Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.6, No.14, 2016

In vitro Inhibitory Studies and Effect of Selected Plant Extracts and Cations on Elastase (EC 3.4.21.11) Activity produced by *Aspergillus niger* and *Aspergillus flavus*

S.A. James*[1]; R. Auta[1]; Efe M. Omwirhiren,Esq.[2]; N. Maxwell[1]
[1]Department of Biochemistry, Faculty of Science, Kaduna State University, Tafawa Balewa Way, P.M. B. 2339, Kaduna, Kaduna State
[2]Department of Chemistry. Federal College of Education, P.M.B. 1041, Zaria, Nigeria.

Abstract
Elastase is a member of the serine protease enzyme family that hydrolytically degrades elastin, a connective tissue component leading to aging and wrinkling of the skin. In this report, we investigated the *in-vitro* inhibitory effects of some cations and n-hexane extract of *Hibiscus cannabinus*, *Vernonia amygdalina*, *Murraya koenigii*, and *Telfairia occidentalis* on the activity of Elastase isolated from *Aspergillus flavus* and *Aspergillus niger*. Elastase was extracted, isolated and partially purified from *Aspergillus flavus* and *Aspergillus niger.* The data obtained in this study demonstrated that the activity of elastase in *Aspergillus flavus* was higher compared to that of *Aspergillus niger* after salting out. The elastase inhibition activities of *Hibiscus cannabinus, Vernonia amygdalina, Murraya koenigii,* and *Telfairia occidentalis* reveal that the leaf extract from *Murraya koenigii,* in *Aspergillus flavus* had more significant inhibition was obtained compared to that of *Aspergillus niger,* with inhibitory effect from 40µg/ml. While that *of Hibiscus cannabinus* showed similar inhibition characteristics to *Murraya koenigii.* Both *Hibiscus cannabinus and Vernonia amygdalina* had an effect at 80µg/ml; all the extracts appeared to have more effects on the enzyme obtained from *Aspergillus flavus* than from the *Aspergillus niger.* The potential of exploring these plant extract as an anti-aging recipe was discussed.
Keywords: Inhibitory studies, Elastase, *Aspergillus flavus*, *Aspergillus niger*, *Hibiscus cannabinus*, *Vernonia amygdalina*, *Murraya koenigii*, and *Telfairia occidentalis*

1. Introduction

Enzymes are energized protein molecules found in all living cells that function as biocatalyst. They catalyze and regulate all biochemical reactions that occur within the human body. They are also instrumental in digestion. Enzymes are exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries (Berg *et al.,* 2007).These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Microorganisms from diverse and exotic environments, extremophiles, are considered an important source of enzymes, and their specific properties are expected to result in novel process applications (Sridhar; 2006).

Of the thousands of known proteins, elastase is not one of the best known. However, it plays an important role as an enzyme where it function as hydrolase (**E.C** 3.4.21.11).

Elastase as the active form of the zymogen proelastase is hydrolytic in enzyme found especially in the pancreatic juice where it catalyzes the hydrolysis of elastin which other proteases can not degrade. Elastin is the protein that makes up the elasticity in tissues (Barros *et al.,* 2012) . Elastin helps keep the elasticity in tissues, where it helps keep skin flexible but tight and stretches to accommodate normal activities like flexing a muscle (Dall, 1999, Mori *et al.,* 2002). Elastase is important as part of our everyday lives, it aids in our digestion when we eat foods such as meat. It also further enhances our understanding of how serine proteases operate (Santillo, 2009).

However, when it is no longer controlled properly, elastase can cause some serious damage to its host. In this sense, elastase is possibly the most destructive enzyme having the ability to degrade virtually all of the connective components in the body. This is one of the features responsible for it broad applications in medical therapy, food processing, and dairy industry (Kothary *et al,* 1984,Kim *et al*., 2009).

