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Antibacterial Activity of the Volatile Oil of *Phyllanthus muellerianus* and Its Inhibition against the Extracellular Protease of *Klebsiella granulomatis*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study was carried out to evaluate the antimicrobial effect of the volatile oil of *Phyllanthus muellerianus* leaves against *Klebsiella granulomatis* and its inhibitory effect partially on the purified and characterized extracellular protease of this bacteria.
Study Design: This involved the use of medicinal plants and pathogenic bacteria.
Place and Duration of Study: Department of Biochemistry, Faculty of Science, Lagos State University, Ojo Lagos State Nigeria, between April, 2014 – September, 2014.
Methodology: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the hydrodistillated volatile oil against this bacterium were estimated by micro-dilution technique. The extracellular protease was partially purified with ammonium sulfate and Sephadex G-100 gel filtration and assayed using casein as substrate with the volatile oil as inhibitor.
Results: The MIC and MBC obtained were 0.5% and ≥0.7% respectively. Gel filtration produced two peaks each for total protein and enzyme activity. Highest fold of 4.67 was achieved with specific activity of 83.3 µmol/min/mg protein as compared to the crude enzyme. The oil competitively inhibited the partially purified extracellular protease of this bacterium with V_{max} = 500

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µmol/min and K_m = 0.2 mg/ml (no inhibitor) and K_m' = 0.8 mg/ml (inhibitor). Optimal activities of this protease were obtained at pH 7.5 and 35°C. None of the metallic chloride salts tested significantly increased the activity of the enzyme. However, Pb^{2+}, Mn^{2+}, Zn^{2+}, K^{+}, Hg^{2+}, Co^{2+}, Ba^{2+} and Mg^{2+} generally inhibited the enzyme activity.

Conclusion: The volatile oil of *Phyllanthus muellerianus* possessed antimicrobial property and inhibited the extracellular protease of *Klebsiella granulomatis*. Further clarification and clinical verification of this oil will prove to be an effective match in combating burns and post-operative wound infections caused by *Klebsiella granulomatis* and similar species.

Keywords: Volatile oil; extracellular protease; Phyllanthus muellerianus; antimicrobial agent; Klebsiella granulomatis.

1. INTRODUCTION

There are evidences pointing towards the use of medicinal plants for human ailments as alternative medicine. With reference to the failing of common antibiotics and the marginal threat they pose on the body systems, the use of natural products as alternative sources of managing or curing infections has been viewed as one of the ways of tackling infectious diseases especially those associated with pathogens [1]. Medicinal plants are of great importance to the health because of the phytoactive components that possess both curative and preventive properties [2,3].

Phyllanthus muellerianus (Euphorbiaceae), as an example of medicinal plant, is an evergreen monoecious, spiny, straggly or climbing shrubs with numerous stems from the base generally found throughout the tropical and sub-tropical regions with high species similarities [4-6]. Several works confirmed the presence of carbohydrate, saponin, flavonoid, tannin, alkaloid, steroid, triterpene, cardiac glycoside and anthraquinone as key phytochemical components of this plant [2,7]. Similar results with concentration-dependent antimicrobial activities on selected human pathogens by this plant have been reported by Brusotti et al. [8] and Assob et al. [9]. The extracts from different parts of this plant have been used for many types of biliary and urinary conditions including kidney and gall-bladder stones; viral infections like hepatitis, cold and flu; liver diseases and disorders like anaemia, jaundice and liver cancer; bacterial infections like tuberculosis, cystitis, tetanus, wound infections, caries, prostatitis, urinary tract infection and venereal diseases [10,11]. It is also widely employed for diabetes, hypertension, pain and fever relief, digestive stimulant, antispasmodic and cellular protective properties [12,13]. Furthermore, the volatile oil, as part of the phytocomponents of this plant, showed a promising results as antioxidant with significant effects as compared to vitamin C or butylated hydroxyltoluene (BHT) [14,15]. Meanwhile, its antimicrobial effect is of interest to this work.

Brusotti et al. [8] showed a weak inhibitory activity of the water extract of this plant against *Clostridium sporogenes* (MIC 900 µg/mL) and many tested bacteria and fungi but when defatted showed competitive growth inhibition between *Clostridium sporogenes* and *Streptococcus* species (an evidence as used in pygmies traditional medicine) but remained inactive against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Surprisingly, few reports have documented the antimicrobial activities of the essential oils from this plant especially the bark but we scarcely observed any report on the leaves of this plant.

