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Inhibition characteristics of peptide extracts of four medicinal plants on activities of bovine trypsin

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ABSTRACT

Aqueous extract of leaves of *Momordica charantia, Hymenocardia acida, Lawsonia inermis* and fruit of *Xylopia aethiopica* have been used in sub Saharan Africa in the management of many viral diseases. Their medicinal properties had been reported to be due to their high antioxidant activities, but limited information is available whether these properties are also due to inhibition or modulation of proteases important in the pathology of viral infections. We report the inhibitory characteristics of peptides extracted from these medicinal plants against bovine trypsin, a serine protease.

Extraction of the peptides was done using standard procedure and their inhibitory activities were measured against bovine trypsin. Aqueous extract of *M. charantia, H. acida, L. inermis* and *X. aethiopica* contain 5.7 ± 0.5 mg/ml, 1.0 ± 0.2 mg/ml, 1.8 ± 0.1 mg/ml and 28.3 ± 4.1 mg/ml of peptides, respectively. Using N_{α}-benzoyl-DL-arginine 4-nitroanilide (BAPNA) as trypsin substrate, a K_m and V_{max} of 0.34 mM and 0.6 μmole/min/mg protein obtained, were altered in the presence of the peptide extracts suggesting the extracts modulate trypsin activity. The inhibition was either competitive, non-competitive or mixed-type of non-competitive inhibition. Inhibition constant (K_i) ranging from 81 to 831±120 μg/ml were obtained using Dixon plot with the peptide extract from *M. charantia* being the most potent.

We concluded that the medicinal and antiviral properties of the extracts could also be due to inhibition or modulation of proteases involved in the pathology of viral infection.

Introduction

The great diversity of plants in tropical Africa offers interesting possibilities of finding novel antiviral compounds of natural origin. Among the medicinal plants known, aqueous leaf extracts of *M. charantia, H. acida, L. inermis* and *X. aethiopica* have been reported to contain many potent antiviral activities. *Momordica charantia*, commonly known as bitter gourd or bitter melon is an economically important medicinal plant and edible vegetable belonging to the Cucurbitaceae family. It is used in folk medicines for the treatment of several ailments- antiviral, antitumor, antimalarial, antidiabetic and antihelmintic agents [1].

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It has been reported that the proteins from this plant are potent inhibitors of several viruses such as dengue virus, human immunodeficiency virus, hepatitis B virus and herpes simplex virus [2,3]. MAP30 protein isolated from bitter gourd, proteolytic fragments of MAP30 and recombinant MAP30 were reported to possess antiviral, anti-HIV and antineoplastic activities [4]. Bitter melon extracts also appear to inhibit the growth of several Gram-negative and Gram-positive bacteria including *P. aeruginosa, E. coli, S. aureus, S. typhi, S. pneumoniae, S. dysenteriae* and *S. moniliformis* [5].

Hymenocardia acida Tul has been used in folk medicine for many years in Nigeria and some other parts of tropical Africa. All parts of the plant are useful as remedies for many ailments [6]. Decoction or infusion of leaves is used in the management of smallpox and together with the roots for deficiency diseases [7]. An extract of the leafy twigs is rubbed in to strengthen sickly children; leaf macerate or leaf decoction is taken to treat stomach ache, trypanosomiasis and coughs. Aqueous and organic extracts of various parts of *H. acida* have shown significant antibacterial activity against *S. aureus, S. pyogenes, S. mutans, S. auricularis, B. subtilis, B. cereus, S. epidermidis, M. kristinae, E. coli, S. poon and S. Marcescens* [8].

Lawsonia inermis Linn of the family Lythraceae is a branched glabrous shrub, cultivated for its leaves. In addition to the leaves, stem bark, flowers, seeds and roots have been used in ethnomedicine. This plant has been used worldwide as a cosmetic agent to stain hair, skin and nails [9]. Chaudhary et al. [10] reported the plant to contain hennadiol, lupeol, laxanthone, esculetin, isoplumbagin, fraxetin, botulin, lacoumarin, two pentacytic triterpenes and flavone glycosides. Also, the plant has been reported to have antimicrobial, analgesic, antiviral, antioxidant, anticancer, hypoglycemic, wound healing, immunostimulatory and hepatoprotective properties [9].

