

Essential Oil of *Syzygium samarangense*; A Potent Antimicrobial and Inhibitor of Partially Purified and Characterized Extracellular Protease of *Escherichia coli* 25922

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Abstract: Volatile oils being secondary metabolites are phytoactive ingredients found in medicinal plants and may be active against various infectious microorganisms. The present study was carried out to evaluate the antimicrobial effect of the volatile oil from the leaf of *Syzygium samarangense* on *Escherichia coli* and its inhibition on the extracellular protease of this organism. The volatile oil inhibited the growth of *Escherichia coli* with IC_{50} of 0.42% (v/v). The extracellular protease of this organism exhibited highest activities at pH 7.0 and 43°C. This enzyme was moderately activated by the chloride salts of Zn^{2+} , K^+ and Cu^{2+} . The set of chloride salts of Ba^{2+} , Pb^{2+} , Hg^{2+} and Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} , Fe^{2+} were, respectively strong and mild inhibitors against the activity of this enzyme. The line weaver burke kinetic plot indicated a competitive mode of inhibition by the volatile oil on the enzyme with V_{max} of $8.33 \times 10^3 \mu\text{mol}/\text{min}$ and the K_m in the absence and presence of the volatile oil (inhibitor) were 0.23 mg/mL and 1.25 mg/mL, respectively. The highest percentage yield during purification was 66.3 and the highest purification fold was 11.9 as compared to the crude enzyme. Sephadex G-100 gel filtration produced one peak each for total protein and enzyme activity. Therefore, the volatile oil from the leaf of *Syzygium samarangense* may possess antimicrobial activity and its inhibitory effect on the extracellular protease of *Escherichia coli* may be one of its modes of action on the pathogenic organisms.

Keywords: Antimicrobial, *Escherichia coli*, extracellular protease, inhibitor, *Syzygium samarangense*, volatile oil

INTRODUCTION

Man, since creation, has been dependent on plants for food, shelter, clothing and medicine. Medicinal plants are herbs that contain phytoactive components known to modern and ancient civilization for their healing properties. These medicinal plants used for disease remedies could be in any usual forms such as infusions, decoctions, tinctures, syrups, infused oils, essential oils, ointments and creams (Leslie, 2004; Green, 2000; Chancal, 2006). *Syzygium samarangense* (*Myrtaceae*) is a deciduous tree commonly known as Semarang apple. The fruits of this plant are used in traditional medicine to cure diabetes (Shahreen *et al.*, 2012). The oils extracted from *Syzygium samarangense* (Blume) contain γ -terpene (28.5%), α -pinene (18.2%) and β -cymene (13.7%) as some of the essential components (Gao *et al.*, 2012). Essential/volatile (ethereal) oils have been found to be one of the active ingredients of the medicinal plants (Singh *et al.*, 2002; Lawless, 1995; Jansen *et al.*, 1987). They are concentrated, hydrophobic liquid containing volatile aromatic compounds (Lima *et al.*, 1993). Some of these aromatic compounds have been isolated and characterized, with antibacterial, antifungal, antiviral and antiprotozoal properties (Danuta, 1998). These oils

give various scents to medicinal plants (Watt *et al.*, 1962). The volatile oils have been used as part of active ingredients in the manufacture of antiseptics and disinfectants (Hoffman, 1987) and they have found their applications in the production of pharmaceutical and household cleaning products (Kurt, 1998).

Escherichia coli (*Enterobacteriaceae*) are enteric, facultative, anaerobic, gram-negative, rod-like bacteria that live in the intestinal tracts of healthy and disease animals (Eckburg *et al.*, 2005). Minimal cell density of *Escherichia coli* at the gastrointestinal tract of man could be beneficial. Symbiotically, vitamin K_2 (Bentley and Meganathan, 1982) in man is partly synthesized by the intestinal fauna colonization of these bacteria. However, higher cell density of these bacteria could be responsible for flatulation, stomach upset, induced diarrhoea, colitis, Urinary Tract Infections (UTI), soft tissue infections, bacteraemia and neonatal meningitis (Todar, 2007). Certain strains like *Enteroinvasive* and *Enterohaemorrhagic Escherichia coli* are highly virulent and pathogenic (Vogt and Dippold, 2002). Virulence factors enable *Escherichia coli* and its strains to colonise and invade adjacent mucosal uro-epithelium tissue layer by invoking inflammatory reactions (Todar, 2007). These human pathogens produce extracellular proteases with which they accomplish their

pathophysiological roles (Holder and Haidaris, 1979). Extracellular proteases are strategically used by these pathogens to digest host cellular surface glycoprotein at the epithelial layer of the gastrointestinal lining thereby facilitating host invasion and colonization (Crowther *et al.*, 1987; Stewart-Tull *et al.*, 1986).

