Membrane Stabilization, PhospholipaseA2, Albumin Denaturation, Protease Inhibition, as viable Mechanisms for the Anti-Inflammatory Effects of Methanol Extract of Rauwofia vomitera Leaves.

Abstract

Background: The body uses inflammation as a defense mechanism to eliminate harmful stimuli like germs, damaged cells, or irritants and to initiate the healing process. However, the ongoing discovery of several medicinal plants and the testing of their bioactivity to produce information that will assist doctors and patients in making informed decisions prior to employing them has been established.

Aims: The study valuate the phosphatelipase A2 ,membrane stabilization, albumin denaturation, protease inhibition, and platelete aggregation activities as viable mechanisms for the anti-inflammatory effects of the methanol extract of *Rauwofia vomitera* leaf (MERVL).

Methodology: The anti-inflammatory effect of (MERVL) was ascertained using the phosphate lipaseA2, membrane stabilization model, albumin denaturation, protease inhibitor, assay.

Place and Duration of the Study: Department of Pharmacology Lab, Enugu State of University of Science and Technology Agbani Nigeria, between March 2021 and August 2021.

Results: MERVL inhibited hypotonicity-induced haemolysis by 27.14, 41.10 and 65.70%, at the concentration of 0.4, 0.8 and , 1.0 mg/mL respectively.The highest percentage of inhibition (67.70%) was noticed at the highest concentration of the MERVL. These results were almost analogous to the standard drug (indometacin) used, as it exhibited concentration dependent inhibition of albumin denaturation. Protease activity was significantly ($P < 0.05$) increased at all concentrations which follow the similar tendency as standard drug used. The results showed that MERVL has anti-inflammatory activities.

Keywords: Rawofia vomitera, Membrane stabilization, Anti-inflammatory, Methanol extract.

Introduction

The body uses inflammation as a defense mechanism to eliminate harmful stimuli like germs, damaged cells, or irritants and to initiate the healing process.[1] The early phases of inflammation are characterized by the production of reactive oxygen species (ROS) and the

recruitment of inflammatory mediators at the site of injury. When the production of ROS surpasses the capacity of antioxidants to reduce it, oxidative stress is unavoidable.[2].Oxidative stress and inflammatory processes are connected. The prolonged release of inflammatory mediators, which might result in oxidative stress, can lead to chronic inflammatory diseases. Anti-proteinases are oxidatively inactivated, whereas pro-inflammatory mediators are generated by gene activation in response to oxidative stress. Previous studies have shown that medicinal plants with anti-inflammatory characteristics can lower oxidative stress and boost immune function.[3]. One of such plants with anti-inflammatory properties is *Rauwofia vomitera* (Apocyanaceae.) leaf. It is a specie of vomitoria in the family of Apocyanaceae. It is also called serpent wood, snake root and swizzle **[**4]. In local Nigerian languages, it is called *asofeyeje* in Western part of Nigeria [5]. Major phytochemical constituents of this plant include alkaloids, glycosides, polyphenols, and reducing sugars [6]. The active alkaloids of *R. vomitoria* include rauwolfine, reserpine, rescinnamine, serpentine, ajmaline serpentinine, steroid-serposterol and saponin [7]. *R. vomitoria* has been used over the years for the treatment of hypertension and mental disorders and it is a common herb used traditionally for psychiatric management in Nigeria [8]. The presence of the phytochemicals in *Rauwolfia vomitoria* are responsible for the medicinal use of the plant in the management of inflammation. For example, several studies have shown that alkaloids lowered lymphocyte proliferation brought about by antigen and mitogen, the cytotoxicity of natural-Killer cell, the synthesis of histamine by mast cells, the release of interleukin-1 by human monocytes and the platelet activating factor action on platelets [9]. Alkaloids such as tetrandine and its analogue, berbamine have been shown to quit prostaglandin and leukotriene release by monocytes and neutrophils in humans. The inhibitory effect of berbarine on inflammation has shown that alkaloids may exert an important activity in chronic inflammation [10]. However, safer and more effective drugs have been developed from medicinal plants on the basis of their ethnomedicinal importance.[11]. Inflammation remains an area of great interest for research, doubtless due to the unavailable of a safer and more effective anti-inflammatory agent. This has led to increase in demand for natural products with anti-inflammatory activity having fewer side effects.