Xylopia aethiopica (Dunal) belonging to the family Annonaceae, is a tree that grows in the forests of tropical and subtropical Africa. The fruits of *X. aethiopica* are widely used in West and Central Africa as both spices to flavor food and drinks and as traditional medicine [11,12]. Fetse *et al.* [11] reported its use in the treatment of different respiratory, digestive, and inflammatory illnesses and infections, including dysentery and malaria. Furthermore, different parts of the plant are employed traditionally, in several therapeutic preparations, like skin and gastrointestinal infections [13]. Plants of the genus *Xylopia* are reported to yield products such as acetogenins, alkaloids, flavonoids, and terpenoids and exhibited anti-measles activity at low concentrations [14].

Most of the studies on these medicinal plants have shown them to prevent oxidative modification by oxygen scavenging or neutralizing free radicals through their antioxidant properties which are linked to the abundance of flavonoids and phenolics. However, the study of Majumdar *et al.* [15] suggests that flavonoids present in the plant extracts could perform roles other than scavenging oxygen radicals; that they can inhibit proteases and modulate their activities in a wide variety of diseases including cancer, viral and bacterial infections. Among several possible cellular proteases known, trypsin and trypsin-like proteases play pivotal roles in the proteolytic activation of a broad range of viruses thus resulting in the virulence and severity of the diseases. Viral proteolytic activation has been established in HIV/AIDS, Ebola, Influenza and COVID-19 etiology [16]. Hence, cellular proteases represent an important target with high therapeutic values in the treatment of these diseases.

In this study, we report the inhibitory properties of peptides extracted from the four medicinal plants on activities of bovine trypsin which was used as a model serine protease.

Materials and methods

Plant materials, collection and authentication

Four different plants including *Momordica charantia, Lawsonia inermis, X. aethiopica* and *Hymenocardia acida* were selected based on their reported antiviral activities. Aerial parts of *M. charantia*, leaves and seeds of *L. inermis* were collected in the period from September 2020 to February 2021 within Ile-Ife, (7°30'56"N 4°31'33"E; 7°28'27"N 4°31'22"E) Osun State. *X. aethiopica* fruits were purchased from Oja-Ife market, Ile-Ife (7°48''38"N 4 °24"56"20"E), Leaves and stem bark of *H. acida* were collected from Awo-Ede, Osun state (7°46'5"N 4°24'13"E). All plants were authenticated at the herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife where voucher specimens IFE 17,952, IFE 17,953, IFE 17,954, 1FE 17,955 were issued respectively, for the plants.

The various plant materials were cleaned and air-dried under the shade at room temperature. After drying, the plant materials were pulverized, weighed, stored in airtight bags and kept under room temperature until required.

Chemicals and reagents

All chemicals were of analytical grade and were purchased from reputable chemical suppliers. Analytical grade trypsin from bovine pancreas was a product of Boehringer-Mannheim, Germany. N_{α}-benzoyl-DL-arginine 4-nitroanilide (BAPNA) was a product of Sigma Chemical Company, St. Louis, USA.

Extraction of peptide

The extraction of peptide from the fruits of *X. aethiopica* was done following the method of Zhang *et al.* [17]. Powdered plant sample (10 g) was lysed in 20 ml of buffer A (50 mM Tris-HCl pH 7, containing 7 M urea), 80 ml of ice-cold acetone and 1 mM 2-mercaptoethanol. The resulting suspensions were allowed to stand for 2 h at −20 °C and centrifuged at 12,000

Table 1
Peptide concentration of different parts of the medicinal plants.