To complement the outcome of Brusotti et al. [8], we examined the antimicrobial activity of the volatile oil of the leaves of *Phyllanthus muelleranus* against *Klebsiella granulomatis* (Enterobacteriaceae) – a genus of non-motile, gram-negative, rod-shaped bacterium [16]. We selected this bacterium because it is implicated in a wide range of diseases notably nosocomial pneumonia, urinary/respiratory tract infections, neonatal septicemia, ankylosing spondylitis, bacteraemia, thrombophlebitis, cholecystitis, diarrhea, wound infection, osteomyelitis, meningitis and soft tissue infections [17-20]. Apart from other pathogenic mechanisms exhibited by this organism to evade the host immune system [11,21], extracellular proteases are used to degrade intestinal host membrane lining glycoproteins or stimulate endocytosis of the host cells thereby facilitating host invasion and cause infections [22]. Apart from the medical awareness and the use of enduring antibiotics, natural products are now employed as active agents against these infectious pathogens. It is

therefore an attempt of this work to assess the antimicrobial activity of the volatile oil of the leaves of *Phyllanthus muellerianus* against *Klebsiella granulomatis* and to examined its inhibitory effect on the extracellular protease of this organism.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Phyllanthus muellerianus plants were obtained at Ojo Local Government Area of Lagos State. Part of the green foliage sample was deposited at the Herbarium of the Botany Department, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria for proper identification and authentication while the remaining was air-dried for 7 days.

2.2 Bacterial Strain

Klebsiella granulomatis was obtained from Nigerian Institute of Medical Research (NIMR), Yaba Lagos State, Nigeria in a disposable petri dish containing nutrients agar maintained at 4°C.

2.3 Chemicals

All chemicals used were obtained from BDH and Sigma-Aldrich Chemicals and they were all of Analytical grade.

2.4 Extraction of Volatile oil of *Phyllanthus muellerianus*

The volatile oil of *Phyllanthus muellerianus* was extracted by hydrodistillation method [23]. Briefly, the seven-day-air-dried leaves were chopped into pieces, weighed and packed, with distilled water (1.5 L), into the 5 L capacity 34/35 quick fit round bottom flask with fixed Clevenger distiller. The volatile oil was collected over a period of 3 hours at 80°C steady temperature in 2 ml *n*-hexane and stored tightly in a sample bottle at 4°C until it was used.

2.5 Antibacterial Activity of the Volatile Oil

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the volatile oil *Phyllanthus muellerianus* leaves against the growth of *Klebsiella granulomatis* were carried out using micro-nutrient-broth dilution spectrophotometric method as described by Akujobi and Njoku [24] with little modifications. A colony of *Klebsiella granulomatis* was added to 200 µl susceptible test broth, previously prepared with 0.5 %v/v Tween-80 [25], containing twofold serial dilutions of the essential oil in a microtitre plate (21.5 cm by 17 cm). The plate was covered and incubated at 37°C for 24 hours. After 24 hours, each inoculum from the microwell was re-inoculated into a fresh nutrient broth and the inhibition of the bacteria was spectrophotometrically monitored at 620 nm using a microplate reader after 18 hours of incubation at 37°C. The average percentage growth inhibition (*%I*) of the bacteria by the volatile oil was estimated using the formula below:

$$\%I = \frac{A_o - A_1}{A_o} \times 100$$

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. In this work, MIC was reported as the average minimum percentage inhibition of the volatile oil observed against the growth of the bacteria under standard conditions, while MBC was the average maximum percentage inhibition of the volatile oil that prevented visible growth of the bacteria under normal conditions. Inhibition was noted as a gradual decrease in the absorbance at 620 nm as compared to the control.

2.6 Extraction of Crude Enzyme from the Isolate

As described by Makino et al. [26] with little modification, a colony of *Klebsiella granulomatis* was inoculated into 5.0 ml of nutrient broth contained in McCartney bottle. The incubated sample (24 hours, 37°C) was centrifuged (Kendros PicoBiofuge, Heraeus) at 9000 rpm for 10 minutes at room temperature and the supernatant was decanted and used as crude enzyme extract.