Aspergillus flavus also known as mould is a plant, animal and human pathogen that produces the carcinogen, aflatoxin. It grows by producing thread-like branching filaments known as hyphae. *Aspergillus niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environment, where its black colonies can be confused with those of stachybotrys (species of which have also been called “black mould”) (Entchev, *et al*., 2001).

Recent development in biotechnology have resulted in the exploitation of new and undiscovered microorganisms and the devising of improved methods for enzyme production and their effect in biological systems.

The present study therefore focuses on the inhibitory effect of cations and some plant extracts on the activity of the enzyme elastase from *Aspergillus flavus* and *Aspergillus niger*.

2 Materials and Methods

2.1 Materials

2.1.1 Sample Collection and Processing

The matured leaves of *Hibiscus cannabinus, Vernonia amygdalina and Murraya koenigii* (i.e. *rama*, bitter leaves and curry leaves respectively) used in the study were purchased from the Kaduna Central market and transported to the Biochemistry Department using a polythene bag. Prior to the commencement of the experiment, the leaves were authenticated at the Herbarium of the Biological Science Department, Ahmadu Bello University, Zaria.

The leaves were chopped into smaller bit with a knife and dried at room temperature (27-30^{O}C) so as to maintain the constituents. After the complete drying, each plant materials were ground using a domestic pistil and mortar and then transferred into an air tight plastic container. Then the powder were stored in refrigerator at (2-8^{O}C) for analysis.

2.2 Reagents

Tween 20, biuret reagent and bovine serum albumin were supplied by Sigma –Aldrich Co. USA. n-hexane, Calcium carbonate and ammonium sulphate were supplied by BDH chemicals, Poole, England . Sodium dihydrogen phosphate, disodium hydrogen phosphate were products of Pharmacia fine Chemicals, Upsala, Sweden. All chemicals and reagents used for the study were of analytical grade and supplied by reputable chemical manufacturers.

2.3 Enzyme Cultivation

2.3.1 Isolation of Microorganism

The microorganism *Aspergillus niger* and *Aspergillus flavus* used was isolated from soil, purified, characterized and identified at the Department of Microbiology, Kaduna State University, Kaduna State, Nigeria. The organism was maintained on potato dextrose agar slant (PDA Slant) at room temperature 25^{o}C to obtain a luxuriant growth.

2.3.2 Preparation of spore suspension

Spore of *A. flavus* and *A. niger* was collected aseptically from (PDA slant) by washing with 10ml of sterilized 0.1% tween 20. Mycelia and other debris are removed by filtration of the spore suspension through sterile glass wool. The concentration of the filtrate was adjusted to 3x10^{7} spore per ml with the tween 20 solution as described by Kothary *et al.*, (1984).

2.4 Preparation of Plant extracts

The plants powder (30g) were subjected to successive maceration with 100ml of n-hexane in a shaker system at room temperature. Then the extract was filtered. The filtrate was subjected to evaporation using water bath so as to obtain dried extract. Each extract were prepared by dissolving 100mg per 10ml of solvent (i.e. n-hexane). Then each dried extract was subjected to elastase inhibitory assay.

2.5 Production of Elastase

Liquid culture medium was required, which was made up of 1.17% yeast carbon base, 0.2% elastin particles, and 0.3% $CaCO_3$. The solution are mixed thoroughly by shaking. The flask is plugged with cotton and autoclave for 20min at 121^{o}C. The sterilized media was cooled to room temperature and inoculated with 4ml of the spore suspension. The culture was incubated at 37^{o}C with shaking at 150 rpm for 72 to 96 hours. The culture broth was filtered through cheese cloth, double whatman 114 filter paper, 1.2μm pore size membrane filter and finally through a 0.45µm pore size membrane filter. Elastase activity and total protein content was determined as appropriate (Kothary *et al*, 1983).