Medicinal Plant	Plant Part	Peptide Concentration (mg/ml±SEM)
M. charantia	Aerial Part	5.7 ± 0.5
L. inermis	Leaves	1.8 ± 0.1
	Seeds	1.7 ± 0.2
X. aethiopica	Fruits	28.3 ± 4.1
H. acida	Stem Bark	15.9 ± 1
	Leaves	0.99±0.2

x g for 30 min at 4 °C. After centrifugation, the supernatant was collected, concentrated and used as crude peptide inhibitor. The extraction of peptide from different parts of *M. charantia, L. inermis* and *H. acida* was done as described by Camargo Filho et al. [18]. Briefly, the powdered samples were homogenized in a solution containing 15 mM sodium monobasic phosphate, 10 mM sodium dibasic phosphate, 1.5% EDTA and 100 mM KCl for 2 h at 4 °C. The homogenate was squeezed through cheesecloth and further centrifuged at 10,000 x g for 30 min at 4 °C. The resulting supernatants were collected, concentrated and stored for use.

Protein/Peptide quantification

The peptide concentration was determined using the method of Bradford with bovine serum albumin as standard protein [19].

Effects of peptide extracts on trypsin activity

The effect of the peptide extracts on trypsin activity was determined by carrying out trypsin activity assay in the presence of peptide extracts at concentration ranging from 0 to the highest concentration that will completely abolish the activity of the enzyme [20] using Nα-benzoyl-DL-arginine 4-nitroanilide (BAPNA) as the substrate. Activity obtained without the peptide extract was taken as 100% trypsin activity. Changes in absorbance was monitored at 405 nm in a spectrophotometer at 30 s interval for 5 min.

Kinetic analysis of bovine trypsin in the presence and absence of peptide extracts

The kinetic parameters including Michaelis constant (K_m) and maximal velocity (V_{max}) were determined using the Enzyme Kinetics Statistical Package add-in on Sigma Plot version 10.0. Aliquots of bovine trypsin (1 mg/ml) solution, were added to a reaction mixture containing varying concentrations of BAPNA (0.09 mM to 0.9 mM) in the absence of the peptide inhibitors and at two different concentrations of the inhibitors. All assays were performed in triplicate at room temperature in 0.1 M phosphate buffer pH 8. The data obtained were fitted into equations representing different inhibition types to determine the pattern of inhibition.

Results

Peptide concentration of the medicinal plants extracts

The soluble peptide concentration was found to be highest in *X. aethiopica* fruit (28.3 ± 4.1 mg/ml). The extracts of other medicinal plants have varied concentrations as shown in Table 1.

Effects of peptide extracts on trypsin activity

Among the different plants screened for trypsin inhibitory activity, *M. charantia* extract was the most potent inhibitor of trypsin activity followed by *L. inermis* (Figs. 1-4). Inhibition constant (K_i) of 81.1 μg/ml was obtained for *M. charantia* aerial part extracts, indicating that extract of *M. charantia* is a very potent inhibitor when compared to extracts from other plants. The concentrations of the plant extracts that caused 50% inhibition of trypsin activity (IC_{50} value), as well as their respective K_i values, are shown in Table 2.

Inhibition by *M. charantia* peptide extract appears to be biphasic and at 900 µg/ml, the activity of trypsin was totally abolished (Fig. 1). *L. inermis* leaf and seed peptide extracts appear biphasic and at about 2 mg/ml and 6 mg/ml, respectively, the activity of trypsin was totally lost (Fig. 2). Same biphasic mechanism was observed for *X. aethiopica* fruit peptide extract (Fig. 3) as well as *H. acida* stem bark and leaf extracts (Fig. 4). Extract concentrations of 7.5 mg/ml, 0.8 mg/ml and 2.0 mg/ml, respectively, resulted into total loss of trypsin activity.