2.7 Determination of Total Protein

Total protein of the enzyme extract was determined using Lowry et al. method [27]. This was done by adding 5 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 1ml of solution “B” (5 g

cupper II tetraoxosulphate VI pentahydrate and 10 g sodium-potassium tartrate in 1 L) to 0.5 ml of crude extract and mixed thoroughly. The solution was allowed to stand for 10 minutes at room temperature and 0.5 ml of freshly prepared Folin Ciocalteau's phenolic reagent (55%v/v) was added. The solution was mixed thoroughly and the absorbance was read at 750 nm after 30 minutes. Bovine serum albumin (BSA) was used as standard protein (0.2 mg/ml).

2.8 Determination of Enzyme Assay

The extracellular protease of *Klebsiella granulomatis* was assayed using Shana et al. [28] method with slight modification. Protease activity was carried out by adding 5 ml of casein solution (0.6%w/v in 0.05 M Tris buffer at pH 8.0) to 0.5 ml of the crude enzyme extract and the mixture was incubated for 10 minutes 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 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 minutes of thorough mixing. The absorbance was read at 750 nm after 30 minutes. L-tyrosine solution (0.2 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 µmol of tyrosine in 1 minute at 37°C. The specific activity was expressed in units of enzyme µmol/min/mg protein.*

2.9 Dialysis

Salting out technique was carried out on the crude enzyme extract. A 55% $(NH_4)_2SO_4$ saturated solution of the crude enzyme extract was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D9402, suitable for ≥ 12 kDa) for 48 hours and thereafter centrifuged (using Kendros PicoBiofuge, Heraeus) at 5000 g for 5 minutes and total protein and enzyme assay were carried out on the sediment after reconstitution as earlier described.

2.10 Gel Filtration

This was carried out by soaking 3 g of Sephadex G-100 (Sigma-Aldrich) in distilled water for 72 hours. The gel was poured into the chromatographic column (28 cm by 1.5 cm column) and formed a bed length of 22 cm with a flow rate of 1.5 ml/hr and this was used to separate the 55% $(NH_4)_2SO_4$ dialysate. A total number of 50 elutions were collected. Each elution contained 3 ml of eluent and for each of the eluent; total protein and enzyme assay were carried out.

2.11 Determination of Optimum pH of the Enzyme Activity

The method adopted was described by Makino et al. with little modification [26]. This was carried out by adding 5 ml of 0.6%w/v casein solution in 0.05 M Tris buffer (pH range of 6 – 9) to 0.5 ml of partially purified enzyme extract and the assay was carried out at 37°C for 10 minutes as earlier described.

2.12 Determination of Optimum Temperature of the Enzyme Activity

As described by Makino et al. [26] with little modification, 5.0ml of 0.6% casein in 0.05 M Tris buffer at pH 8.0 was mixed with 0.5ml of the partially purified enzyme extract and the assay was carried out at temperature range of 30 – 60°C for 10 minutes. The reaction was stopped and enzyme activity was assayed at each stage of temperature as earlier described.

2.13 Inhibitory Assay

The method adopted was described by Makino et al. with slight difference [26]. Briefly, 0.5 ml of the partially purified enzyme extract and 0.1 ml of 3.5%v/v of the volatile oil in 0.5%v/v Tween 80 (BDH) 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 and the reaction mixture was incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 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 and the procedure was repeated without an inhibitor.

2.14 Effect of Metallic Ions

Following the method described by Jahan et al. with little modification [29], the activity of this partially purified enzyme was carried out in the presence of 1 mM chloride solutions of Mg^{2+}, Fe^{2+}, Ca^{2+}, Co^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, K^{+}, Zn^{2+}, Ba^{2+} and Cu^{2+}. Briefly, to 0.5 ml of the crude enzyme

extract, 1 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, as substrate, were concurrently added and the reaction mixture was incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium chloride and 0.33 M acetic acid mixed in ratio 1:2:3. Protease assay was then carried out at each of the inhibitory stage as earlier described.