2.6 Enzyme Purification

Ammonium sulphate precipitation

Crude elastase in the culture supernatant fluid was precipitated by adding solid $(NH_4)_2SO_4$ at 40-80% saturation. 188g/l of solid ammonium sulphate was added slowly with continuous stirring for 20-30 min. The precipitated proteins were collected by centrifugation at 14,000 rpm for 30 minutes. The resultant pellet was suspended in 20 mM sodium phosphate buffer pH 7.0 and dialyzed against the same cold buffer for 18 hours at 20% (v/v). The dialysate was centrifuge for 20 min at 8000 rpm and 4^{o}C and the supernatant was retained while the pellet was resuspended in 20 mM sodium phosphate buffer pH 7.0 and centrifuge again for 20 min at 8000 rpm. The supernatant with those from the first centrifugation were combined and the pellet discarded. Aliquot of the supernatant were retained for protein concentration and elastase activity determination.

2.7 Determination of Total Protein Content

The total protein content of the enzyme was determined according to Bradford (1976) using bovine serum albumin as standard as reported by Kothary *et al.*, (1985).

2.8 Measurement of Elastase Activity

Determination of kinetic constants (K_m and V_{max})

The kinetic constants K_m and V_{max} for the enzyme was determined from lineweaver-burk (double reciprocal) using spectrophotometric assay from the elastase activity as calculated by Kothary *et al*, (1984).

Effect of inhibitors on Elastase activity

Aliquot of the enzymes 1.0ml and 0.2ml of varying concentration of the inhibitor (of plant extracts) were incubated with the substrate of varying concentration. The elastase activity was determined and the inhibition constant k_1 determined from the double reciprocal plot.

2.9 Effect of Cations on Elastase Activity

Exactly 1.0 ml of the enzymes and 0.2ml of 0.02M of the cations (Mg^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}) were incubated with 1.0 ml of the substrate and elastase activity assayed calorimetrically.

3.0 Results

Inhibitory studies and effect of plant extract and cations as isolated from *Aspergillus flavus* and *Aspergillus niger* yielded the result shown below:

The spores obtained from the fungi after incubation was 740,000/ml for the *Aspergillus flavus* and 660,000/ml for *Aspergillus niger*. The total crude protein obtained from the fungi is 0.876µmol/ml for *Aspergillus flavus* and 0.93µmol/ml for *Aspergillus niger*. While the concentration after first purification by salting out gave a concentration of total protein 0.696µmol/ml for *Aspergillus flavus* and 0.676µmol/ml for *Aspergillus niger*. The result obtained shows that the enzyme activity for *Aspergillus flavus* is 0.696µmol/ml and 0.676µmol/ml for *Aspergillus niger* (see figure 1).

