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Assessment of Antibacterial Activity of Essential Oil Extracted from Leaves of *Thaumatococcus danielli* (Benn.) Benth. in Light of its Inhibitory Impact on Extracellular Protease of *Shigella dysenteriae*

Adeola A. Segun[1*], Folorunso O. Samuel[1] and Akintobi T. Aminat[1]

[1]Department of Biochemistry, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria, P. O Box 0001 LASU Post Office, Ojo Lagos State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author AAS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FOS and ATA managed the analyses of the study. Author FOS managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study was carried out to assess the antimicrobial effect of the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. leaves against selected enteric pathogens and its possible inhibition against a partially purified and characterized extracellular protease of *Shigella dysenteriae.*
Study Design: The volatile oil was used as antibacterial agent against eight enteric pathogens and as potential inhibitor to a partially purified extracellular protease of *Shigella dysenteriae*.
Place and Duration of Study: Department of Biochemistry, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria; between May – September 2013.

**Corresponding author: Email: femtolasse@yahoo.com, segun.adeola@lasu.edu.ng, femtolasse@yahoo.com;*

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 $(NH_4)_2SO_4$ salting-out followed by gel filtration chromatography. Enzyme assay was carried out with casein (as substrate) and volatile oil (as inhibitor).
Results: The average inhibition zones ranged 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 18.4µmol/min/mg protein were obtained after Sephadex G-75 gel filtration of the enzyme extract. The oil competitively inhibited the partially purified extracellular protease of *Shigella dysenteriae* with V_{max} = 8.33 ∆A/min, K_m = 0.85mg/ml (no inhibitor) and apparent K_m' = 1.54mg/ml (inhibitor). Optimal activities of this protease were obtained at pH 7.5 and 30ºC.
Conclusion: Though, it may not be clarified whether the oil interacted with the substrate/enzyme. Nevertheless, there was an inhibition of the extracellular protease of *Shigella dysenteriae* by the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. It was therefore envisaged that elaborate work on the inhibition of this type of enzyme in pathogens by essential oil would be a platform of arresting infection caused by bacteria.

Keywords: Thaumatococcus danielli (*Benn.) Benth*; *volatile oil; antimicrobial; Shigella dysenteriae; extracellular protease.*

ABBREVIATIONS

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; K_m: Affinity constant; V_{max}: Reaction velocity

1. INTRODUCTION

The use of medicinal plants for treating and managing infectious diseases has continued to witness dramatic turnout in recent past. Though the usage has been very peculiar to the impecunious populace but the intrinsic ability of these plants within their niche to withstand infection is of interest [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 to treat diseases [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 others 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 infections, diarrhea, cardiovascular diseases, upper respiratory infections, urinary tract infections, cancer, malaria and many other infections [4,5]. *Thaumatococcus danielli (Benn.) Benth.* is a notable rhizomatous perennial tropical medicinal plant of African 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 [6-8]. This plant, together with its fruits, has been commonly named as *Miraculous fruit, Miraculous berry* and *Katamfe/Katempfe.* In Africa, it is called *Serendipity berry*. In Nigeria, it is popular referred to as *Soft cane* and among the Yoruba tribes in the Southwestern part of Nigeria, it is called “Ewe gbodogi” [4]. 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) isolated from this plant has demonstrated antifungal activities against *Candida albicans, Neurospora crassa, Trichoderna reesei* and *Cercospora beticola*. Anthony et al. [11] has determined volatile constituents of the essential oil of this plant. In his report, twenty-one constituents were characterized representing 95.8% of the total oil

with significant antioxidant and insecticidal properties. The predominant 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%). Unfortunately, the organic and inorganic solvent extraction of the leaves of this plant failed to elicit significant antibacterial activity [12]. Similarly, Adegunloye et al. apparently assigned the bacterial load of food wrapped with leaves to the poor handling of the leaves [13].

Shigella dysenteriae 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 transmissions have been responsible for epidemic of shigellosis in the developing countries noted for unhygienic practises. Combating pathogenic organisms is paramount to human health. Bacteria, one of the major human pathogens, exhibit pathogenicity in their host by releasing toxins and extracellular proteases.