3. RESULTS AND DISCUSSION

Antimicrobial and inhibitory effects of the volatile oil extracted from the leaves of *Phyllanthus muellerianus* were carried out against *Klebsiella granulomatis* and its extracellular protease respectively. In this work, 550 g dried leaf sample yielded 0.9 ml of concentrated volatile oil. The MIC and MBC of this oil as estimated based on the average growth inhibition against this pathogen were 0.5% and ≥0.7%. This showed that more than 50% oil dilution is needed to inhibit *Klebsiella granulomatis* growth and more than 70% oil dilution will be needed to kill the growth of this bacteriam.

It appeared that this organism has shown positive responses to some other medicinal plant oils such as cider oil, cinnamon oil, lemon oil and vetiver oil [30]. Brusottia et al. [31] confirmed activities of some functional biological molecules isolated from the stem extract of this plant. They confirmed ε-isoelemicin and α-cadinol as antifungal while caryophyllene oxide as antibacterial: a work similar to Onocha et al. [32] and Doughari & Sunday, [33]. Though, the phytochemical analysis of the plant was undone, but we complement this effort by confirming the antibacterial activity of the volatile oil from the leaves of this plant.

Fig. 1 shows the elution chromatogram of Sephadex G-100 of extracellular protein previously dialyzed with 55% ammonium sulphate saturated solution. The chromatogram shows two peaks each for both total protein and enzyme activity.

The purification profile of the extracellular protein of *Klebsiella granulomatis* is shown in Table 1. The highest specific activity of the enzyme achieved by Sephadex G-100 was 83.3 µmol/min/mg protein while the highest purification fold and yield were 4.67 and 83.3 respectively.

Figs. 2 and 3 show the effect of pH and temperature on the activity of the extracellular protease of *Klebsiella granulomatis*. This enzyme showed optimal activities of 2.04 x 10^2 µmol/min and 1.62 x 10^2 µmol/min at pH 7.5 and 35°C respectively. There were steady decreases in the activities of this protease immediately after their optimal activities.

Apart from its pathological roles during infections extracellular protease of *Klebsiella granulomatis* may contribute to the survival of this nosocomial pathogen because of its activities at optimal conditions [22,34] and this infers that the organism can survive relatively warm and mild alkaline environments (alkalophilic protease), heat disinfection and drying (Figs. 2 and 3). Dienst and Bronwell confirmed that *Klebsiella granulomatis* was pathogenic only for human and that because of this it could only thrive maximally at 37°C and pH 7.2 [35]. Essentially, the volatile oil competitively inhibited the activity of the extracellular protease of *Klebsiella granulomatis* (Fig. 3), and this may constitute one of the ways by which this oil exhibited its antimicrobial activity. Generally, essential oils are lipophilic with phytoconstituents capable of disrupting the topology and the architectural design of the cell membrane leading to cell lysis and inhibiting extracellularly secreted proteolytic enzymes – that fashion the cleaves of host functional proteins.

Table 1. Purification profile of extracellular protein from *Klebsiella granulomatis*

Purification	Total activity (µmol/min)	Total protein (mg/ml)	Specific activity (units/mg)	%Yield	Purification fold
Crude enzyme	2067	115.75	17.85	100	1.0
55% $(NH_4)_2SO_4$ precipitation	1102.4	177.76	6.2	53.3	0.35
Sephadex G-100 gel filtration	1722.5	20.67	83.3	83.3	4.67

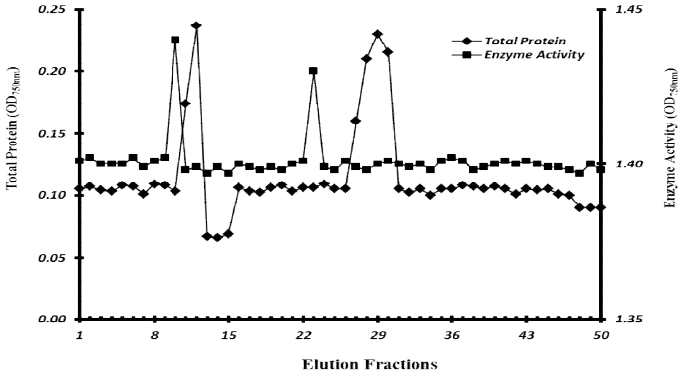

Fig. 1. Sephadex G-100 elution chromatogram of the dialyzed extract of protein from *Klebsiella granulomatis*