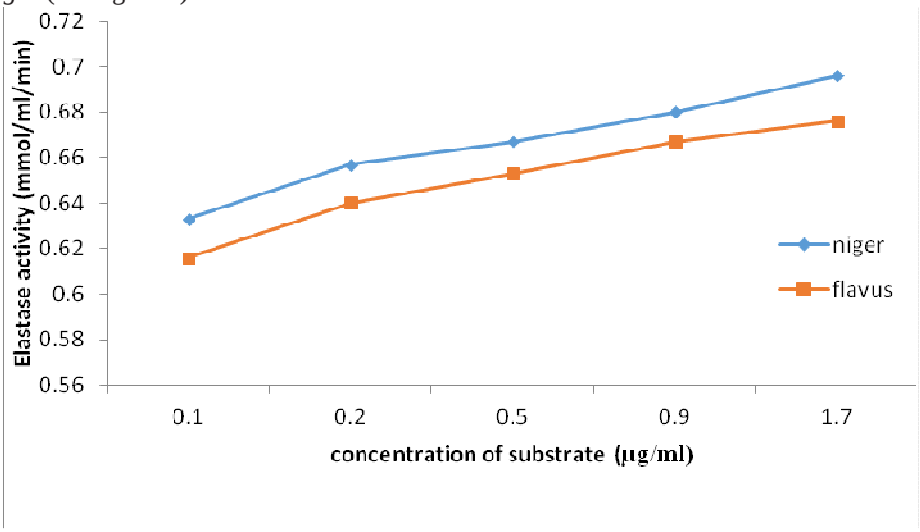

Figure 1: Elastase activity in *Aspergillus flavus* and *Aspergillus niger*

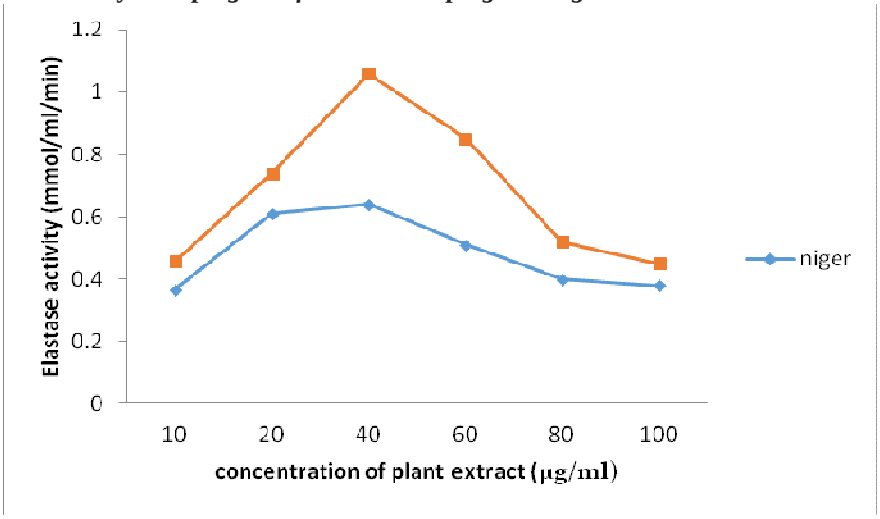

Figure 2: Elastase activity in *Aspergillus flavus* and *Aspergillus niger* with *Murraya koenigii* at different concentrations

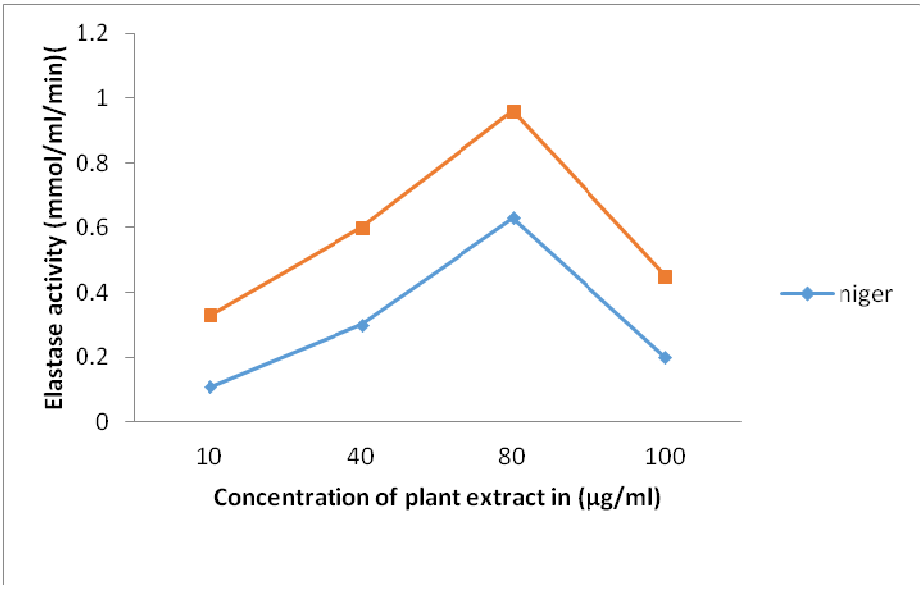

Figure 3: Elastase activity in *Aspergillus flavus* and *Aspergillus niger* with *Hibiscus cannabinus* extract at different concentrations

