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RESEARCH ARTICLE

Partial Purification and Characterization of Extracellular Protease of *Proteus vulgaris* and its Inhibition by the Volatile Oil of *Syzygium samarangense*

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ABSTRACT

Background: The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial infections. However, in the recent time, unrelenting effort to explore the medicinal importance of plants has become a major concern to scientists because of the less toxic and reliable phytopharmacoactive components of these plants.

Purpose: The present study was carried out to evaluate the growth inhibitory effect of the volatile oil of *Syzygium samarangense* leaves against *Proteus vulgaris* and to examine the mode of inhibition of this oil on partially purified extracellular protease of this pathogen.

Procedures: The volatile oil was extracted by hydrodistillation from air-dried leaves of *Syzygium samarangense*. The inhibitory effect of the oil was tested against the growth of *Proteus vulgaris* under favourable conditions using spectrophotometric method in nutrient broth. Similarly, the mode of inhibition against partially purified and characterized extracellular protease of this nosocomial opportunistic enteric pathogen was determined from Lineweaver Burke plot. The activity of this protease was also assessed based on the effect of different chloride salt solutions.

Findings: The volatile oil of *Syzygium samarangense* inhibited *Proteus vulgaris* with IC_{50} of 0.42%v/v. The enzyme had optimum activity at pH 7.5 and 45°C. This enzyme was relatively stable at pH range of 7.0 - 8.0. The activity of this enzyme was moderately activated by K^+ but most inhibited by Co^{2+}. Double reciprocal plot of this enzyme showed a competitive inhibition by the volatile oil with V_{max} of 8.33 x 10^3µmol/min and the K_m in the absence and presence of volatile oil were 0.23mg/ml and 1.25mg/ml respectively. Highest percentage yield was 35.9 while the highest purification fold was 4.49.

Conclusion: This study has shown that the relative stability of the protease between pH 7.0 – 8.0 confirmed one of the reasons why this pathogen is responsible for most of the urinary tract infections. The volatile oil extracted from the leaves of *Syzygium samarangense* possessed antimicrobial activity and its inhibition on the extracellular protease of *Proteus vulgaris* may just be one of its numerous modes of antibacterial activity.

Keywords: volatile oil, *Syzygium samarangense,* antibacterial, extracellular protease, *Proteus vulgaris*.

1. INTRODUCTION:

The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial infections. Down the ages, essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened for their potential usage as alternative remedies for the treatment of many infectious diseases (1). World Health Organization (WHO) noted that majority of the world's population depends on

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traditional medicine for primary healthcare (2). Medicinal and aromatic plants, which are widely used as medicine, constitute a major source of natural organic compounds (phytoconstituents) and these include essential oils, alkaloids, tannins, saponins, terpenes, flavonoids (3). Though the synthetic drug 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 actions of synthetic drugs (4).

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. Essential oils have been shown to possess antibacterial, antifungal, antiviral, anticancer, insecticidal, antioxidant properties (5, 6) food preservatives properties (7) aromatherapy properties (8) and fragrance/aroma useable in industries (9). *Syzygium samarangense* (syn: *Eugenia javanica*) is a deciduous tree commonly known as Semarang apple. Several *Syzygium species* have been reported to possess antibacterial (10, 11), antifungal (11) and anti-inflammatory (12, 13) activities. Eugenol (85.3%) (14) has been found as one of the key components of the essential oil of *Syzygium samarangense* and this is mostly responsible for its pharmacological actions (15). The remarkable antibacterial activity exhibited by this volatile oil can be attributed to the synergetic effects of the antimicrobial agents present in it (10).

Proteus vulgaris (*Enterobacteriaceae*) is a rod-shaped, peritrichous flagellated, gram-negative, anaerobic bacterium that inhabits the intestinal tracts of humans and animals (16). It can be found in soil, water and faecal matter. It is an opportunist pathogen of human. It is known to cause urinary tract infections (UTI) and wound infections. Virulence factors are molecules expressed and secreted by pathogens (bacteria, viruses, fungi and protozoa) that enable them to suppress the host immune response and elicit their pathogenicity (17). Human pathogens produce extracellular and intracellular proteases with which they accomplish their physiological roles (16). Extracellular protease is capable of digesting insoluble nutrient materials outside the cell for absorption into the cell. Pathologically, these set of enzymes, indirectly referred to as enzyme virulence factor (18), are commonly used by pathogen to degrade host glycoproteins found lining the epithelial tissues. These proteases play crucial role in numerous pathological processes such as inflammations, arthritis, cancer and a number of degenerative diseases (19). Purification and characterization of microbial proteases are prerequisite for understanding their roles in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. To this extent, rapid and sensitive techniques for the detection, purification and characterization of microbial proteases are highly desirable (20, 21). This work is therefore designed to study the antimicrobial effect of the volatile oil of *Syzygium samarangense (Blume)* and the mode of inhibiting the extracellular protease of *Proteus vulgaris*.

