

Original Research Article

PARTIAL PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEASE OF *Shigella dysenteriae* AND ITS INHIBITION BY THE VOLATILE OIL OF *Thaumatococcus danielli*

ABSTRACT

Aims: This study was carried out to assess the antimicrobial effect of the volatile oil of *Thaumatococcus danielli* leaves against selected enteric human pathogens and its inhibition against the partially purified and characterized extracellular protease of *Shigella dysenteriae*.

Study design: Hydrodistillation of the volatile oil of *Thaumatococcus danielli*, eight enteric human pathogenic bacteria, sensitivity, purification and enzyme assays.

Place and Duration of Study: Sample: Department of Microbiology, Nigeria Institute for Medical Research, Yaba, Lagos Nigeria, between May – September, 2013.

Methodology: The volatile oil was extracted by hydrodistillation. The sensitivity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the volatile oil against the pathogens were determined by disc-diffusion and micro-dilution techniques. The extracellular protease of *Shigella dysenteriae* was partially purified by dialysis and gel filtration. Enzyme assay with casein and volatile oil as substrate and inhibitor respectively.

Results: The average inhibition zones range from 16 – 30 mm. *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Enterococcus faecalis* remained insensitive to the oil. *Shigella dysenteriae* and *Proteus vulgaris* showed highest and lowest sensitivities respectively. Highest purification fold of 2.63 with specific activity of 18.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein were obtained. A peak each for total protein and enzyme activity was revealed by gel filtration. The oil competitively inhibited the partially purified extracellular protease of *Shigella dysenteriae* with $V_{\text{max}} = 8.33 \Delta\text{A}/\text{min}$, $K_m = 0.85\text{mg}/\text{ml}$ (no inhibitor) and apparent $K_m' = 1.54\text{mg}/\text{ml}$ (inhibitor). Optimal activities of this protease were obtained at pH 7.5 and 30°C.

Conclusion: The result has shown effective inhibition of the extracellular protease of *Shigella dysenteriae* by the volatile oil of *Thaumatococcus danielli* as one of its ways of antibacterial effect. The volatile oil of this plant, therefore, will be a promising natural product with numerous pharmaceutical effects especially as antibiotic against enteric human pathogens.

Keywords: volatile oil, extracellular protease, *Thaumatococcus danielli*, antimicrobial agent, *Shigella dysenteriae*

1. INTRODUCTION

There are continuous developments in the pharmacological importance of medicinal plants. Recently, interest in drugs derived from higher plants has increased with the manifestation of diverse chemical compounds of pharmacological impacts being isolated from medicinal plants [1,2]. The World Health Organization (WHO) has made an estimation that about 80% of the people in the developing countries of the world rely on local traditional medicines involving the use of plant extracts as sources of drug [3]. The medicinal importance of these plants lies in the phytochemical components such as polyphenols, sterols, flavonoids, terpenes, saponins, alkaloids, volatile oils, phytols, lycopenes and many more that are usually found in their leaves, flowers, stems, bark, roots and buds. Most of these had had their pharmaceutical impacts in the treatment of pathogenic/worm infections, diarrhea, cardiovascular diseases, upper respiratory infections, urinary tract infections, cancer, malarial and many other infections [4,5]. *Thaumatococcus danielli* is a notable rhizomatous perennial tropical medicinal plants of Africa origin known for being the natural source of thaumatins (variants *I* and *H*), an intensely sweet protein which is of interest in the development of sweeteners/taste modifier [6,7,8]. The aril of this plant is traditionally used

for sweetening bread, over-fermented palm-wine and sour foods [9]. The fruit is used as a laxative and the seed as an emetic and for pulmonary problems [6]. In traditional medicine, the leaf sap is used as antidote against venoms, stings and bites. Worthy of mention was the old use of aqueous decoctions of the leaf of this plant against metrorrhagia [10]. Similarly, an osmotic stress protein called osmotin (similar to that found in tobacco plant cells) had been isolated from this plant and it has demonstrated selective antifungal activities against *Candida albicans*, *Neurospora crassa*, *Trichoderma reesei* and *Cercospora beticola*. Anthony et al, [11] has evaluated some chemical components of the volatile oils of this plant, representing 95.8% of the total oil significant antioxidant and insecticidal properties. The predominantly component was octadecamethylcyclononasiloxane (48.3%) while others were phytol (12.9%), 6, 10, 14-trimethyl-2-pentadecanone (9.3%), 4-methyl-2-octadiene (4.5%), (ε)-3-eicosene (3.1%) and hexadecanoic acid (2.8%)