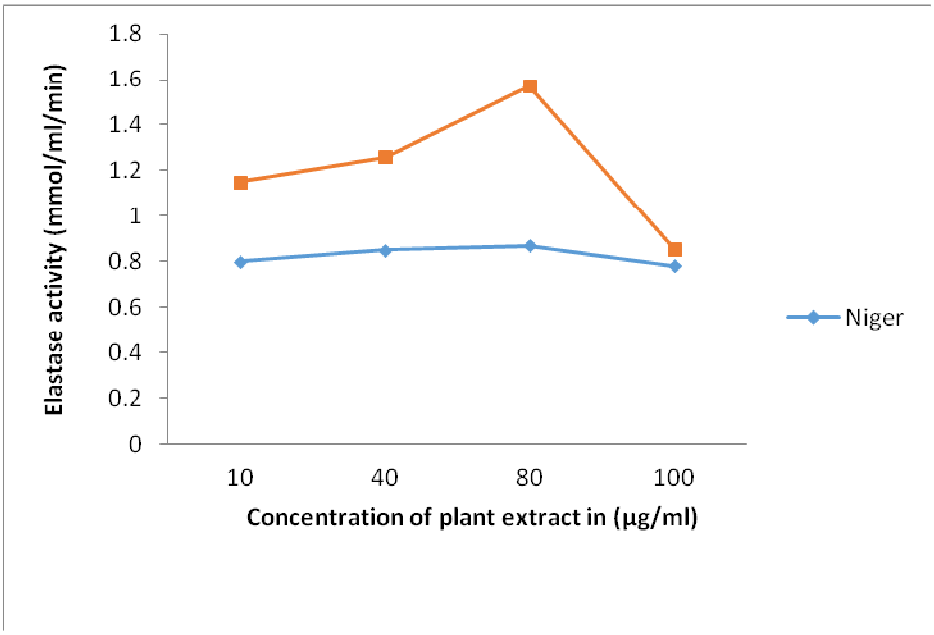

Figure 4: Elastase activity in *A.* niger and *A.* Flavus with *Vernonia amygdalina* extract at different concentrations