Materials and Methods

Plant materials: Freshly leaves of Rawuofia vomitera was sourced within the school premises of the Enugu State University of Science and Technology in March, 13, 2021 at the time this research was conducted. The freshly leaf was authenticated by Mr. A. O. Ozioko of the International Centre for Ethnomedicine and Drug production (InterCEED), Nsukka Nigeria. Voucher specimen of the plant with No. INTERCEED/002 was deposited at the InterCEED Herbarium.

Chemicals and reagents:

Chemicals used for this study were of the analytical grade and products of May and Baker England, British Drug House (BDH) England, Fluka Germany, Burgoyne, India, Harkin and Williams, England and Sigma Aldrich.

Preparation of plant material

The plant was collected, washed, and shade-dried. The dried leaves was pulverized into the powdered form using a mechanical grinder. A weighed quantity (1000 g) was macerated in 2.5 L absolute methanol using a maceration flask. It was allowed to stand for 72 h with frequent stirring, and then filtered into a flatbottomed flask with the aid of a muslin cloth. The filtration was done using Whatman No 1 filter paper . The extract was concentrated using rotary evaporator at a temperature of 45°C to obtain the crude ethanol extract. The concentrated extract was stored at a temperature of 2-4^{0C} in a labeled sterile reagent bottle in the refrigerator.

Anti inflammatory assays:

Methanol Extract of *Rauwolfia vomitoria* leaves on Phospholipase A2 (PLA2) Activity: The effect of MERVL on PLA2 activity was carried out using method of Vane [12] with little modifications by Enechi et al. [13]. Fresh human blood samples collected from healthy individuals were centrifuged at 3000 rpm for 10 min and the supernatant (plasma) was discarded. The red cells were washed thrice with equal volume of normal saline and reconstituted as a 40% (v/v) suspension with normal saline. Fungal enzyme preparation was obtained from Aspergillus nigerstrain culture. Aspergillus niger was cultured using a nutrient broth for 72 h at room temperature. The culture was transferred into test tubes containing 3 ml of phosphate buffered saline and centrifuged at 3000 rpm for 10 min. The fungal cells constituted the pellet, while the supernatant was used as crude enzyme preparation. HRBC (0.2 ml), CaCl2 (0.2 ml), 0.2 ml crude enzyme preparation, and varying concentration of normal saline and the fraction (0.2-1.0 mg/ml) were incubated at 37°C for 1 hr. Control tube contained HRBCs, CaCl2, and crude enzyme preparation. The blanks were treated with 0.2 ml of boiled enzyme separately. The incubated reaction mixture was centrifuged at 3000 rpm for 10 min. A measured quantity of the supernatant (1.5 ml) was diluted with 10 ml of normal saline and the absorbances were the solution was taken at 418 nm. Prednisolone was used as standard drug which is an inhibitor of phospholipase A2. The percentage maximum enzyme activity and percentage inhibition were calculated using the expression:

$$\% \text{ Maximum activity} = \frac{Absorbance of test}{Absorbance of control} \times \frac{100}{1}$$

$$\% \text{ Inhibition} = 100 - \% \text{ maximum activity of the enzyme.}$$

Methanol Extract of *Rauwolfia vomitoria* leaves on Platelet aggregation inhibition (Manaharan etal, 2011, Murugan,2014[14-15]: The platelet rich plasma with 1.2 x 107 platelet cells for each assay was re-suspended in pH 7.4 Tris buffer. The platelet aggregation was recorded as absorbance values of spectrophotometer measurement. To determine the *in vitro* inhibition of platelet aggregation, different concentrations of methanolic extract o*f Rawuofia vomitera* (0.2, 0.4, 0.6, 0.8, 1.0 µg/mL) in isosaline were used. The platelet aggregation was induced with ADP at a concentration of 1mM. indometacin was used as the standard. The absorbance was recorded after 5minutes at 660nm. Control was taken without the extract. The activity was calculated using the formula: (Control – Test)/Control x 100.