Extracellular proteases have been proposed as virulence factors [15] responsible for the digestion of host intestinal lining glycoprotein, attacking antibodies and host zymogens, facilitating host cell attachment, integration, invasion and colonization [16,17]. *Shigella* species generally employed toxins and extracellular proteases [17,18] 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 [19-21]. Antidiarrheal agents such as loperamide, diphenoxylate as well as some antibiotics that shared common mechanisms of action have failed to treat shigellosis [22]. Resistance to these pharmacological agents represents a severe medical problem, which might be resolved by using new generation of antibiotics, having a different mechanism of action. Bacterial protease inhibitors represent such possibility, because many specific as well as preponderant proteases have been elaborated in bacteria [23]. The extracellular protease of infectious bacteria has been majorly classified as either serine or metallo-proteases. Blocking the activities of these proteases and similar toxins with appropriate inhibitors is highly important especially in the curing, prevention and management of infectious diseases. Inhibitors can prevent the growth of bacteria and unfavourably alter the activities of some key enzymes responsible for the survival of this pathogen. Protease inhibitors have been significantly recognized as strong chemotherapeutic agents and can be produced naturally (plants, fungi and other bacteria) or synthetically.

Presently, the rate of bacterial infection has continued to increase with little or no promising remedy. Shigellosis and bacillary dysentery, as caused by *Shigella dysenteriae,* are very rampant in overcrowding environments of major cities in underdeveloped countries. Very many antibiotics are failing because of pathogen resistant. The urgent demand for active and relatively nontoxic antibiotics has geared up research into the antibiotic importance of medicinal plants. In our previous study, *Shigella dysenteriae* has been inhibited by the volatile oil of *Camellia sinensis* [5]. In view of this and with the hope of opening a platform to finding a more active antibiotic, this work was designed to evaluate the antimicrobial effect of the essential oil extracted from the leaves of *Thaumatococcus danielli* (Benn.) Benth. against eight enteric pathogens *vis-à-vis* the possibility of inhibiting the extracellular protease of *Shigella dysenteriae*.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Thaumatococcus danielli (Benn.) Benth. 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 seven days. 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, Enterococcus 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℃. 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* (Benn.) Benth

The volatile oil of *Thaumatococcus danielli* (Benn.) Benth. was extracted using hydrodistillation method [24]. Briefly, 400g of the seven-day-sun-dried *Thaumatococcus danielli* leaves were chopped into pieces and soaked with 3L distilled water inside 5L capacity 34/35 quick fit round bottom flask with fixed Clevenger distiller. The volatile oil was collected over a period of 3 hours at steady temperature of 80ºC over 2ml *n*-hexane. The collected oil was tightly stored in a sample bottle at 4℃ until it was used.

2.5 Cell Culture

The bacteria used in this work were specifically and differentially cultured for optimum growth. *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 enteritidis* and *Shigella dysenteriae* were cultured in *Salmonella-Shigella* Agar (SSA) while *Staphylococcus aureus* was cultured in Mannitol Agar (MA). A colony of each of the organisms (≈1.0 x 10^6cfu) was incubated at 37ºC for 24 hours.

2.6 Assessment of Antibacterial Activity of the Volatile Oil of *Thaumatococcus danielli* (Benn.) Benth

The antibacterial test of the volatile oil of the leaves of *Thaumatococcus danielli* (Benn.) Benth. against eight human enteric pathogenic bacteria was carried out using agar disc microdiffusion method as described by NCCLS [25] 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℃ for 24 hours. The sensitivity of the organisms to the volatile oil was an indication of the clear zones found around the paper disc placed on the agar.

2.7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Volatile Oil of *Thaumatococcus danielli* (Benn.) Benth

The MIC and MBC of the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. leaves against eight enteric pathogens under standard conditions were carried out using microbroth dilution method as described by Wannissorn et al. [26] with little modifications. A colony of each of the organisms was added to 200µL of appropriate test broth containing two-fold serial dilution of the volatile oil in a microtitre plate (21.5 cm x 17 cm). The test broth was prepared using 0.5%v/v Tween 80 as broth diluent. The first well of the plate (A1) contained 100 µL as initial volume of the broth. Subsequently, 100 µL of the oil was added to this well before serial dilution was made across the well. The plates were covered taped and incubated at 37ºC for 18 hours.

Inoculating loop was dipped into the broth in each well (twelve wells in each row) and streaked on a freshly prepared appropriate agar where MIC and MBC were determined. The essence of this was to find out which of the well will yield microbial growth on this freshly prepared agar. 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 standard conditions.

2.8 Extraction of Extracellular Protease from *Shigella dysenteriae*

As described by Makino et al. [27] 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 37ºC. The inoculum was centrifuged (Kendros Pico Biofuge, Heraeus) at 9000g for 10 minutes at 20ºC. The supernatant was decanted and used as crude enzyme extract.