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains (Kapil, 2005). The emergence of Multiple Drug Resistant bacteria (MDR) has become a major cause of failure in the treatment of infectious disease by antibiotics (Livermore, 2002; Costerton and Anwar, 1994; Gibbons *et al.*, 2003, 2004; White *et al.*, 2002; Sanders *et al.*, 1977). The need for alternative natural antibiotics may be one of the ways of complementing the presence failing drugs. This study aims at assessing the antimicrobial effect of the volatile oil of *Syzygium samarangense* and its mode of inhibition on partially purified extracellular protease of *Escherichia coli*.

MATERIALS AND METHODS

Collection of plant material: Identified and authenticated leaves of *Syzygium samarangense* were obtained as green foliage from the botanical garden of Lagos State University, Ojo Lagos State, Nigeria. The leaf sample was air-dried for a week.

Microorganism: The *Escherichia coli* 25922 used in this study was obtained from the Department of Microbiology, Nigeria Institute of Medical Research (NIMR) Yaba, Lagos State, Nigeria. The isolate was maintained at 37°C in a disposable petri dishes containing nutrient agar for 24 h and then stored at 4°C.

Extraction of volatile oil of *Syzygium samarangense* by hydrodistillation: This was done according to the procedure of Lawrence and Reynolds (1993). Briefly, 600 g of the dried leaves of *Syzygium samarangense* were introduced 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°C for 3 h and the oil was collected over 2 mL *n*-hexane. The oil was kept tightly in a sample bottle and stored at 4°C until it was used.

Bacteria growth inhibition and determination of IC₅₀ of the volatile oil: The antimicrobial activity of the volatile oil extracted from *Syzygium samarangense* was tested against the growth of *Escherichia coli* 25922 and the inhibitory concentration required to clear off 50% of the bacterial growth was estimated. This was done by using microbroth dilution technique in nutrient broth following a modified method described by Akujobi and Njoku (2010). Briefly, a colony of the organism was added to 200 µL of susceptible test broth (prepared with 0.5% v/v Tween-80) containing two-fold serial dilutions of the volatile oil in the microtitre

plate (21.5 cm by 17 cm). The plate was covered and incubated under anaerobic condition at 37°C for 24 h. After 24 h, each inoculum from the microwell was re-inoculated into a fresh nutrient broth and growth inhibition of the bacteria was spectrophotometrically determined at 620 nm using a microplate reader after 18 h of anaerobic incubation at 37°C. The degree of percentage growth inhibition was estimated using the formula:

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

where,

A_o = The absorbance of the well in the absence of volatile oil

A_1 = The absorbance of the well in the presence of volatile oil

Production of extracellular protease: This was done according to the procedure of Makino *et al.* (1981). *Escherichia coli* 25922 was re-inoculated under anaerobic condition into 5.0 mL freshly prepared nutrient broth in McCartney bottle and this was incubated at 37°C for 24 h. The dirty cloudy microbial broth formed was centrifuged at 9000 rpm for 10 min. The supernatant of this microbial broth was stored in a sample bottle at 4°C until it was used. This supernatant was used as a crude source of extracellular protease.

Protein determination: Total protein of the crude enzyme extract was determined using Lowry *et al.* (1951) method. This was done by adding 5.0 mL of alkaline solution containing a mixture of 50 mL of solution X (20 g sodium trioxocarbonate IV and 4 g sodium hydroxide in 1L) and 1mL of solution Y (5 g copper II tetraoxosulphate VI pentahydrate and 10 g sodium-potassium tartrate in 1L) to 0.1 mL of crude enzyme extract and mixed thoroughly. The 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 after 30 min. Bovine Serum Albumin (BSA) was used as standard protein (0.20 mg/mL).

Enzyme assay: The extracellular proteolytic activity of *Escherichia coli* 25922 was assayed using Folin and Ciocalteau (1927) method. This 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 Ciocalteu'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) was used as standard for the protease activity. A unit of protease activity was defined as the amount of enzyme required to liberate 1.0 μ mol of tyrosine in 60 sec at 37°C. The specific activity was expressed in units of enzyme μ mol/min/mg protein.

Determination of optimum pH of the enzyme activity: The method adopted was described by Makino *et al.* (1981) with little modification. This was carried out by adding 5.0 mL of 0.6% w/v casein solution in 0.05 M Tris buffer (pH ranges from 6.0-8.5), as substrate, to 0.1 mL of the crude enzyme extract and the enzyme assay was carried out at 37°C for 10 min as earlier described.