Effect of Methanol Extract of *Rauwolfia vomitoria leaves* on albumin denaturation The method of Mizushin and Kobyashi[16] with minor modifications was used. The reaction mixture consisted of test extract and 1% aqueous solution of bovine albumin fraction; pH of the reaction

mixture was adjusted using a small amount of HCl at 37^{oC}. The extract sample was incubated at 37^{oC} for 20 min and then at 51^{oC} for 20 min, after cooling the samples, the turbidity was read spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition (%) = [{Abs control - Abs sample}/Abs control] x 100

Effect of Methanol Extract of *Rauwolfia vomitoria leaves* on protease activity The test was performed according to the method described by(Oyedapo 1995, Sakat, 2010 [17-18] with minor modification. The reaction mixture (2 mL) was containing 0.06 mg trypsin, 20 Mm Tris HCl buffer (pH 7.4) and 1 mL test sample of different concentrations (0.1-0.5 µg/mL). The mixture was incubated for an additional 20 min. 2 mL of 70% perchloric acid was added to arrest the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of protease inhibitor activity was calculated as: Percentage inhibition (%) = [{Abs control - Abs sample}/ Abs control] x 100

Methanol Extract of *Rauwolfia vomitoria leaves on* HRBC membrane stabilization assay [19]: Blood was collected freshly and mixed with equal volume of Elsevier's solution. It was then centrifuged at 3000 rpm for 15 minutes. The packed cells were washed with isosaline and a 10 % suspension was made with isosaline. Different concentrations of methanolic extract of R. vomitera (0.2, 0.4, 0.6, 0.8, 1.0 µg/mL) were prepared in isosaline. To 0.5 mL of the extract, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension was added and incubated for 30 minutes at 37^{0C} and then centrifuged at 3000 rpm for 20 minutes. Absorbance was measured at 560 nm. Indometacin was used as the standard and control was taken without the extract served as negative control. The activity was calculated using the formula: (Control – Test)/Control x 100.

Statistical analysis The data obtained were analysed using a one-way analysis of variance (ANOVA) in Statistical Product and Service Solution (SPSS) version 22.0 and presented as Mean ±SD. Mean values with $p < 0.05$ were considered significant.

The result in figure1 shows the effect of MERVL on phospholipase A2 activity. The MERVL significantly ($p<0.05$) inhibited the PLA2 at different concentrations. The highest percentage inhibition of the extract, 67.67%, was observed at the highest concentration 1.0 mg/ml. The standard drug, indometacin are inline with the plant extract.

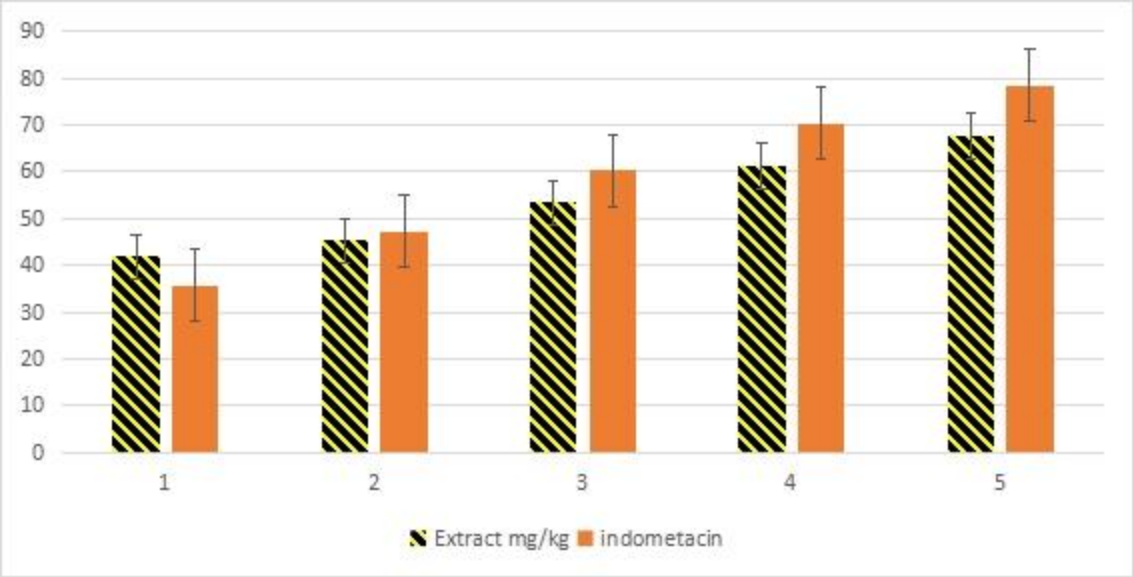

Figure 1: Effect of Methanol Extract of *Rauwolfia vomitoria* on PLA2

The result in fig2 shows the effect of MERVL on membrane stabilization. The extract showed a significant ($p<0.05$) percentage inhibition of hyponicity-induced lysis of HRBC at 1.0 mg/ml compared to other concentrations. However, as the concentrations of the extract increased 1.0 mg/ml, the percentage inhibition also increases maximally at 68.5 % . This is also in resemblant with standard drug (indometacin) with percentage inhibition 70.2% at concentrations of 1.0 mg/ml.