2.9 Partial Purification of the Extracellular Protease of *Shigella dysenteriae*

2.9.1 Salting-out

Salting out technique was carried out on the crude extracellular protease extract of *Shigella dysenteriae* [28]. A 100% saturated solution of $(NH_4)_2SO_4$ was prepared (4.1M ≈ 541.8g/L) at 25ºC. The crude enzyme extract (at 0% initial $(NH_4)_2SO_4$ saturation) was dialyzed (using SIGMA Dialysis Tubing Cellulose Membrane, D9402) with 35% $(NH_4)_2SO_4$ saturated solution for 48 hours at 25ºC and centrifuged at 5000g for 5 minutes at 20ºC. Subsequently, 55% and 65% $(NH_4)_2SO_4$ saturations were again carried out on the respective previous supernatant in order to maximize protein yield from the extract. In each case, total protein and enzyme assay on the residue were carried out as earlier described.

2.9.2 Gel filtration

The soaked gel (3g Sephadex G-75) was poured into the chromatographic column (28.0cm by 1.5 cm column) and formed a bed length of 22 cm with a flow rate of 1.5ml/min and this was used to separate 3ml (sample load) of 65% $(NH_4)_2SO_4$ precipitate. Tris buffer (0.05M, pH 8.0), at 25ºC, was used to wash the column, equilibrate it and as elution buffer. A total number of fifty fractions were collected. Total protein and enzyme activity were carried out on each of the 3ml fraction as earlier described. Fractions with significant enzyme activities were pooled together and concentrated for further kinetic studies.

2.10 Determination of Total Protein

Total protein of the enzyme extract was determined using Lowry et al, method [29]. 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 0.5ml of crude extract and mixed thoroughly. The solution was allowed to stand for 10 minutes at 20ºC 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 with concentrations ranging from 0.1 - 1.0mg/ml.

2.11 Determination of Enzyme Activity

The extracellular protease of *Shigella dysenteriae* was assayed using a method described by Shana et al. [30] with slight modification. Protease activity was carried out by adding 5.0ml of casein solution (0.2 - 1.0%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 37ºC. 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 vortex mixing. The absorbance was read at 750nm after 30 minutes. L-tyrosine solution (0.1 - 1.0mg/ml) was used as standard for the protease activity. *One unit of protease activity was defined as the amount of enzyme required to liberate 1.0µmol of tyrosine in 1 minute at 37℃. The specific activity was expressed in units of enzyme µmol/min/mg protein.*

2.12 Optimum pH of the Enzyme Activity

The method adopted was described by Makino et al, [27] with little modification. This was carried out by adding 5.0ml of casein solution (0.2 - 1.0%w/v) 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℃ for 10 minutes as earlier described.

2.13 Optimum Temperature of the Enzyme Activity

As described by Makino et al, [27] with little modification, 5.0ml of casein solution (0.2 - 1.0%w/v) 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℃ for 10 minutes. The reaction was stopped and enzyme activity was carried out at each stage of temperature as earlier described.

2.14 Assessment of Inhibitory Effect of Extracted Essential Oil on Extracellular Protease of *Shigella dysenteria*e

The method adopted was described by Makino et al. [27] with slight alteration. Concisely, 0.5ml of the partially purified enzyme (pooled fractions) 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 37ºC 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. The mixture was filtered and enzyme activity was carried out on 1.0ml of the filtrate. This procedure was again repeated but without inhibitor.

3. RESULTS AND DISCUSSION

The volatile oil obtained from the leaves of *Thaumatococcus danielli* (Benn.) Benth. was tested for antimicrobial activity on eight different human enteric pathogenic bacteria. This essential oil was further used as possible inhibitor against the activity of extracellular protease of *Shigella dysenteriae* as likely evidence of its antimicrobial effect through enzyme inhibition.

The results of microbial sensitivity test, MIC and MBC of the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. are shown in Table 1. The zones of inhibition 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).