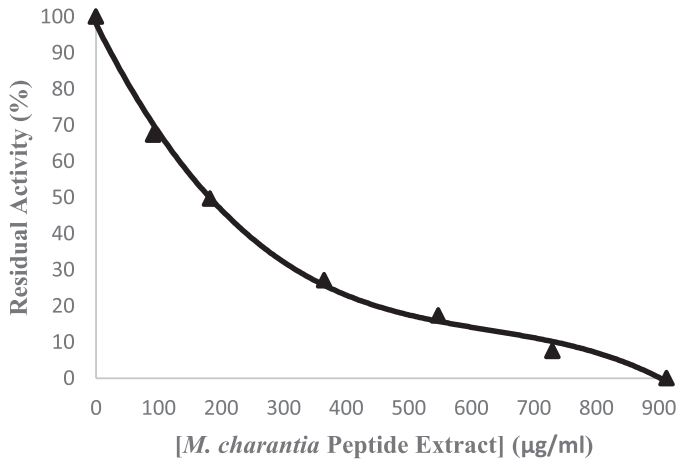

Fig. 1. Trypsin inhibitory activity of *M. charantia* peptide extract. The percentage (%) residual trypsin activity is plotted as a function of *M. charantia* aerial part (▲) peptide extract at a fixed enzyme concentration using BAPNA as substrate.

Table 2
Trypsin inhibition parameters of peptide extract of different parts of the medicinal plants.

Inhibitor Source	IC_{50} (mg/ml)	K_i (mg/ml)	Pattern of Inhibition
Aerial part of *M. charantia*	0.168±0.012	0.081±0.003	Mixed inhibition
L. inermis leaves	0.411±0.033	0.231±0.014	Mixed inhibition
L. inermis seeds	1.03±0.06	0.117±0.04	Mixed inhibition
X. aethiopica fruits	1.19±0.07	0.831±0.12	Competitive inhibition
H. acida leaves	0.604±0.02	0.119±0.01	Non-competitive inhibition
H. acida stem bark	0.205±0.01	0.108±0.008	Non-competitive inhibition

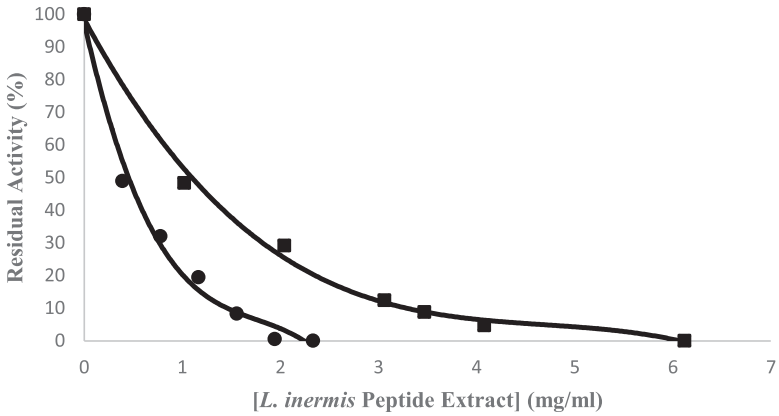

Fig. 2. Trypsin Inhibitory Activity of *L. inermis* Peptide Extract. The percentage (%) residual trypsin activity is plotted as a function of *L. inermis* leaves (●) and *L. inermis* seeds (■) peptide extract at a fixed enzyme concentration using BAPNA as substrate.

Kinetic analysis of bovine trypsin in the presence and absence of peptide extracts

Kinetic pattern of bovine trypsin in the presence and absence of inhibitors showed different forms of inhibition on double reciprocal plots (Figs. 5-10). Peptide extracts of *H. acida* leaves and stem bark were non-competitive inhibitors of trypsin while *X. aethiopica* fruits extract was a competitive inhibitor. Mixed inhibition was observed when peptide extracts of the aerial parts of *M. charantia* and *L. inermis* leaves and seeds were used as inhibitors of trypsin activity.

Discussion

A variety of bioactive compounds have been isolated from the medicinal plants used in this investigation and preliminary findings from our earlier study revealed that the plants contain flavonoids, peptides, phenolics, and glycosides as bioactive compounds [21].