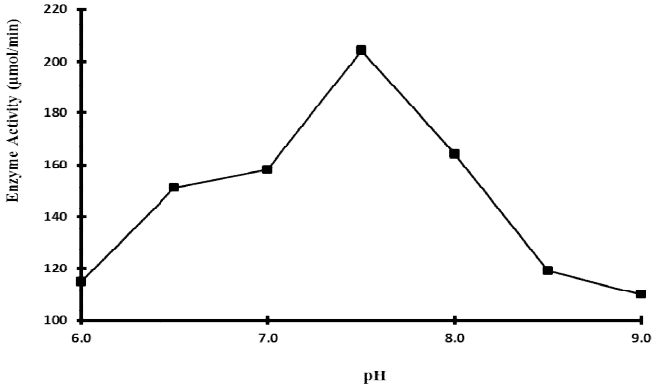

Fig. 2. Effect of different pH on the activity of extracellular protease of *Klebsiella granulomatis*

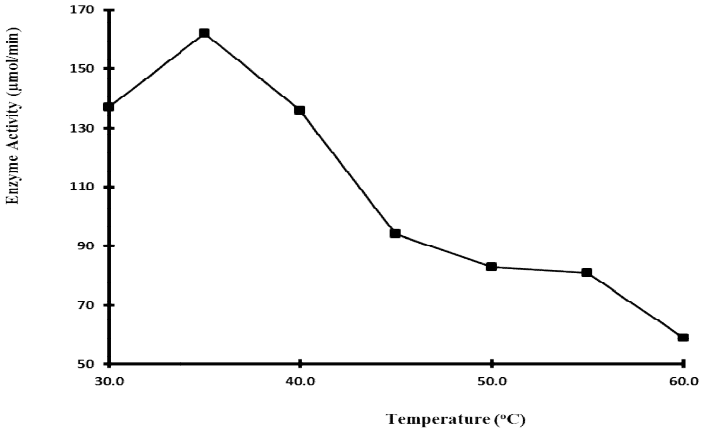

Fig. 3. Effect of temperature on the activity of extracellular protease of *Klebsiella granulomatis*

The caseinolytic kinetics of extracellular protease of *Klebsiella granulomatis* in the absence and presence of volatile oil of *Phyllanthus muellerianus* as inhibitor is displayed in Fig. 4. The volatile oil exhibited a competitive inhibition with the substrate at the active site of the enzyme as indicated on the Lineweaver Burke plot. Competitively, the V_{max} of the enzyme was 500 µmol/min. The enzyme speed of reaction appeared not to be affected by the inhibitor because this value remained unaltered both in the absence and presence of inhibitor. However, the K_m in the absence of inhibitor was 0.2 mg/ml, but this value as a result of the presence of inhibitor, K_m, increased to 0.8 mg/ml.

Partial purification adopted in this work presented the extracellular protease of *Klebsiella granulomatis* as a dimeric protein. Though the essential oil was not able to alter the maximum reaction rate reached by this partially purified protein, but it altered the substrate affinity by unnecessarily increased the K_m´, forestalling this means that the substrate concentration must be increased (Fig. 4).

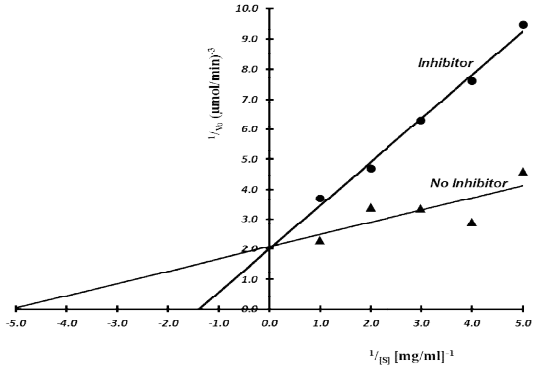

Fig. 4. Lineweaver Burke kinetic plot of the effect of volatile oil of *Phyllanthus muellerianus* (as inhibitor) against the activity of extracellular protease of *Klebsiella granulomatis*

Fig. 5 shows the effect of different metallic chloride salt solutions on the activity of the extracellular protease of this pathogen. By direct comparison, Fe^{2+} appeared to have petite positive activation on the activity of this enzyme as compared to K^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} (Fig. 5), though, this activity may not be too significant when compared to the control. Meanwhile, all other metallic ions (Co^{2+}, Pb^{2+}, Hg^{2+}, Ba^{2+} and Ca^{2+}) unrestrainedly lowered the enzyme activity as compared to the control.