The essential oil from this plant inhibited the growth of five pathogenic organisms out of eight tested. The report in this work literally contradicted the findings of Ojekale et al. [12], who described lack of antimicrobial activity of the organic and aqueous extracts of the leaves of this plant and Adegunloye et al. [13], who examined the effect of handling on microbial load of foods wrapped in leaves. 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 difference in these results might be due to extraction method of phytoconstituents of this plant. However, we cannot ascertain which of the exact component(s) of the oil inhibited the growth of these bacteria, but this effect may be attributed to the presence of alkanols, esters and alkanoic acids containing components of the essential oil of this plant [12]. There were limited reports on the antimicrobial activity of the essential oil of the leaves of this plant. Nevertheless, secondary metabolites such as tannins, alkaloids, saponins, flavonoids, anthraquinones, cardenolides, steroids, anthocyanoside and cardiac and cyanogenic glycosides found in this plant may also contributed to the antimicrobial activity of this plant [31,11].

Table 1. Assessment of antimicrobial activity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. against eight human enteric pathogenic bacteria (Data were read in duplicate and the average was used)

Organisms	Inhibition zone (mm)	MIC (%v/v)	MBC (%v/v)
Escherichia coli (25922)	18.0	0.10	≥0.20
Klebsiella pneumoniae (4352)	-	-	>100
Proteus vulgaris	16.0	0.10	≥0.20
**Staphylococcus aureus (25923)*	-	-	>100
Pseudomonas aeruginosa (27853)	18.0	1.56	≥3.12
Shigella dysenteriae (13313)	30.0	0.10	≥0.20
Enterococcus faecalis	-	-	>100
Salmonella enteritidis	18.0	1.56	≥3.12

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

The summary of the partial 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.

Fig. 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 a peak each for both total protein and enzyme activity. The activity of this enzyme and protein content were found in almost the same region of the fractions.

The extracellular protease of *Shigella dysenteriae* was partially purified. Increase in the ionic concentration of ammonium tetraoxosulphate VI salt used increased the protein yield. Furthermore, most of the extracellular proteins released from this pathogen in the *Salmonella-Shigella* broth medium were enzymes as revealed by the chromatogram. Fractions 10 – 13 were pooled for extracellular protease activity while fractions 11 – 17 were pooled for evaluating total protein. Indisputably, very many proteins may be present as shown on the peak. The peak has revealed that the present proteins may have closely range molecular weight and only further works like size-exclusion and ion-exchanged chromatography and 2D PAGE would be suitable to elucidate this protein mixture.

Figs. 2 and 3 show the effects of pH and temperature on the activities of the extracellular protease of *Shigella dysenteriae*. The enzyme showed optimal activities at pH 7.5 and 30℃ respectively. The activity of this enzyme sharply declined immediately after the optimal conditions Figs. 2 and 3. This implied that the extracellular protease of *Shigella dysenteriae* was highly unstable at optimal conditions; any alteration in the optimal conditions will reduce the catalytic effects of this enzyme due to denaturation of the enzyme by extreme conditions such as high pH and temperature. A report by Gorden & Small [32] 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 for few hours [33]. Previous report by Folorunso et al. [5] showed optimal activity at pH 8.0 and 43.4℃ for the extracellular protease prepared from *Shigella dysenteriae*. Similarly, Adeola et al. [34] have reported pH 8.0 and temperature 44℃ as optimal conditions for the extracellular protease of *Shigella flexneri*. There were consistencies in the last two reports. We envisaged that strain types and media variability could discreetly account for this variations as compared to the present report. Notwithstanding, Louboudy et al. [35] have confirmed that most of the water-associated pathogenic bacteria thrived well at temperature range of 10-55℃ and pH 4.0-12 and respectively spoiled foods or caused infections at these conditions.

The caseinolytic kinetics of extracellular protease of *Shigella dysenteriae* in the absence and the presence of volatile oil of *Thaumatococcus danielli* (Benn.) Benth. as inhibitor is displayed in Fig. 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 8.33ΔA/min. The reaction velocity in this work was measured as increased in absorbance per unit time (ΔA/min). The reaction velocity appeared not to be affected by the inhibitor because this value remained unaltered. However, the K_m in the absence of inhibitor was 0.85mg/ml, but this value because of the presence of inhibitor, K_m', increased to 1.54mg/ml.