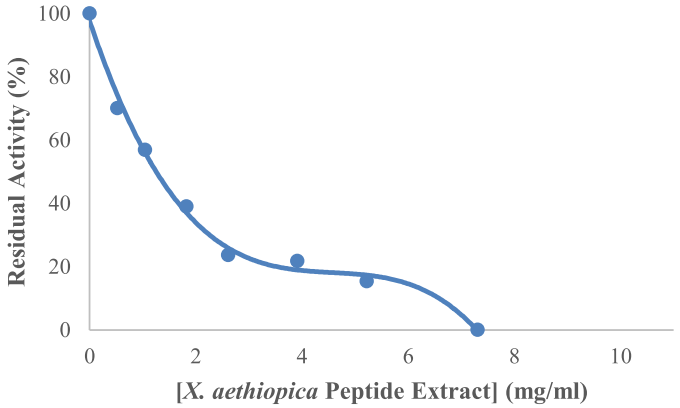

Fig. 3. Trypsin inhibitory activity of *X. aethiopica* fruit peptide extract. The percentage (%) residual trypsin activity is plotted as a function of *X. aethiopica* fruit peptide extract at a fixed enzyme concentration using BAPNA as substrate.

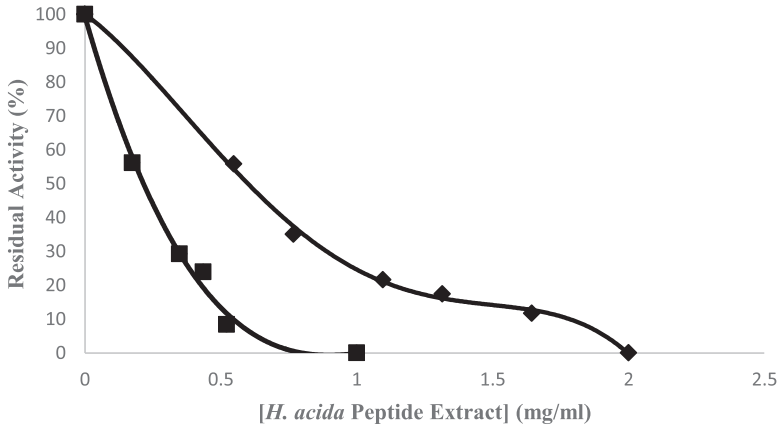

Fig. 4. Trypsin inhibitory activity of *H. acida* peptide extract. The percentage (%) residual trypsin activity is plotted as a function of *H. acida* stem bark extract (■) and *H. acida* leaves (♦) peptide extract at a fixed enzyme concentration using BAPNA as substrate.

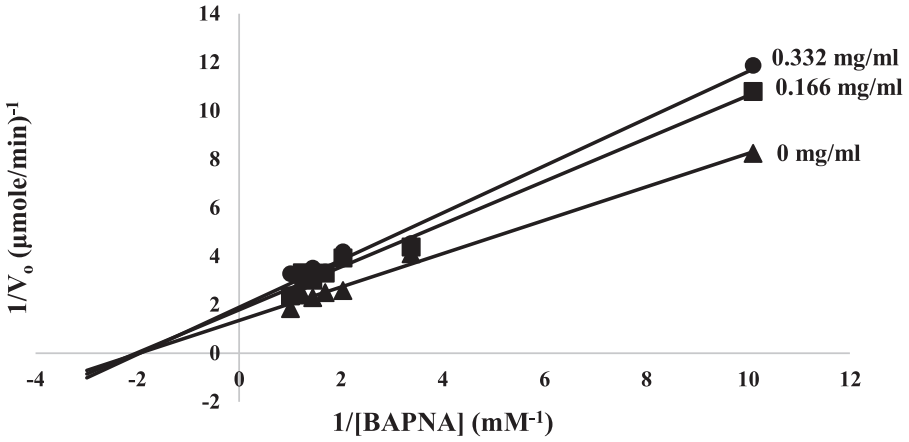

Fig. 5. Double reciprocal plot for the inhibition of bovine trypsin in the presence of aerial parts of *M. charantia* peptide extract.