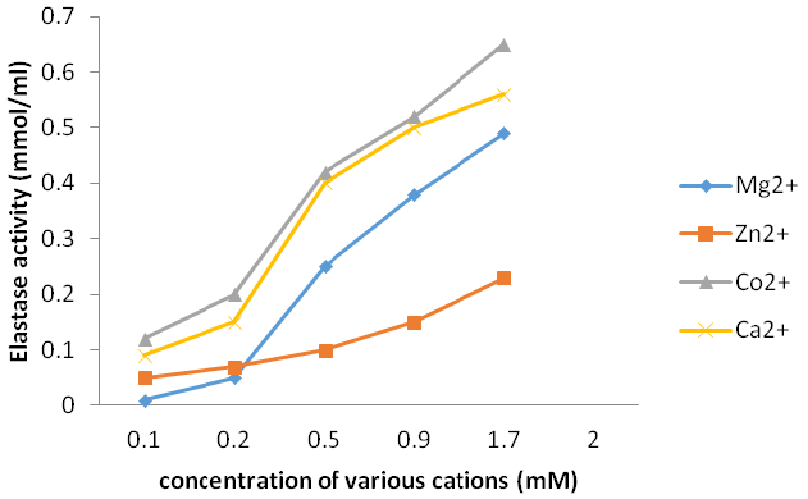

Figure 5: Elastase activity of *A. flavus* with various cations

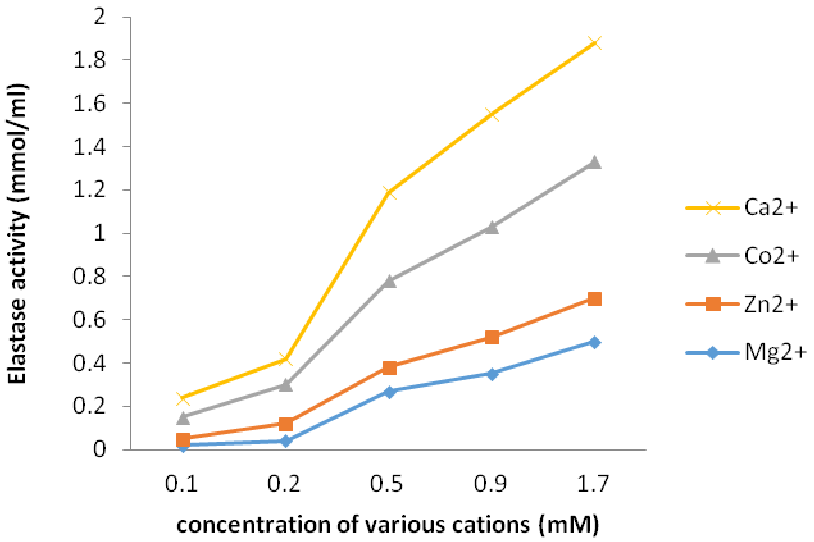

Figure 6: Elastase activity of *A. niger* with various cations

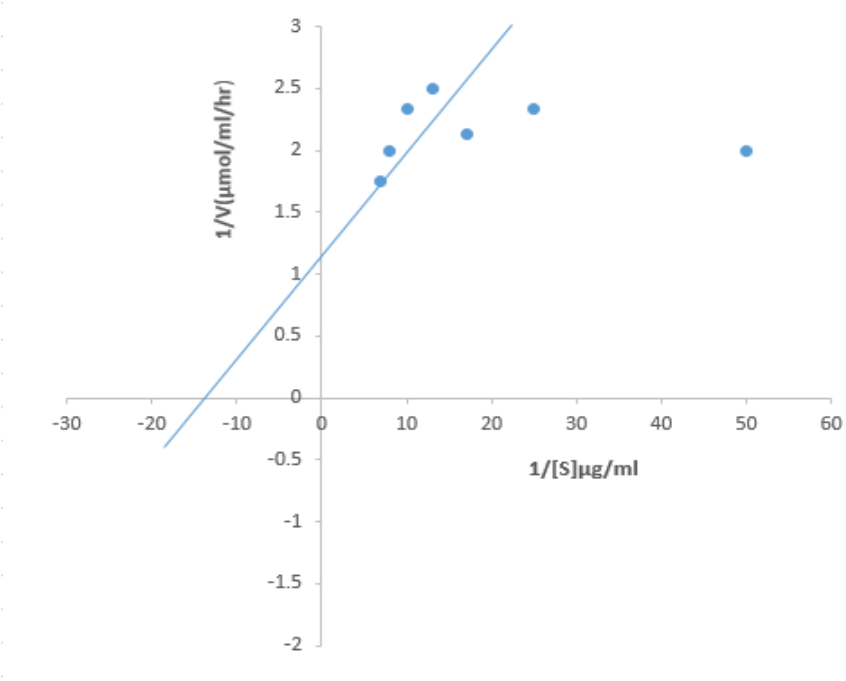

Fig 7: Double reciprocal plot of elastase using elastin as substrate

Results shows that *Murraya koenigii* had effect on the level of enzyme activity at 0.42 mmol/ml/min at 40 µg/ml and was continuously reducing for *Aspergillus flavus* (see figure 2). While for *Aspergillus niger*, it has an effect on the level of activity at 0.64 mmol/ml/min at a reaction with extract of 40 µg/ml and also increases with increase in plant extract to 60, 80 and 100µg/ml.

Also *Hibiscus cannabinus* had an effect on the level of activity of the enzyme elastase (see figure 3) at 0.33 mmol/ml/min at a concentration of 80 µg/ml and continuously reducing the enzyme activity on increasing in the extract for *Aspergillus flavus*. While for *Aspergillus niger*, it has an effect on the level of activity of elastase at 0.63 mmol/ml/min at a reaction with the plant extract of 80µg/ml and same as *Aspergillus flavus* on increasing the extract concentration to 100 µg/ml.