2. MATERIALS AND METHODS

2.1. Collection of plant materials:

Identified and authenticated leaves of *Syzygium samarangense* (Local Apple) were obtained as green foliage from the Botanical garden of Lagos State University, Ojo Lagos State, Nigeria. The leaves were air-dried for a week.

2.2. Microorganism:

Proteus vulgaris used in this work was obtained from the department of Microbiology, Nigeria Institute of Medical Research (NIMR) Yaba, Lagos State, Nigeria. The isolate was maintained at 37^{o}C in a disposable petri dishes containing nutrient agar for 24 hours and then stored at 4^{o}C.

2.3. Extraction of volatile oils of *Syzygium samarangense* by hydrodistillation:

This was done according to the procedure of Lawrence & Reynolds (22). Briefly, 600g of air-dried leaves of *Syzygium samarangense* were introduced into the 5 L 34/35 Quick fit round bottom flask containing 1.5 L de-ionized water with fixed Clevenger. The oil was extracted at a steady temperature of 80 ^{o}C for 3 hours and the oil was collected over 2 ml *n*-hexane. The oil was kept tightly in a sample bottle and stored at 4 ^{o}C until it was used.

2.4. Production of extracellular protease:

This was done according to the procedure of Makino et al, (23). A colony of *Proteus vulgaris* was re-inoculated under anaerobic condition into 5.0 ml freshly prepared nutrient broth in McCartney bottle and this was incubated at 37^{o}C for 24 hours. The dirty cloudy microbial broth formed was centrifuged at 9000 rpm for 10 minutes. The supernatant of this microbial broth was stored in a sample bottle at 4^{o}C until it was used. This supernatant was used as a crude source of extracellular protease.

2.5. Bacteria growth inhibition and determination of IC_{50} of the volatile oil:

The antimicrobial activity of the volatile oil extracted from *Syzygium samarangense* was tested against the growth of *Proteus vulgaris* and the inhibitory concentration required to clear off 50% of the bacterial growth was estimated. This was done by using microbroth dilution method in nutrient broth following a method described by Akujobi & Njokwu (24) with slight modification. 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 volatile oil in the microtitre plate (21.5cm by 17cm). The plate was covered and incubated under anaerobic condition at 37^{o}C for 24 hours. After 24 hours, each inoculum from the microwell was re-

inoculated into a fresh nutrient broth and growth inhibition of the bacteria was spectrophotometrically determined at 620nm after 18 hours 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 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.

2.6. Protein determination:

Total protein of the microbial crude extract was determined using Lowry et al, (25) method. This was done by adding 5.0ml of alkaline solution containing a mixture of 50ml of solution X (20g sodium trioxocarbonate IV and 4g sodium hydroxide in 1L) and 1ml of solution Y (5g cupper II tetraoxosulphate VI pentahydrate and 10g sodium-potassium tartrate in 1L) to 0.1ml of crude enzyme 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 protein (0.20mg/ml).

2.7. Enzyme assay:

The extracellular proteolytic activity of *Proteus vulgaris* was assayed using Folin & Ciocalteau, (26) method. This was carried out 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 extract and the mixture was incubated for 10 minutes at 37°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 60 seconds at 37°C. The specific activity was expressed in units of enzyme µmol/min/mg protein.

2.8. Determination of optimum pH of the enzyme activity:

The method adopted was described by Makino et al, (23) with little modification. This was carried out by adding 5.0ml of 0.6 %w/v casein solution in 0.05M Tris buffer (pH ranges from 6.0 - 8.5), as substrate, to 0.1ml of the crude enzyme extract and the enzyme assay was carried out at 37°C for 10 minutes as earlier discussed.

2.9. Determination of optimum temperature of the enzyme activity:

As described by Makino et al, (23), 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 - 65°C for 10 minutes. The reaction was stopped and enzyme activity was carried out at each stage of temperature.

2.10. Inhibitory assay:

The method adopted was described by Makino et al, (23) with a slight difference. 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°C for 10 minutes. The reaction 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. Protease assay was carried out as earlier described. The procedure was repeated without an inhibitor.