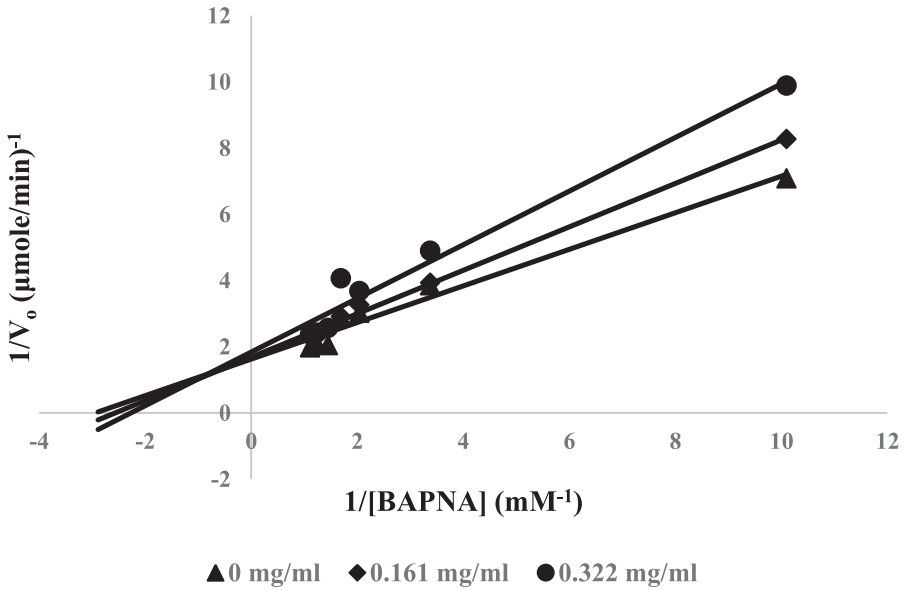

Fig. 6. Double reciprocal plot for the inhibition of bovine trypsin in the presence of *L. inermis* leaves peptide extract.

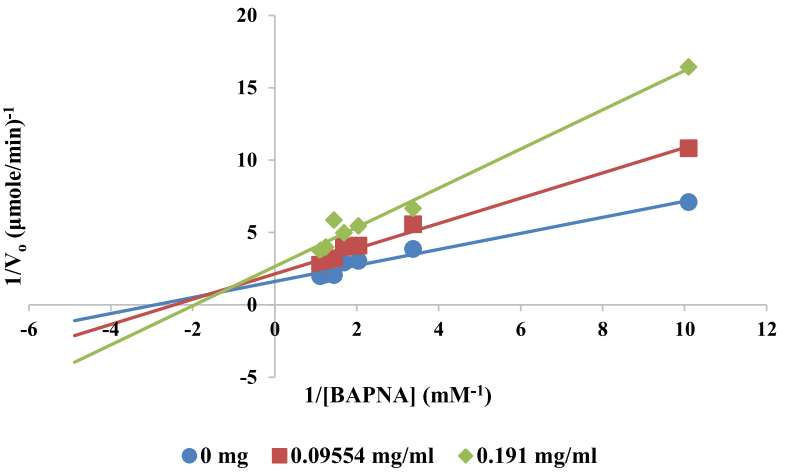

Fig. 7. Double reciprocal plot for the inhibition of bovine trypsin in the presence of *L. inermis* seeds peptide extract.

The mechanism of the pharmacological actions of extracts from these plants had hitherto been adduced to abundance of antioxidants (such as flavonoids) present in the extract thus mopping up the free radicals that could cause cellular injury. The literature is awashed with this perspective [15]. Limited information is available whether the pharmacological actions were due to inhibition of key proteases by some of the phytoconstituents- peptides, flavonoids, alkaloids, tannins etc. present in the plants. We have focused our attention on peptide components of the plants in this first report.

