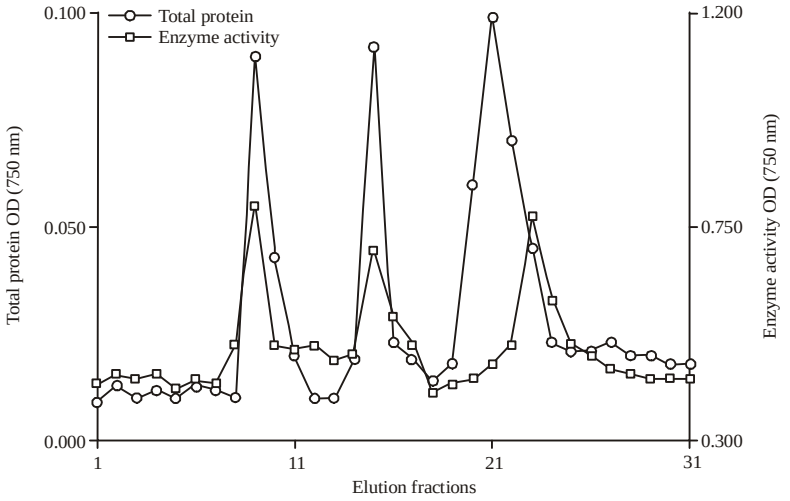

Fig. 7: Elution fractions obtained from DEAE- cellulose ion exchange chromatography. Formation of multiple peaks indicated the presence of multiple subunit proteins

DISCUSSION

The volatile oils of *Chromolaena odorata* has shown to be more potent antimicrobial agent compared to numerous clinical antibiotics. The volatile oil from the stems and leaves of *Chromolaena odorata* inhibited the growth of *Pseudomonas aeruginosa* with an inhibition zone of 32.0±1.0 and 37.50±2.5 mm, respectively. This is in line with the finding of Jahan *et al.* (2007), who confirmed that *Azadirachta indica* oil (neem oil) was highly sensitivity to isolate of *Pseudomonas aeruginosa*. The organism has also been found to be susceptible to cider oil, cinnamon oil, lemon oil and vetiver oil. In the studies carried out by Prabuseenivasan *et al.* (2006), *Pseudomonas aeruginosa* susceptible to different types of essential oils from nineteen medicinal plants. Out of twenty-one plants tested, cinnamon oil had the highest inhibition against *Pseudomonas aeruginosa* with inhibition zones of 33.3 mm and the MIC was >8.0% while basil oil had the least inhibition of 8.2 mm.

The activities of the extracellular protease of *Pseudomonas aeruginosa* under the influence of pH and temperature have probably revealed why this organism can thrive well even in the human gastrointestinal tracts. It was obvious that this organism may not survive the gastric conditions except there were other means of protections adopted by this organism to survive this condition. High *in vivo* abundance of amino acid decarboxylases (GadB and AdiA) and protein disaggregation chaperones (HdeA, HdeB and ClpB) were indicative of a coordinated bacterial survival response and enhancer of pH homeostasis in the cytoplasm of these pathogens to acid stress in human GIT (Pieper *et al*., 2009). Begum *et al*. (2007) in their work to characterize the intracellular protease of *Pseudomonas aeruginosa,* the protease showed highest activities at pH 8.0 and 50°C. Reports have shown that most enteric and opportunistic pathogenic organisms thrive well in pH range 6-9 and temperature range of 30-50°C.

The volatile oil from the stems and leaves of *Chromolaena odorata* competitively inhibited the extracellular protease indicating that these oils were potentially capable of reducing the catalytic activity of the extracellular protease of *Pseudomonas aeruginosa.* The competitive nature of the inhibition showed that these oils might serve as a template for developing new antimicrobial drug that can be targeted against this protease.

The Sephadex G-100 gel filtration revealed a peak each for both total protein and total enzyme activity while Sephadex G-75 further separated this protein into three peaks for protein and two peaks for enzyme activity. By this separation, it may be inferred that the extracellular protease of this organism may likely be a multiple subunit protein (further investigations suggested). The DEAE-cellulose anion exchanger revealed multiple peaks, lending credence to the existence of probably more than one extracellular proteases or a multiple subunit protein. The extracellular protease of this pathogen may likely possess net negative charge. Further work on this is needed to reveal the molecular weight of these subunit proteins.

The volatile oil from the stems and leaves of *Chromolaena odorata* possessed antimicrobial activities against some enteric pathogenic bacteria tested in this work. It has been shown that these oils demonstrated competitive inhibition against the kinetics of the extracellular protease of *Pseudomonas aeruginosa,* a pathogenic microorganism whose virulence is indirectly aided by the secretion of some extracellular proteases.

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