2.11. Effect of metallic ions:

Following the method described by Jahan et al, (27) with little 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+}, Pb^{2+}, Zn^{2+} and K^{+}. Briefly, to 0.1ml of the crude enzyme extract, 1.0ml of each chloride salt solution and 5.0ml of different concentration of casein solution (0.2 - 1.0 %w/v) in 0.05M Tris buffer at pH 8.0 were concomitantly added together and the reaction mixture was mixed and incubated at 37°C for 10 minutes. The reaction 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. Protease assay was carried out as earlier discussed.

2.12. Dialysis:

The crude enzyme extract was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D9402), at room temperature, with 55%w/v saturated solution of ammonium sulphate for 48 hours in 0.05 M Tris buffer solution (pH 8.0). The solution was centrifuged (Kendros PicoBiofuge, Heraeus) at 5000 rpm for 10 minutes to separate the protein residue. After reconstituting in Tris buffer, both total protein and protease activities were assayed.

2.13. Gel filtration:

Three gram of Sephadex G-100 was soaked in 100ml Tris buffer for 72 hours. The soaked gel was poured into a capillary tube (20 x 2) cm^2 with a flow rate of 0.33 ml/min. Sodium azide salt (1.0g) was added to the top of the gel overnight to prevent bacterial growth. Two ml of

separated 55% w/v ammonium sulphate dialysate was introduced on top of the gel and was eluted with Tris buffer (0.05M, pH 8.0, at room temperature). Fifty fractions of 3ml each were collected. Protein and enzyme activity were determined at 280nm in each eluent.

3. RESULTS

The volatile oil form the leaves of *Syzygium samarangense* inhibited the growth of *Proteus vulgaris* as shown in **Figure 1**, with IC_{50} of 0.42%v/v.

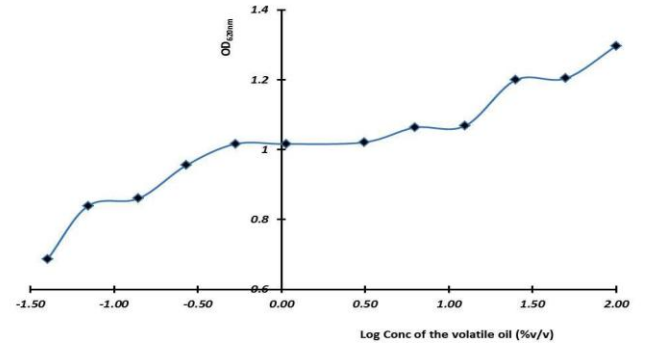

Figure 1: Growth inhibition of *Proteus vulgaris* by the volatile oil of *Syzygium samarangense*. The estimated IC_{50} of the volatile oil against the growth of Proteus vulgaris was 0.42%v/v

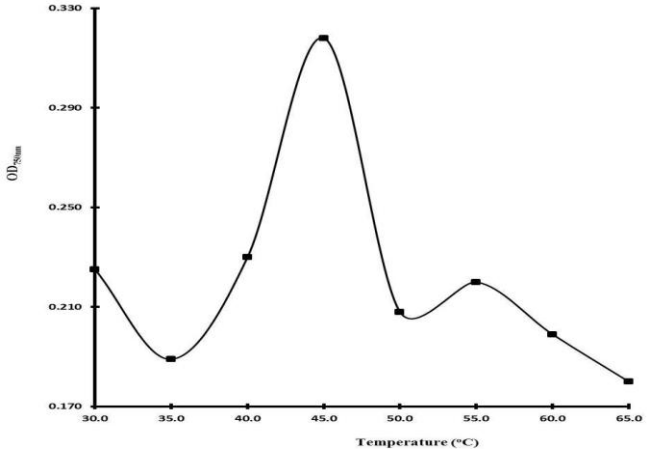

Figure 2: Effect of temperature on the activity of extracellular protease of *Proteus vulgaris.*

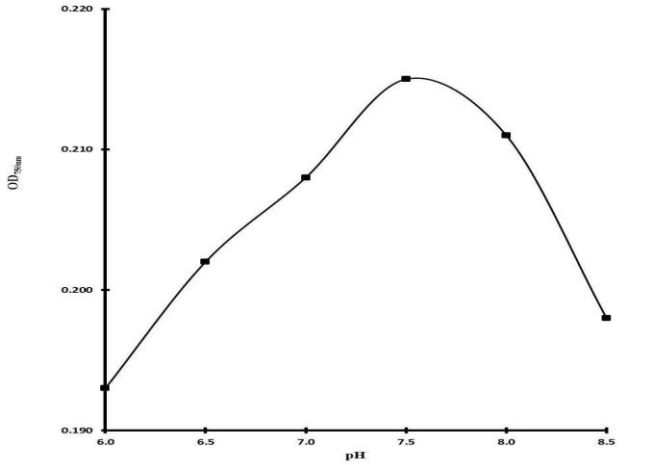

Figure 3: Effect of pH on the activity of extracellular protease of *Proteus vulgaris.* This enzyme exhibited optimum activity at pH 7.5 Meanwhile its activity was moderately high between pH 7.0 and 8.0.