The methods adopted for the peptide extraction in this study were able to efficiently extract the peptides in the medicinal plants. The peptides extracted from all species of medicinal plants tested, showed inhibitory activity against trypsin in a concentration-dependent manner. The highest inhibition was observed for peptides extracted from *M. charantia* having a relatively low K_i of 81 μg/ml and peptide concentration of 5.7 $\pm$ 0.5 mg/ml compared to others. This may suggest that the use of aqueous crude extract of these plants may modulate proteolytic cleavage of serine proteases via inhibition when used. At K_i ranging from 81 - 831 μg/ml, all of the peptides extracted from the four medicinal plants effectively inhibited the activity of trypsin suggesting the possibility of this also happening *in vivo*.

The peptides extracts showed linearity in trypsin activity inhibition whereas some show biphasic curve indicating that different types of peptides are involved in the inhibitory action. Furthermore, the biphasic mechanism suggests changes in the enzyme as a result of the peptide inhibitors. All the peptides extracted from the different plant parts showed broad-

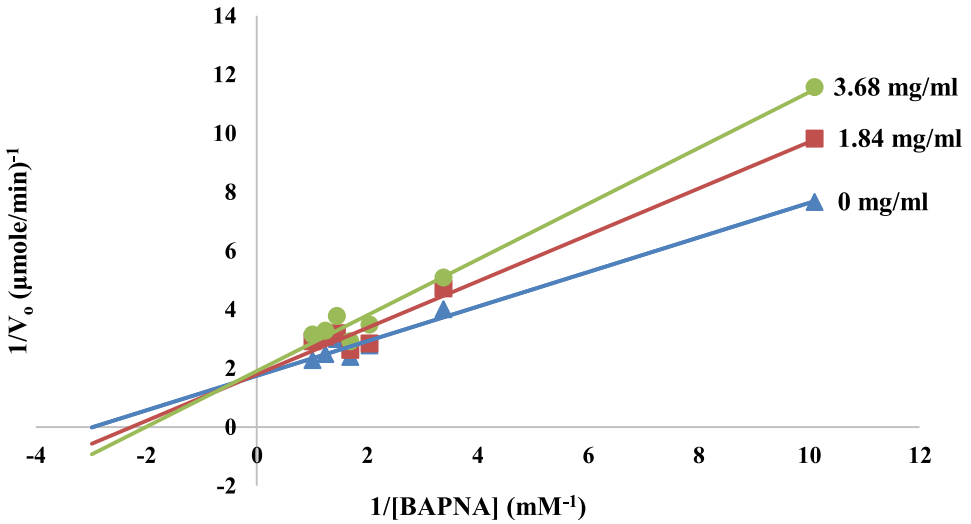

Fig. 8. Double reciprocal plot for the inhibition of bovine trypsin in the presence of *X. aethiopica* fruits peptide extract.

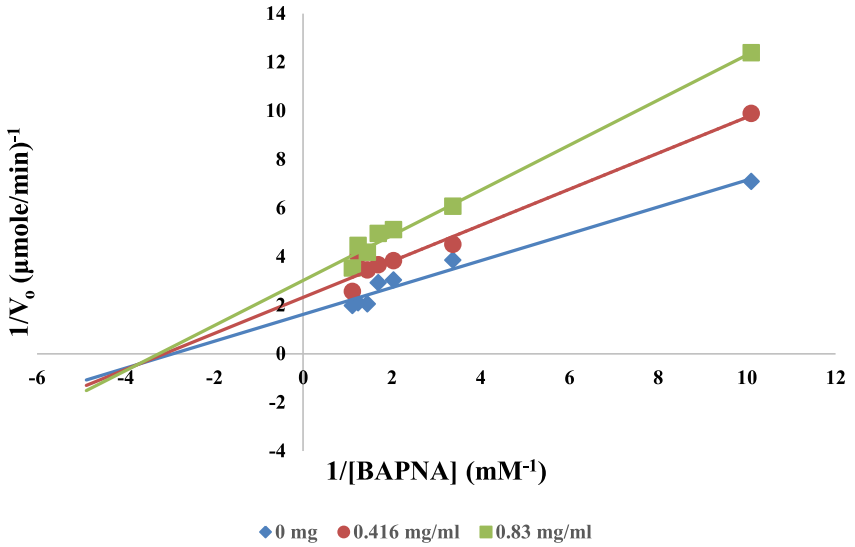

Fig. 9. Double reciprocal plot for the inhibition of bovine trypsin in the presence of *H. acida* stem bark peptide extract.