Combating pathogenic organisms are very paramount to human health. Bacteria, one of the major human pathogens, exhibit their pathogenicity by the release of toxins, most of which are membrane located toxic-proteins with catalytic activities. Extracellular proteases have been proposed as part of the necessary proteins responsible for the digestion of host intestinal lining glycoprotein, attacking antibodies and host zymogens, facilitating host cell attachment, integration, invasion and colonization [12,13]. *Shigella dysenteriae*, amongst other species, are pathogenic gram-negative, rod shaped, non-motile, non-spore-forming, non-encapsulated facultative anaerobes capable of causing bacillary dysentery (shigellosis) characterized by symptoms like abdominal pain, diarrhea, fever, vomiting, mucus/bloody stools, and chronically, haemolytic uremic syndromes and thrombocytopenia purpura [14]. *Shigella* pathogen is transmitted from person-to-person by faecal-oral route. Foodborne and waterborne transmission have been responsible for epidemic of shigellosis in the developing nations noted for unhygienic practises. Antidiarrheal agents such as loperamide, diphenoxylate as well as some antibiotics have failed to treat shigellosis due to pathogen resistance [15]. *Shigella* species generally employed toxins such as proteases and outer membrane surface expressed proteins [13,16] to withstand heat shock, induced inflammation, facilitate the activity of type III secretory systems, alter host cell receptors, evade neutrophil engulfment and induction of complement cascades, promoting biofilm formation and quorum sensing [17,18,19,20].

The urgent demand for active and relatively nontoxic antibiotics has geared up research into the pharmacologic importance of medicinal plants. Previously, *Shigella dysenteriae* has been inhibited by the volatile oil of *Camellia sinensis* [5]; Ojekale et al, [21] had reported antibacterial and antifungal failures of the extracts of this plant against major pathogenic food spoilers. In other to further evaluate the medicinal significance of *Thaumatococcus danielli*, this work is designed to assess the antimicrobial and inhibitory activities of the volatile oil of this medicinal plant against *Shigella dysenteriae* and its extracellular protease.

2. MATERIAL AND METHODS

2.1 Collection of Plant Material

Thaumatococcus danielli plants were obtained at Igbo Elefon in Ogun State, South-western part of Nigeria. The sample was gotten as green foliage and was sun-dried for 4days. The green foliage sample of the plant was deposited at the Herbarium of the Botany Department, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria for proper identification and authentication.

2.2 Microorganisms

A gram positive bacterium (*Staphylococcus aureus*) and seven gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter faecalis*, *Shigella dysenteriae*, *Pseudomonas aeruginosa* and *Salmonella enteritidis*) were obtained from Nigerian Institute of Medical Research (NIMR), Yaba Lagos State, Nigeria in disposable petri dishes containing nutrients agar maintained at 4°C. All the bacteria were human enteric pathogenic strains.

2.3 Chemicals

All chemicals used in this work were obtained from BDH and Sigma-Aldrich Chemicals and they were all of Analar Grade.

2.4 Extraction of Volatile Oil of *Thaumatococcus danielli*

The volatile oil of *Thaumatococcus danielli* was extracted by hydrodistillation method [22]. Briefly, 400g of the four-day-sun-dried *Thaumatococcus danielli* leaves were chopped into pieces and packed, with distilled water (3L), into the 5L capacity 34/35 quick fit round bottom flask with fixed Clevenger distiller. The volatile oil was collected over a period of 3hours at 80°C steady temperature in 2ml *n*-hexane and tightly stored in a sample bottle at 4°C until it was used.