The inhibition studies with *Vernonia amygdalina* (see figure 4) shows that the plant had an effect on the level of activity of the enzyme elastase at 0.7 mmol/ml/min at a concentration of 80µg/ml and reduces the enzyme activity on increase of extract for *Aspergillus flavus*. While for *Aspergillus niger*, it has an effect on the level of activity of elastase at 0.87 mmol/ml/min at a reaction with the plant extract of 80µg/ml and same as *Aspergillus flavus* on increasing the extract concentration to 100µg/ml.

The cationic inhibitory study on elastase was employed using Mg^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} from aliquots of preparations. Results shows higher activity for the enzyme with Co^{2+} and Ca^{2+} when compared with other cations in both fungi as enzyme activity increases with the increase in substrate concentration (see figure 5 and 6).

4 Discussion

Elastase was isolated from *Aspergillus flavus* and *Aspergillus niger*. Elastase is a serine protease with three main amino acids (aspartate, histidine and serine) at its active sites, which make up the catalytic triad that work together to create a nucleophilic catalysis (Berg *et al*, 2007). Also, the enzyme has a binding site for Zn^{2+} and Mg^{2+} that function as a cofactor, hence also known as a metalloproteinase. This divalent cations Zinc is necessary for the enzymatic reactivity while Magnesium serve for the stability of the protein structure of the enzyme during catalysis. Moreover, it has been observed that apart from Zinc, the second divalent cation could varied in various species of microorganism for example Ca^{2+} is noted in *Pseudomonas aeruginosa* and *Bacillus thermoproteolyticus* (Olson and Ohman, 1992; Hase and Finkelstein, 1993). From this present studies the cationic activity for elastase extracted from the two fungi rises from a concentration of 0.2mM as against the K_m of 0.071, where all the divalent cations used act as an activator in an increasing order of Co > Ca > Mg > Zn for *A. flavus* and Ca > Co > Zn > Mg for *A. niger*. Also the studies by Furlan *et al*., (1996), shows that other chelating agent will inhibit the release of elastase, but calcium and magnesium will not, but found to activate elastase activity. The preference of this divalent ion influences the amino acid structural arrangement in the

polypeptide structure of the protein nature of the elastase where these ion exhibit various geometric coordination structure with amino acid residue of the enzyme (Rulíšek and Vondrášek, 1998).

The inhibitory effect of various plant extract shows that n-hexane extract of *Murraya koenigii*, *Hibiscus cannabinus* and *Vernonia amygdalina* exhibited a significant competitive inhibition; with greater effect on enzyme extracted from *A. niger*. At concentration of 40µg/ml and above there was a variation in the level of activity of theses enzyme in the fungi, which could be influenced by the presence of alkaloids or tannin present in the plants (James *et al*., 2014). The findings from present study suggests that the inhibition of elastase activity may be a valuable method to protect against skin aging. It is for this purpose that the plant extract under consideration were found to possess elastase inhibition activities. This is particularly instructive as these extracts exhibit the potential of preventing the loss of skin elasticity and thus reduce skin wrinkling, sagging and aging(Black,2005,Masie *et al*.,2009) . Elastase activity increases with age, resulting in a reduction in elastin and further decreasing the elasticity of skin, which causes the appearance of wrinkles and stretchmarks (Mori *et al.*,2002, Tamada, 2009).

Moreover, the optimum activity obtained at the same plant extract concentration is possibly due to the fact that both enzymes sources are from same family.

5 Conclusion

The present study demonstrated that n-hexane extracts of *Hibiscus cannabinus*, *Vernonia amygdalina*, *Murraya koenigii*, and *Telfairia occidentalis* possesses elastase inhibition effects. At present, numerous cosmetic companies utilize elastase inhibitors and other antioxidant ingredients in antiwrinkle, skin-lightening and antiaging products. The results of the current study therefore provide greater knowledge and compelling evidence for the rational exploration of these indigenous plants as a source of cosmetic materials. Further investigations will focus on assessment of the biological activity of these plant extracts in vivo and on chemical identification of the major active components responsible for the inhibitory effects of these plants extracts in preventive treatment for cutaneous wrinkling and ageing.