Determination of optimum temperature of the enzyme activity: As described by Makino *et al.* (1981), 5.0 mL of 0.6% w/v casein in 0.05 M Tris buffer at pH 8.0 was mixed with 0.1 mL of crude enzyme extract and the enzyme assay was carried out at temperature range of 30-60°C for 10 min. The reaction was stopped and enzyme activity was carried out at each stage of temperature.

Inhibitory assay: The method adopted was described by Makino *et al.* (1981) with a slight difference. Briefly, 0.1 mL of the crude enzyme extract and 0.1 mL 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 0.05 M Tris buffer at pH 8.0 and the reaction mixture was mixed and incubated at 37°C for 10 min. The reaction 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. Protease assay was carried out as earlier described above. The procedure was repeated without an inhibitor.

Effect of metallic chloride salts: Following the method described by Jahan *et al.* (2007) with little modification, the extracellular protease activity was carried out in the presence of 1.0 mM chloride salt solutions of Hg²⁺, Pb²⁺, Ca²⁺, Cu²⁺, Ba²⁺, Co²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Zn²⁺ and K⁺. Briefly, to 0.1 mL of the crude enzyme extract, 1.0 mL of each chloride salt solution and 5.0 mL of different concentration of casein solution (0.2-1.0% w/v) in 0.05 M Tris buffer at pH 8.0 were concomitantly added and the reaction mixture was mixed and incubated at 37°C for 10 min. The reaction 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. Protease assay was carried out as described above.

Dialysis: The crude enzyme extract was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D

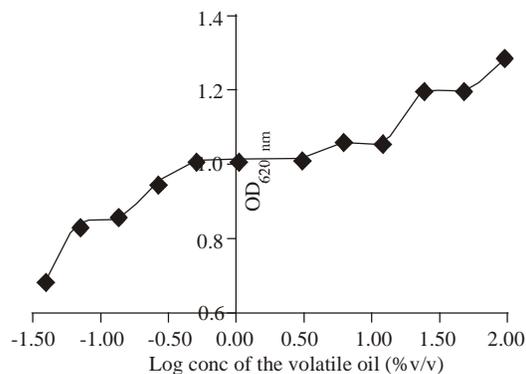


Fig. 1: Growth inhibition of *Escherichia coli* 25922 by the volatile oil of *Syzygium samarangense*

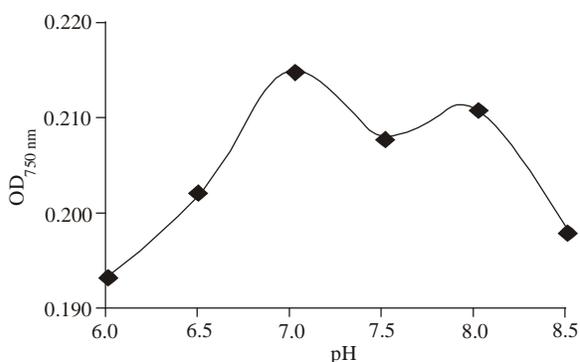


Fig. 2: Effect of pH on the caseinolytic activity of extracellular protease of *Escherichia coli* 25922

9402), at room temperature, with 55% w/v saturated solution of ammonium sulphate for 48 h in 0.05 M Tris buffer solution (pH 8.0). The solution was centrifuged (Kendros Pico Biofuge, Heraeus) at 5000 rpm for 10 min to separate the protein residue. After reconstituting in Tris buffer, both total protein and protease activity were assayed.

Gel filtration: Three gram of Sephadex G-100 was soaked in 100 mL Tris buffer for 72 h. The soaked gel was poured into a capillary tube (20×2) cm² with a flow rate of 0.33 mL/min. Little sodium azide salt was added to the top of the gel overnight to prevent bacterial growth. Five mL of separated 55% w/v ammonium sulphate dialysate was introduced on top of the gel and was filtered using Tris buffer (0.05 M, pH 8.0, at room temperature) as mobile phase. Fifty fractions of 3 mL each were collected. Protein and enzyme activity were determined at 750 nm.

RESULTS

The volatile oil from the leaves of *Syzygium samarangense* inhibited the growth of *Escherichia coli* 25922 as shown in Fig. 1. The IC₅₀ of this oil as estimated from the graph was 0.42% (v/v).