2.5 Cell Culture

The bacteria used in this work were specifically and differentially cultured for optimum growth condition. *Escherichia coli* and *Pseudomonas aeruginosa* were cultures in Mueller Hinton Agar (MHA). *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterococcus faecalis* were cultured in the Nutrient Agar (NA). *Salmonella enteritis* and *Shigella dysenteriae* were cultured in *Salmonella-Shigella* Agar (SSA) while *Staphylococcus aureus* was cultured in Mannitol Agar (MA). All organisms were incubated at 37°C.

2.6 Sensitivity Test

The sensitivity test of the volatile oil of the leaves of *Thaumatococcus danielli* against eight human enteric pathogenic bacteria was carried out using agar disc microdiffusion method as described by NCCLS [23] with little modification. A 5mm diameter paper disc previously soaked in the volatile oil was picked with sterile tong and gently placed on the corresponding media, which have been surface-spread with a colony of each of the organisms. The plates were immediately covered and incubated at 37°C for 24 hours. The sensitivity of the organisms to the volatile oil was an indication of the clear zones found around the paper disc.

2.7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Volatile Oil

The MIC and MBC of the volatile oil of *Thaumatococcus danielli* leaves against eight enteric pathogens under favourable conditions were carried out using microbroth dilution method as described by Wannissorn et al, [24] with little modifications. A colony of each of the organisms was added to 200 µL of corresponding susceptible test broth (with 0.5 %v/v Tween 80 as diluent) containing two-fold serial dilution of the volatile oil in a microtitre plate (21.5 x 17 cm²). The plates were covered, taped and incubated at 37 °C for 18 hours. The broth from each well was re-inoculated on a freshly prepared appropriate agar where MIC and MBC were determined. The MIC was interpreted as the lowest concentration of the oil sample that prevented visible growth of the microorganism in the required medium at standard conditions. The MBC was considered as the lowest concentration of the oil that yielded no colonial growth of the sub-cultured microorganisms in the required medium under favourable conditions.

2.8 Extraction of Crude Enzyme from the Isolate

As described by Makino et al, [25] with little modification, a colony of *Shigella dysenteriae* was inoculated into 5.0ml of *Salmonella-Shigella* broth inside McCartney bottle. It was incubated for 24 hours at 37°C. The inoculum was centrifuged (Kendros PicoBiofuge, Heraeus) at 9000rpm for 10 minutes at room temperature. The supernatant was decanted and used as crude enzyme extract.

2.9 Total Protein

Total protein of the enzyme extract was determined using Lowry et al, method [26]. 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 copper II tetraoxosulphate VI pentahydrate and 10g sodium-potassium tartrate in 1L) to 0.5ml of crude extract and mixed thoroughly. The solution was allowed to stand for 10 minutes at room temperature and 0.5ml of freshly prepared Folin Ciocalteu'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 (0.2mg/ml).

2.10 Enzyme Assay

The extracellular protease of *Shigella dysenteriae* was assayed using a method described by Shana et al, [27] with slight modification. Protease activity was carried out by adding 5.0ml of casein solution (0.6%w/v in 0.05M Tris buffer at pH 8.0) to 0.5ml 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 Ciocalteu's phenolic reagent after 10 minutes of vortex mixing. The absorbance was read at 750nm after 30 minutes. L-tyrosine solution (0.2mg/ml) was used as standard for the protease activity. *A unit of protease activity was defined as the amount of enzyme required to liberate 1.0μmol of tyrosine in 1 minute at 37°C. The specific activity was expressed in units of enzyme μmol/min/mg protein.*

2.11 Dialysis

Salting out technique was carried out on the crude enzyme extract. A 35% (NH₄)₂SO₄ saturated solution of the crude enzyme extract was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D9402) for 48 hours and thereafter centrifuged (using Kendros PicoBiofuge, Heraeus) at 5000 g for 5 minutes. Subsequently, 55% and 65% (NH₄)₂SO₄ were sequentially carried out on the residue of previous dialysate. In each case, total protein and enzyme assay on the residue were carried out as earlier described.