Figures 2 and 3 show the effects of temperature and pH on the activity of extracellular protease of *Proteus vulgaris.* This enzyme exhibited optimal activities at 45°C and pH 7.5. However, the activity of this enzyme was moderately high between pH 7.0 and 8.0.

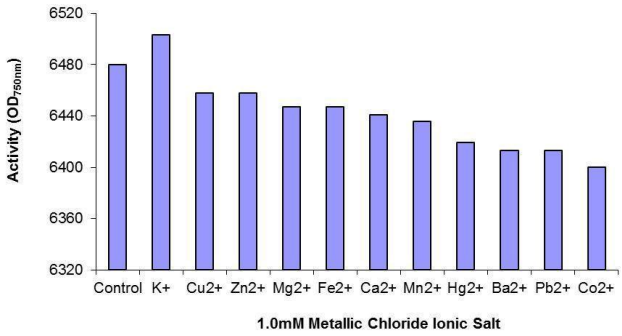

Figure 4: Effect of metallic chloride ions on the activity of extracellular protease of *Proteus vulgaris* Chloride salt of K^+ moderately activated this enzyme. The activity of this enzyme gradually reduced from Cu^{2+} to Co^{2+} chloride salts. Co^{2+} showed highest catalytic inhibition.

Figure 4 shows the effect of metallic chloride ions on the activity of this extracellular protease. K^+ moderate activated this enzyme. Cu^{2+} showed lowest catalytic inhibition against the activity of this protease.

The kinetic inhibition of the volatile oil of *Syzygium samarangense* against the caseinolytic activity of the extracellular protease of *Proteus vulgaris* was shown in **Figure 5.**

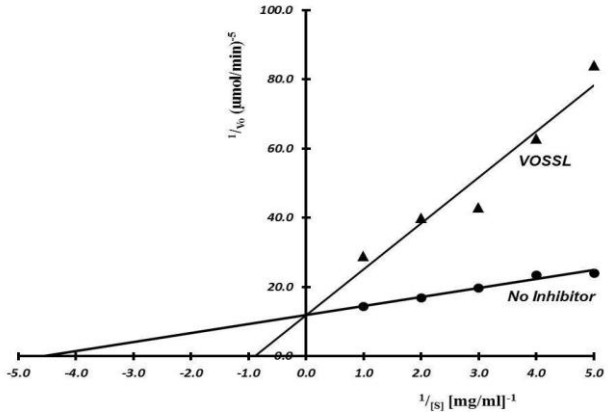

Figure 5: Line weaver burke plot showing the kinetic inhibition of the volatile oil of *Syzygium samarangense* leaves (VOSSL) on the activity of the extracellular protease of *Proteus vulgaris.* The volatile oil showed a competitive inhibition. The V_{max} = 8.33 x 10^3 µmol/min and the K_m in the absence and presence of inhibitor = 0.23 mg/ml and 1.25 mg/ml respectively.

From the line weaver burke plot, the volatile oil as inhibitor, exhibited competitive inhibition with V_{max} of 8.33 x 10^3 µmol/min and the K_m in the absence and presence of volatile oil as inhibitor were 0.23 mg/ml and 1.25 mg/ml respectively.

The purification profile of the crude extracellular protease of *Proteus vulgaris* is shown in **Table 1.** The highest percentage yield obtained during purification was 35.9 and 4.49 as the highest purification fold as compared to the crude extract. The highest enzyme activity obtained was 345.0µmol/min/mg protein.

Purification Steps	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg protein)	Percentage Yield	Purification Fold
Crude enzyme	125	9600	76.8	100	1
55% $(NH_2)SO_4$ precipitation	48	7700	160.8	80.2	2.09
Sephadex G-100	10	3450	345.0	35.9	4.49

Table 1: Purification profiles of extracellular protease of *Proteus vulgaris.* The highest purification fold obtained was 4.49 with specific activity of 345.0µmol/min/mg protein.