The essential oil as potential inhibitor offered a clue to predict that it either altered the rate of protease reaction by competitively inhibiting the enzyme as shown in Fig. 4 or probably directed its effect on the substrate as ligand-substrate making it inaccessible to the active site of protease. The microbial extracellular protease offers opportunity for the pathogen to digest proteins and their conjugated molecules externally for nutrients absorption, barrier penetration and adherence. This implies that essential oil may be capable of preventing these vital processes needed by the pathogen to survive in their host. The essential oil may contain component(s) very similar to the enzyme substrate and can fit into the active site of the enzyme thereby preventing the enzyme reaction. On the other hand, the oil might have reacted with the substrate to create a conjugated molecule, which can no longer be recognized by the active site of the enzyme. Consequently, further study needs to be done to establish which of these suggestions the essential oil of this medicinal plant supports. Meanwhile, the results

in this work have confirmed the present of protease inhibitor against the extracellular protease of *Shigella dysenteriae* in the essential oil of *Thaumatococcus danielli* Benn.) Berth., the evidence which needs explicit works for further appraisal. Most naturally occurring protease inhibitors prevent the action of microbial serine proteases. The approaches to protease inhibition have been shown to include formation of stable complexes with the enzyme, blocking the active site/action of the enzyme, deactivation of the active site, chemical modification of the active sites and chelation of co-factors [36]; all these are practically possible with the use of active component(s) of essential oil of medicinal plants.

Therefore, the volatile oil of *Thaumatococcus danielli* (Benn.) Benth. has shown to be an active antimicrobial agent against enteric pathogenic bacteria and its provisional inhibitory effect against the extracellular protease of *Shigella dysenteriae* was nonetheless an open way of curbing pathogenic bacteria.

Table 2. Partial 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% $(NH_4)_2SO_4$ precipitation	49	372.40	7.60	96.7	1.09
55% $(NH_4)_2SO_4$ precipitation	40	336.90	8.42	87.5	1.20
65% $(NH_4)_2SO_4$ precipitation	28	300.75	10.74	78.1	1.53
Sephadex G-75 gel filtration	10	184.24	18.4	47.85	2.63

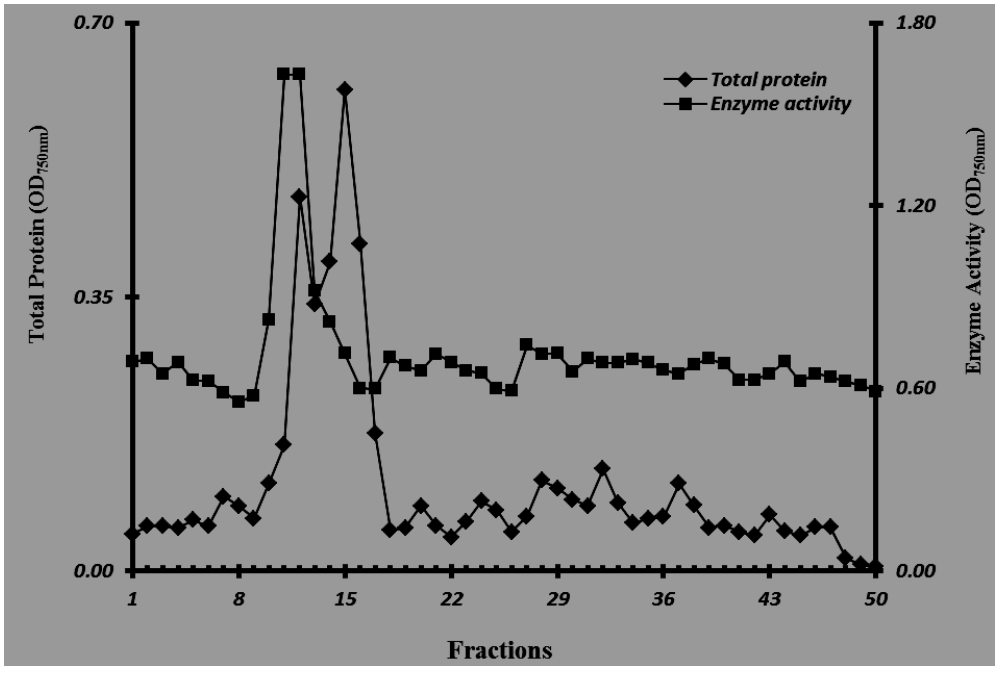

Fig. 1. Sephadex G-75 elution chromatogram of the extracellular protease of *Shigella dysenteriae* (Fractions 10 – 13 were pooled and tagged as "partially purified enzyme")