2.12 Gel Filtration

This was carried out by soaking 3.0 g of Sephadex G-75 in distilled water for 72 hours. The gel was poured into the chromatographic column (28.0 cm by 1.5 cm column) and formed a bed length of 22 cm with a flow rate of 1.5 ml/min and this was used to separate the 65% (NH₄)₂SO₄ 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. Eluents with significant enzyme activities were pooled together for further kinetic studies.

2.13 Optimum pH of the Enzyme Activity

The method adopted was described by Makino et al, [25] with little modification. This was carried out by adding 5.0ml of 0.6%w/v casein solution in 0.05M Tris buffer (pH range of 6.5–9.0) to 0.5ml of the enzyme extract and the enzyme assay was carried out at 37°C for 10 minutes as earlier described.

2.14 Optimum Temperature of the Enzyme Activity

As described by Makino et al, [25] with little modification, 5.0ml of 0.6% casein in 0.05M Tris buffer at pH 8.0 was mixed with 0.5ml of the enzyme extract and the enzyme assay was carried out at temperature range of 25–60°C for 10 minutes. The reaction was stopped and enzyme activity was carried out at each temperature as earlier described.

2.15 Inhibitory Assay of the Volatile Oil of *Thaumatococcus danielli*

The method adopted was described by Makino et al, [25] with slight alteration. Concisely, 0.5ml of the partially purified enzyme extract and 0.1ml of 3.5%v/v of the volatile oil in 0.5%v/v Tween 80 solution were added concurrently to different concentration of casein solution (0.2–1.0%w/v) in 0.05M Tris buffer (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 and the procedure was repeated without an inhibitor.

3. RESULTS AND DISCUSSION

The volatile oil obtained from the leaves of *Thaumatococcus danielli* was tested for antimicrobial activity on eight different human enteric pathogenic microorganisms. With reference to the extracellular protease of *Shigella dysenteriae*, the inhibition kinetic of this enzyme by the volatile oil was elucidated in order to predict the antimicrobial pattern of the oil on the pathogen.

The microbial sensitivity test, MIC and MBC of the volatile oil of *Thaumatococcus danielli* are shown in **Table 1**. The zones of inhibition obtained ranged from 16 – 30mm with *Proteus vulgaris* and *Shigella dysenteriae* having the lowest and highest zones of inhibition respectively. *Klebsiella pneumoniae*, **Staphylococcus aureus* and *Enterococcus faecalis* were resistant to the volatile oil of this plant. The MIC and MBC results further buttressed the insensitivities of the three resistive bacteria. Among the most inhibited bacteria were *Escherichia coli*, *Proteus vulgaris* and *Shigella dysenteriae*, all having an inhibition concentration of 0.10 (%v/v).

Table 1. Antimicrobial sensitivity test, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the volatile oil of *Thaumatococcus danielli* against eight enteric human pathogenic bacteria under favourable conditions

organisms	Inhibition zone (mm)	MIC (%v/v)	MBC (%v/v)
<i>Escherichia coli</i>	18.0	0.10	≥0.20
<i>Klebsiella pneumoniae</i>	-	-	>100
<i>Proteus vulgaris</i>	16.0	0.10	≥0.20
<i>*Staphylococcus aureus</i>	-	-	>100
<i>Pseudomonas aeruginosa</i>	18.0	1.56	≥3.12
<i>Shigella dysenteriae</i>	30.0	0.10	≥0.20
<i>Enterococcus faecalis</i>	-	-	>100
<i>Salmonella enteritis</i>	18.0	1.56	≥3.12

*A gram positive enteric bacteria. Others were gram negative bacteria

The summary of purification profile of the extracellular protease of *Shigella dysenteriae* is shown in **Table 2**. The highest specific activity of the enzyme achieved by Sephadex G-75 was 18.4μmol/min/mg protein while the highest purification fold and yield were 2.63 and 47.85 respectively.