In addition, the result on the effect of different chloride salt solutions on the activity of this enzyme supported the evidence that Fe^{2+} may be necessary for the survival of this pathogen. Dong et al, in their work stated that formation of biofilms on biological and non-biological materials including epithelial tissues, water pool, iron water pipes, hospital equipment by nosocomial pathogens involved Fe^{2+} [36] and this facilitates pathogen-resistant, antibody disguise and camouflaging [37]. One of the survival strategies of this organism through this mechanism is to connect to the source of iron [38]. Therefore, every possible source of activator for the survival of this pathogen and other similar pathogens are thus germane in combating the resulting infections.

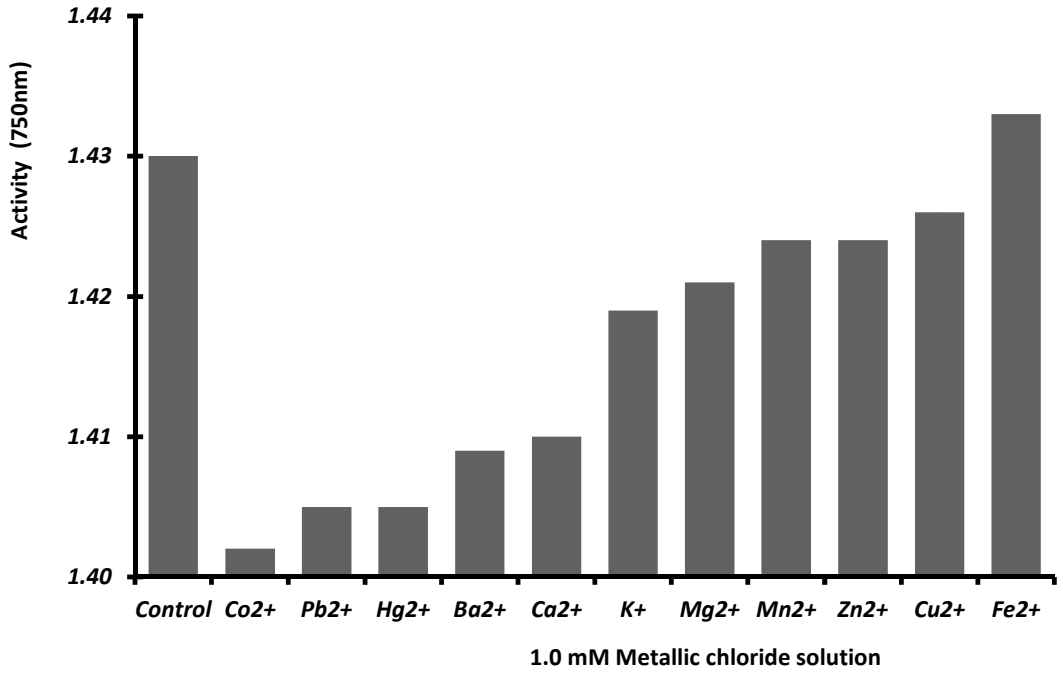

Fig. 5. Inhibitory effect of different metallic chloride salt solution on the activity of the extracellular protease of *Klebsiella granulomatis* at 37°C

Thus, volatile oils of medicinal plants are potential source of antimicrobial agents, which need intensive research into their clinical usages. Identification, isolation, characterization and purification of these agents are paramount to the development of Alternative Medicine. Insight into the strategic inhibition of the extracellular proteases of nosocomial pathogens is therefore suggested.

4. CONCLUSION

The volatile oil of *Phyllanthus muellerianus* showed promising results as antimicrobial and inhibitor of *Klebsiella granulomatis* and its extracellular protease machinery respectively. Further studies are needed to evaluate the antimicrobial activity of this oil against a wide range of microorganisms including multidrug-resistant bacteria. The climax of it is the isolation and purification of the phytoactive component(s) of the essential oil and this unswervingly may intersect the foundation of new natural drugs which would serve a purpose of treating and controlling pathogenic infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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