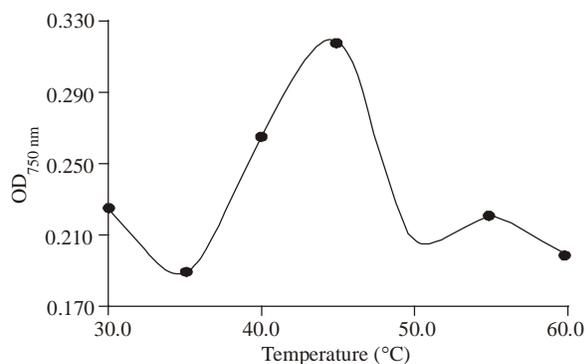


Fig. 3: Effect of temperature on the caseinolytic activity of extracellular protease of *Escherichia coli* 25922

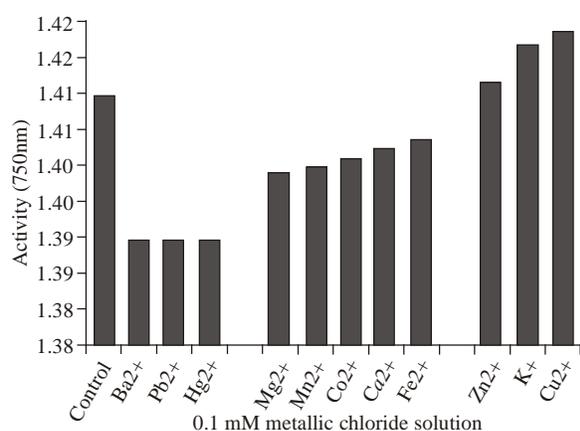


Fig. 4: Effect of metallic chloride ions on the caseinolytic activity of extracellular protease of *Escherichia coli* 25922

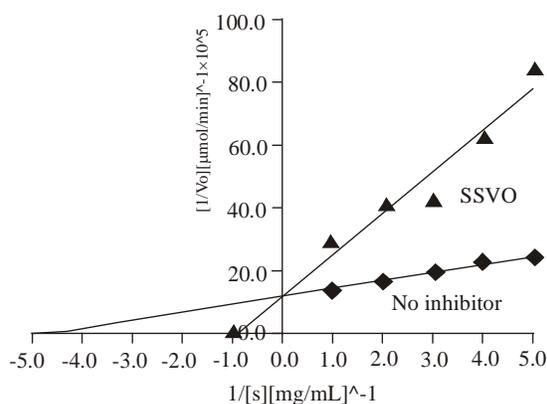


Fig. 5: Line weaver burke plot showing the kinetic inhibition of *Syzygium samarangense* volatile oil (SSVO) on the caseinolytic activity of extracellular protease of *Escherichia coli* 25922

Figure 2 and 3 show the effects of pH and temperature on the activity of extracellular protease of *Escherichia coli* 25922. This enzyme exhibited optimal activities at 7.0 and 43°C, respectively. However, the

activity of this enzyme was moderately high between pH 6.7 and 8.2. There was a steady increase in the enzyme activity between 40-45°C.

Figure 4 shows the effect of metallic chloride ions on the activity of this extracellular protease. Ba²⁺, Pb²⁺ and Hg²⁺ were strong inhibitors of this enzyme. Mg²⁺, Mn²⁺, Co²⁺, Ca²⁺ and Fe²⁺ were mild inhibitors. Conversely, Zn²⁺, Cu²⁺ and K⁺ were moderate activators of this enzyme.

The kinetic inhibition of the volatile oil of *Syzygium samarangense* against the caseinolytic activity of the extracellular protease of *Escherichia coli* is shown in Fig. 5. From this line weaver burke plot, the volatile oil as inhibitor exhibited competitive inhibition with V_{max} of 8.33 × 10³ μmol/min and the K_m in the absence and presence of volatile oil were 0.23 and 1.25 mg/mL, respectively.

The purification profile of the crude extracellular protease of *Escherichia coli* is shown in Table 1. The highest percentage yield obtained during purification was 66.3 and 11.9 as the highest purification fold as compared to the crude extract.

Figure 6 shows the chromatogram for Sephadex G-100 elution profile. One peak each was obtained for both total protein and enzyme activity.

DISCUSSION

In this study, the volatile oil from the leaves of *Syzygium samarangense* was used to inhibit the growth of *Escherichia coli* under anaerobic condition. In addition, the kinetic activity of the extracellular protease of this organism was examined under the influence of this essential oil as inhibitor in order to determine one of the ways by which this plant exhibits its antimicrobial activity. This volatile oil showed a noticeable growth inhibition of *Escherichia coli* under favourable condition. Venkata and Venkata Raju (2008) have been able to show the antibacterial effect of the organic extract of the fruits of this plant against both gram negative and positive bacteria. Joji and Beena (2011), after isolating the components of the volatile oil from the leaves of this plant, suggested that the broad antimicrobial activity of this plant may have been as a result of synergistic effect of the phytoconstituents present in this oil. Eucalyptin was one of the two flavonoids successfully extracted from *Syzygium alternifolium* species (Pulla *et al.*, 2005) and this flavonoid was reported to possess antimicrobial activity (Takahasi *et al.*, 2004) against some gram positive and negative pathogenic bacteria. The infusing volatile oil as an antimicrobial agent generally has ability to disrupt cell membrane thereby increasing their permeability to this oil and causes the biological functions of key proteins to be inhibited and this is followed by cell lysis.