Table 2. Purification profile of the extracellular protease of *Shigella dysenteriae*

Purification	Total protein (mg/ml)	Total activity (μmol/min)	Specific activity (μmol/min/mg protein)	%Yield	Purification fold
Crude enzyme	55	385.00	7.00	100	1.00
35% (NH ₄) ₂ SO ₄ precipitation	49	372.40	7.60	96.7	1.09
55% (NH ₄) ₂ SO ₄	40	336.90	8.42	87.5	1.20

precipitation					
65% $(\text{NH}_4)_2\text{SO}_4$ precipitation	28	300.75	10.74	78.1	1.53
Sephadex G-75 gel filtration	10	184.24	18.4	47.85	2.63

Figure 1 shows the elution chromatogram of Sephadex G-75 obtained from gel filtration of the extracellular protease of *Shigella dysenteriae* previously dialyzed on 65% ammonium sulphate saturated solution. The chromatogram shows two peaks each for both total protein and enzyme activity. The activity of this enzyme and the protein contents were found in almost the same region of the elutions.

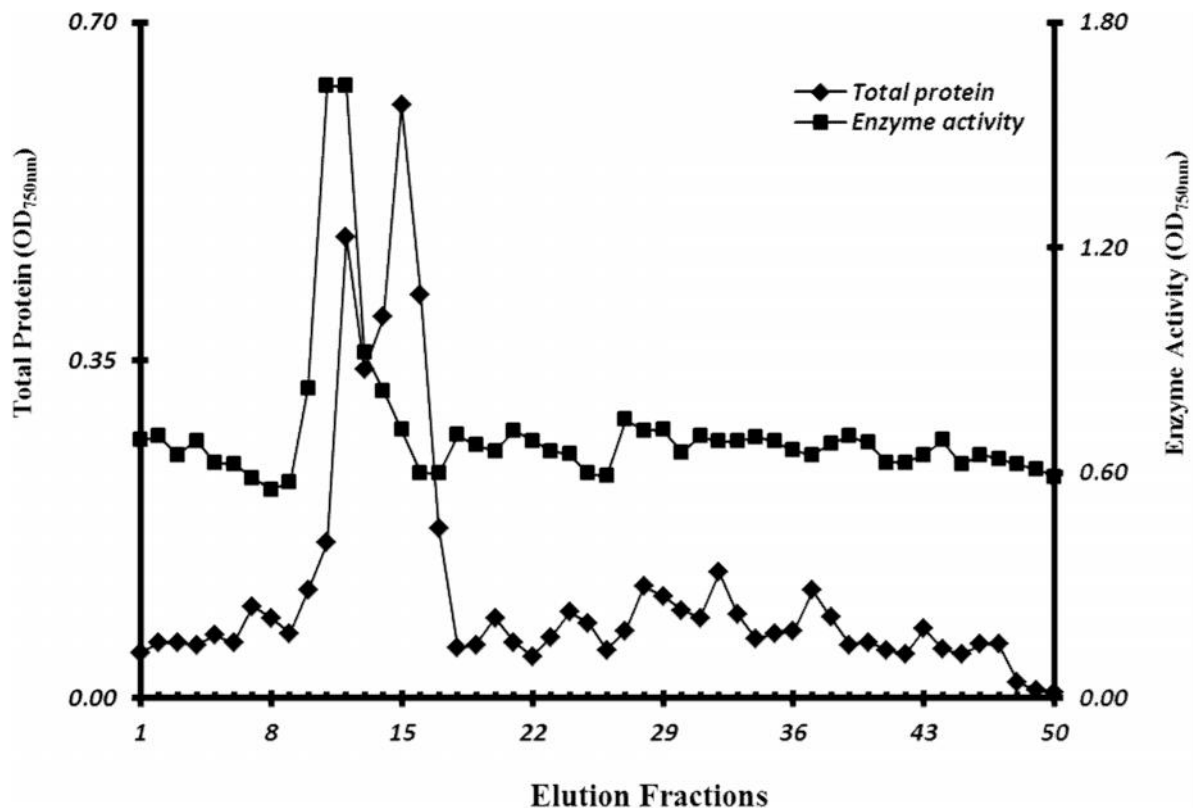


Figure 1. Sephadex G-75 elution chromatogram of the extracellular protease of *Shigella dysenteriae*

Figures 2 and 3 show the effects of pH and temperature on the activities of the extracellular protease of *Shigella dysenteriae*. This enzyme showed optimal activities at pH 7.5 and 30°C respectively. Careful comparison of the activities of this enzyme under the effects of pH and temperature showed sharp drops in the activities of this protease immediately after their optimal points.