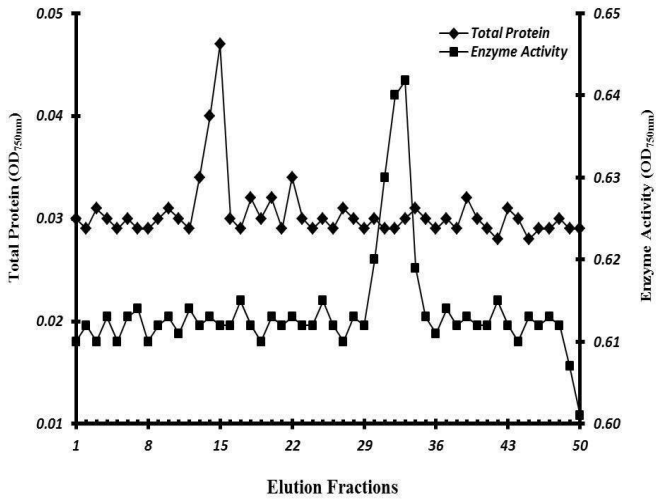

Figure 6: Elution profile obtained from Sephadex G-100 gel filtration One peak each was obtained for both total protein and enzyme activity

4. DISCUSSION

The volatile oil of the leaves of *Syzygium samarangense* has been shown to inhibit the growth of *Proteus vulgaris* under anaerobic conditions. Joji & Jose (28) had shown that the volatile oil extracted from the leaves of *Syzygium samarangense* has pronounced antimicrobial activity against wide range of both grams positive and negative bacteria. Similarly, Venkata & Venkata (29) had studied the *in vitro* antimicrobial properties of the extract from *Syzygium alternifolium* and *Syzygium samarangense* fruits, against certain bacterial and fungal strains using disc diffusion method. The antibacterial activity of this medicinal plant was as a result of the high content of eugenol present in its volatile oil (13). By Joji & Jose, the remarkable antibacterial activity exhibited by the leaf oil of this plant can be attributed to the synergic effect of the antimicrobial agents present in it. The components of this oil based on their finding include α-selinene, β-selinene, γ-terpinene, β-caryophyllene, β-gurjunene, α-thujene and terpinene -4-ol.

The survival of this opportunistic pathogen in human urinary tract and bladder was due to its ability to withstand the pH range of 7.0 – 8.0 and this is one of the reasons why this pathogen is responsible for urinary tract infections and other form of nosocomial-associated infections. Similarly, this pathogen is capable of surviving relatively high temperature. This attribute is common to most nosocomial pathogens because they can survive on hospital equipment and utensils. In this study, extracellular protease from *Proteus vulgaris* exhibited highest activity at 45°C. Generally, the optimal activities of extracellular protease of enteric pathogens are usually found between 35 - 50°C (30).

Metallic ions are necessary for enzyme and protein functions. Most of them are prosthetic groups, either loosely or tightly bound to the protein. In this work, the chloride salt of potassium has shown to be a moderate activator of the activity of extracellular protease of *Protease vulgaris*. This has shown that this enzyme may require metallic ions to function very well under favourable conditions; this is a special case of metalloproteinases (19). Among the tested metallic chloride ions, Co^{2+} exhibited highest inhibition against the activity of this enzyme. This invariably showed that while some metallic ions are good activators for enzyme activity others could be inhibitors. Competitive mode of inhibition exhibited by the volatile oil of *Syzygium samarangense* against the caseinolytic activity of the extracellular protease of *Proteus vulgaris* may have further revealed the antimicrobial nature of this medicinal plant. The oil may have contained a component of similar structure with the true substrate of this enzyme thereby competing with this substrate for the active site of the enzyme. Further works may have been needed to elucidate the biochemical mechanism of this inhibition; whether reversible or irreversible. Interestingly, understanding the nature of this type of inhibition would definitely pave way to the development of chemotherapeutic drugs, which could be directed against pathogenic infections.

Purification and characterization of this extracellular protease could be one of the ways to understanding the pathogenicity of some infectious bacteria. In this work, the partial purification of the crude extract revealed highest percentage yield of 35.9 as compared to the crude extract and the highest purification fold obtained was 4.5. Only one peak each was revealed by Sephadex G-100 for both total protein and enzyme activity.

The variation in the susceptibility of pathogenic microbes to essential oils from medicinal plants could be attributed to their intrinsic properties that are related to the permeability of their cell surface to this volatile oil (31). Due to the emergence of the antibiotic resistant pathogens, plants are being looked upon as an excellent alternative to combat the spread of multi drug resistant pathogens. This study has demonstrated that the essential oil from the leaf of *Syzygium samarangense* is an effective natural antibiotic, which needs to be assessed with the

conventional antibiotics to combat pathogenic microorganisms.

5. ACKNOWLEDGEMENT

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Conflict of Interest: None Declared

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