Table 1: Summary of purification procedures

Purification steps	Total protein (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	Percentage yield	Purification fold
Crude enzyme	126	8600	68.30	100	1.00
55% $(\text{NH}_4)_2\text{SO}_4$	65	8500	130.8	98.8	1.90
Gel filtration	7	5700	814.3	66.3	11.9

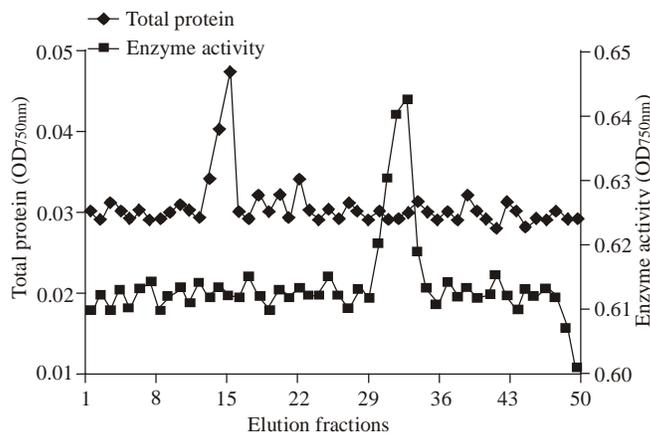


Fig. 6: Sephadex G-100 gel filtration elution profile

The relatively high and stable activity of the extracellular protease of this organism at pH 6.7-8.2 could have been one of the reasons why this organism survives in the fore and the hind guts of GIT especially in warm blooded animals. More importantly, this type of protease is likely to be neutral protease (Fig. 2). Ordinarily, this type of organism hardly cause infections but prolong flora habitation and acquired genotypic variation can make them virulence and therefore capable of causing infections such as gastroenteritis, Urinary Tract Infections (UTI) and neonatal meningitis. In rarer cases, virulence strains are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and gram-negative pneumonia (Todar, 2007). Similarly, the activity of this extracellular protease increased steadily between 40-45°C (Fig. 3). Some strains of *Escherichia coli* have been found to thrive well at 49°C (Fotadar *et al.*, 2005). This enzyme therefore might just be one of the strategic proteins that make this organism to survive relatively high temperature and this is persistently correlated to the characteristics of this bacterium as contaminant of body openings (vagina, anus, nostrils, ears and mouth), dairy products, food, meat, fish and confectioneries. The present result may not totally support the extracellular protease of *Escherichia coli* to be metalloenzyme, because none of the metallic chloride used in this study was able to significantly stimulate the activity of this enzyme more than the control (native enzyme) when subjected to casein hydrolysis. The activity of this enzyme in the presence of Zn^{2+} , Cu^{2+} and K^+ was not different from the control. Ba^{2+} , Pb^{2+} and Hg^{2+} strongly inhibited the activity of this enzyme while Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} and Fe^{2+} mildly inhibited the enzyme.

The volatile oil of *Syzygium samarangense* competitively inhibited the activity of extracellular protease of *Escherichia coli* 25922 indicating that this volatile oil is potentially capable of reducing the catalytic activity of the extracellular protease of *Escherichia coli* by binding to the active site of the enzyme thereby preventing the real substrate from binding and consequently reduce the affinity of the enzyme for the substrate. This may be possible as a result of structural resemblance of the component (s) of the volatile oil and the enzyme substrate. This may be an open way discovery of phytoactive drug capable of arresting similar gram-negative facultative human GIT pathogenic organisms.

The highest purification fold obtained as compared to the extract was 11.9. The highest specific activity was 814.3 $\mu\text{mol}/\text{min}/\text{mg}$ protein. The elution chromatogram revealed a peak each for both total protein and enzyme activity. Further works are needed to elucidate on the nature and molecular weight of this protein.

The essential oil from the leaf of *Syzygium samarangense* was found to be active against *Escherichia coli* 25922 and can be used as either bacteriostatic or bactericidal agent to prevent or treat bacterial infections. This study has shown *Syzygium samarangense* to be a source of compound(s) with possible antimicrobial activities and more pharmacological investigations will be necessary to validate its medical applications.

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