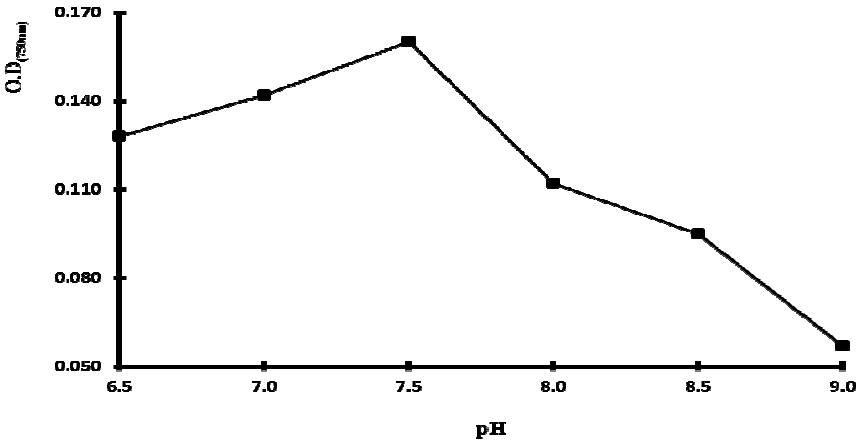

Fig. 2. Effect of different pH on the activity of extracellular protease of *Shigella dysenteriae*

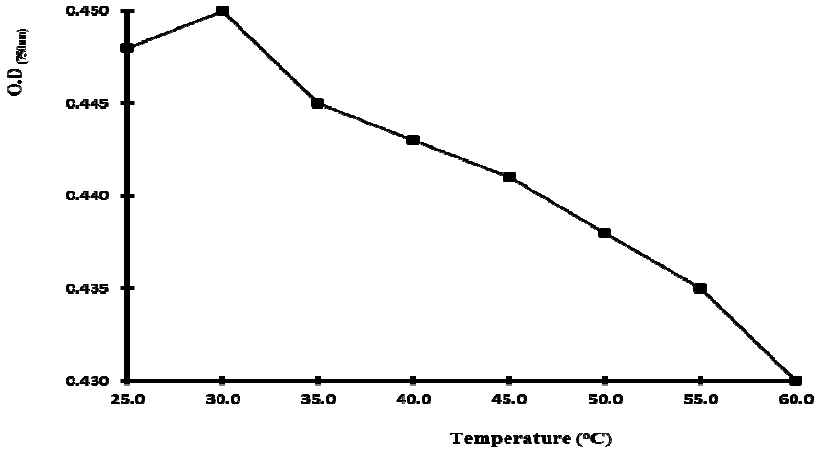

Fig. 3. Effect of temperature on the activity of extracellular protease of *Shigella dysenteriae*

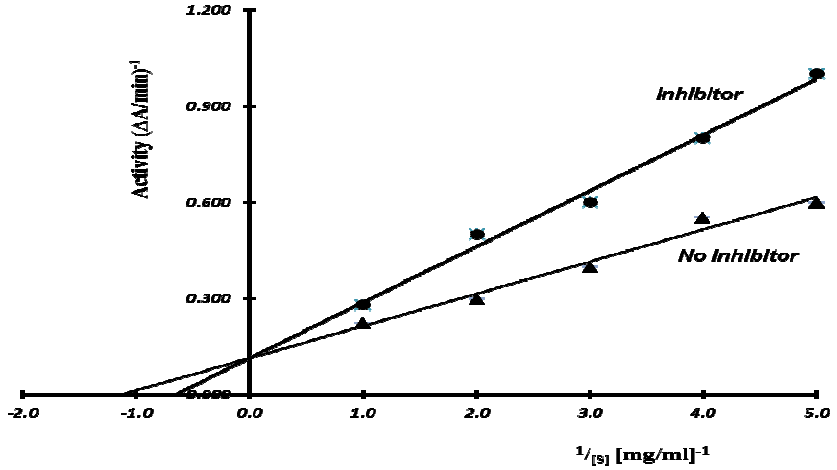

Fig. 4. Lineweaver Burke kinetic plot of the effect of volatile oil of *Thaumatococcus danielli* (Benn.) Benth. (as inhibitor) against the caseinolytic activity of the extracellular protease of *Shigella dysenteriae*

4. CONCLUSION

The volatile oil of *Thaumatococcus danielli* (Benn.) Benth. inhibited the growth of *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Shigella dysenteriae* and *Salmonella enteritidis*. The oil also assumed competitive inhibition against the extracellular protease of *Shigella dysenteriae*. Intricate studies are therefore needed to evaluate the antimicrobial activity of this essential oil against a wide range of infectious microorganisms including multidrug-resistant bacteria. More works are also encouraged to direct antimicrobial agents against the extracellular protease of pathogenic bacteria.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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