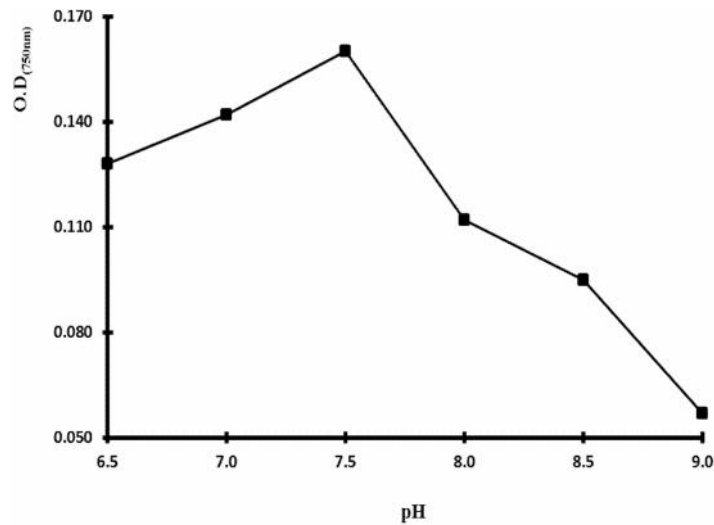


Figure 2. Effect of different pH on the activity of extracellular protease of *Shigella dysenteriae*

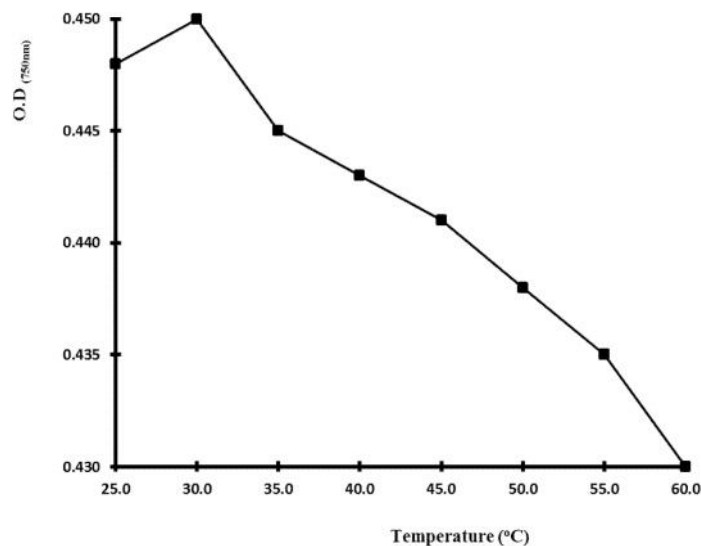


Figure 3. Effect of temperature on the activity of extracellular protease of *Shigella dysenteriae*

The caseinolytic kinetics of extracellular protease of *Shigella dysenteriae* in the absence and the presence of volatile oil of *Thaumatococcus danielli* as inhibitor is displayed in **Figure 4**. The volatile oil exhibited a competitive inhibition on the enzyme as indicated on the Lineweaver Burke plot. The V_{max} of the enzyme was $8.33\Delta A/\text{min}$. The enzyme speed of the 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 0.85mg/ml , but this value as a result of the presence of inhibitor, K_m' , increased to 1.54mg/ml .