spectrum trypsin inhibition potential. Figs. 5-7 showed that these extracts exhibited a mixed type of non-competitive inhibition with respect to the substrate indicating that the extracts affected the affinity of the enzyme for the substrate without binding at the active site. In Figs. 9 and 10, the pattern of trypsin inhibition of peptide extracts from *H. acida* stem bark and leaves, respectively, were found to be non-competitive suggesting that the extracts might be able to enter the catalytic site of trypsin, but could not bind or that the peptide inhibitors and the substrate bind at different sites on the enzyme. The competitive mode of inhibition observed in Fig. 8 where inhibitor increases K_m and V_{max} remains unaffected, suggests that the peptide inhibitors as well as the substrate compete for the same binding site on the enzyme.

In conclusion, the results provide the first report that the different parts of *M. charantia, H. acida, L. inermis* and *X. aethiopica* studied, contain peptides that are potent trypsin inhibitors with *M. charantia* peptides being the most potent. The pattern of inhibition suggests different mechanisms are involved in their inhibitory activities. We speculate that the therapeutic efficacy of the plants particularly in viral disease management may be due to inhibition of proteases involved in the disease pathology.

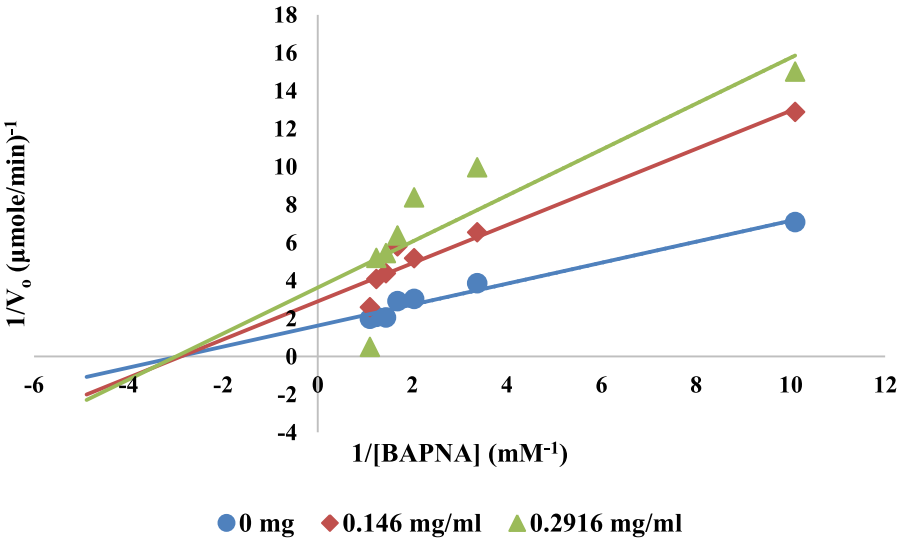

Fig. 10. Double reciprocal plot for the inhibition of bovine trypsin in the presence of *H. acida* leaves peptide extract.

Ethical approval

Not applicable

Informed consent

Not applicable

Declaration of Competing Interest

The authors declare no competing interest

CRediT authorship contribution statement

Oladoyin Grace Famutimi: Methodology, Software, Investigation, Data curation, Visualization, Formal analysis, Writing – original draft. **Isaac Olusanjo Adewale:** Conceptualization, Validation, Resources, Supervision, Funding acquisition, Writing – review & editing. **Kehinde Rofiat Adegoke:** Methodology, Formal analysis, Investigation, Data curation.

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