6 Conflict of Interest Statement

The authors declare no conflict of interest.

Acknowledgement

We acknowledged our colleagues in Biochemistry Department of Kaduna State University, Kaduna, Nigeria respectively.

References

Barros, S.C, Martins, J.A, Marcos, J.C, Cavaco-Paulo, A.(2012) Characterization of potential elastase inhibitor-peptides regulated by a molecular switch for wound dressings applications. *Enzyme Microb Technol.*, 50:107–114.

Berg J. M., Tymoczko, J. L. and Stryer L. (2007). *Biochemistry,* 6th Edition. W. H Freeman: New York. Pp 6-10

Black, L. (2005). Effects of elastase on the mechanical and failure properties of engineered elastase-rich matrices. *Journal of enzyme Application and Physiology*. 98: 1434-1441

Dall, A. (1999). Elastase Substrate Specificity Tailored through Substrate-Assisted Catalysis and Phage display. *Protein Engineering.* Pp *12* (11), 981-987

Entcheva, P., Liebl, W., Johann, A., Hartsch, T. and Streit, W. R. (2001). Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia. *Appl Environ Microbiol*. 67: Pp 89–99

Furlan, M., Robles, R., and Lammle, B. (1996). Partial Purification and Characterization of a Protease from Human Plasma Cleaving von Willebrand Factor to Fragments Produced by In Vivo Proteolysis. *Blood,* 87(10):

Hase,C C. and Finkelstein, R. A. (1993). Bacterial Extracellular Zinc-Containing Metalloproteases *Microbiol. Mol. Biol*. Rev. 57 (4): 4823-837

Kim, J.H., Byun, J.C., Bandi, A.K.R., Hyun, C.G, Lee, N.H. (2009) Compounds with elastase inhibition and free radical scavenging activities from *Callistemon lanceolatus*. *J Med Plant Res.*, 3:914–920.

James, S.A, Ladan, M.J., Auta, R., Audu ,J. and Garba, A. (2014) Biochemical Response Of Normal Albino Rats to the Addition of Aqueous Leaves Extract Of *Hibiscus Cannabinus* and *Murraya Koenigii* in Rats Drinking Water, *Science World Journal* 9(1): 34 – 38

Kothary M. H. (1983). *Ph.D. thesis*. Rutgers University, New Brunswick, N.J.

Kothary, M. H., Chase T, Jr., and Macmillan J.D. (1984). Correlation of elastase production by some strains of Aspergillus fumigatus with ability to cause pulmonary invasive aspergillosis in mice. *Infect. Immun.* 43:320-325

Masie, A. (2009). Elastase-Dependent Live Attenuated Swine Influenza A Viruses Are Immunogenic and Confer Protection against Swine Influenza A Virus Infection in Pigs. *Journal of virology*, *83* (19), 10198

Mori, M., Ikeda, N., Kato, Y.(2002) Inhibition of elastase activity by essential oils *in vitro. J. Cosmet Dermatol.*1(4):183-187.

Olson J C. and Ohman D E. (1992). Efficient Production and Processing of Elastase and LasA by *Pseudomonas aeruginosa* Require Zinc and Calcium Ions. *Journal of Bacteriology*, 174(12): 4140-4147 http://jb.asm.org/content/174/12/4140 06172016 06:25

Rulíšek, L, and Vondrášek, J. (1998). Coordination geometries of selected transition metal ions (Co2+, Ni2+, Cu2+, Zn2+, Cd2+, and Hg2+) in metalloproteins. *Journal of Inorganic Biochemistry*, 71(3): 115-127

Santillo, T. and Karma, M.(2009). Role of indigenous enzymes in proteolysis of casein in caprine milk. *International Dairy Journal. 19* (11), 655-660

Sridhar- Rao P. N. (2006). Introduction to mycology. Oxford press London, pp 156-160.

Tamada, K. T. (2009). Combined High-Resolution Neutron and X-ray Analysis of Inhibited Elastase. *Journal of the American Chemical Society*. 131(31): 11033-11040