Figure 4. Lineweaver Burke kinetic plot of the effect of volatile oil of *Thaumatococcus danielli* (as inhibitor) against the caseinolytic activity of the extracellular protease of *Shigella dysenteriae*

Medicinal plants have been explored for numerous medical purposes with promising results. The volatile oils of medicinal plants, in recent time, have attracted numerous scientific researches for pharmacomedicine and cosmetic purposes. In this view, the antimicrobial effect of the volatile oil of *Thaumatococcus danielli* against eight human enteric pathogenic bacteria was reported. Furthermore, its inhibition against the extracellular protease of *Shigella dysenteriae* has probably supported our hypothesis that antibiotics do not only inhibit the β -lactamase and associated enzymes but also membrane located toxigenic proteins and extracellularly released proteolytic enzymes. The essential oil from this plant inhibited the growth of five pathogenic organisms out of eight tested. Among the notable secondary metabolites found in this plant were tannins, alkaloids, saponins, flavonoids, anthraquinones, anthocyanoside and cardiac and cyanogenic glycosides [28,11]. Both organic and inorganic extract of this plant were not able to produce significant antimicrobial effects against major representatives of pathogenic food poisoning bacteria and fungi [21]. Contrarily, the volatile oil of this plant has shown 62.5% antimicrobial activity against eight pathogenic organisms. *Escherichia coli*, *Proteus vulgaris* and *Shigella dysenteriae* were the most inhibited microorganisms.

The extracellular protease of *Shigella dysenteriae* was partially purified and characterized. Increase in the ionic concentration of ammonium tetraoxosulphate VI salt used increased the protein yield from the crude enzyme extract. Furthermore, most of the extracellular proteins released from this pathogen were enzymes with catalytic effects as revealed by the chromatogram. The activity of this enzyme sharply declined after the optimal conditions (**Figures 2 & 3**). This implied that the protein was highly unstable at optimal conditions; any little alteration from the optimal conditions will reduce the catalytic effects of this enzyme. Few reports are available on the mechanisms of survival of this pathogen in the gastrointestinal environment. A report by [29] has indicated that *Shigella* species are better able to survive the acidic conditions encountered in the stomach than are other enteric pathogens such as *Salmonella* species. Certainly, most isolates of *Shigella*, unlike other *Enterobacteriaceae*, survive acidic treatment at pH 2.5 for at least 2 hours [30]. Folorunso et al, [5] have reported optimal activities of the same enzyme from the same pathogen at pH 8.0 and 43.4°C. Similarly, Adeola et al, [31] have reported pH 8.0 and temperature 44°C as optimal conditions for the extracellular protease of *Shigella flexneri*. Louboudy et al, [32] have confirmed that most of the water-associated pathogenic bacteria thrived well at temperature range of 10-55°C and pH 4.0-12 and respectively spoiled foods or caused infections at these conditions.

Purifications adopted in this work may have presented the extracellular protease of *Shigella dysenteriae* as dimeric protein. The volatile oil of *Thaumatococcus danielli* competitively inhibited the caseinolytic activity of the extracellular protease of *Shigella dysenteriae*. Though this oil was not able to alter the maximum rate of enzyme reaction, but it altered the substrate affinity by gratuitous increased in K_m , forestalling this means that the substrate concentration must be amplified.

Hence, the volatile oil of *Thaumatococcus danielli* has shown a promising result as antimicrobial agent against enteric pathogenic bacteria and its inhibitory effect against the extracellular protease of *Shigella dysenteriae* was nonetheless one of the ways of arresting pathogenic bacteria.

4. CONCLUSION

The volatile oil of *Thaumatococcus danielli* (Benth.) inhibited the growth of *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Salmonella enteritis* and further confirmed to have inhibited the extracellular protease of *Shigella dysenteriae*. Further studies are therefore needed to evaluate the antimicrobial activity of this oil against a wide range of infectious microorganisms including multidrug-resistant bacteria. The climax of it is the isolation and purification of the phytoactive ingredient(s) of the essential oil, which can be used for drug template.

COMPETING INTERESTS

The authors of this work wish to declare that there is no competing interest among them.

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479 480 DEFINITIONS, ACRONYMS, ABBREVIATIONS

- 481 MIC: Minimum Inhibitory Concentration
- 482 MBC: Minimum Bactericidal Concentration
- 483 Km: Affinity constant
- 484 V_{max}: Reaction rate