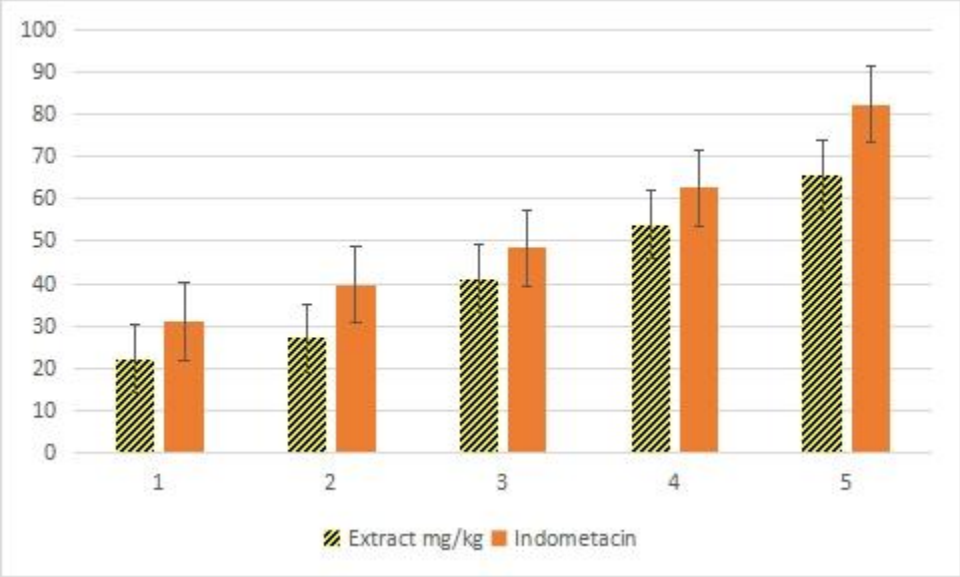

Figure 2 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Membrane Stabilization

The result in fig 3 showed the effect of MERVL on albumin denaturation. The MERVL exhibited a concentration dependent of inhibition of protein (albumin) denaturation. The extract exhibited significant (P<0.05) percentage inhibition at different concentrations. The highest percentage of inhibition (62.06%) was observed at the highest concentration of the MERVL . These results were measurable to the standard drug (indometacin) used as it also exhibited concentration dependent inhibition of albumin denaturation.

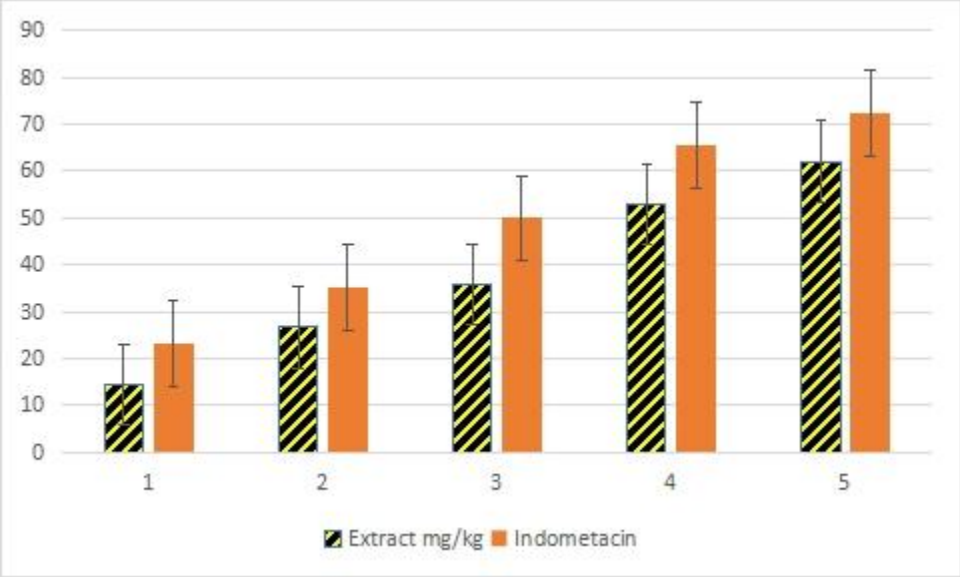

Figure 3 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Albumin Denaturation

The results in fig 4 shows the effect of MERVL on Protease activity. The concentration of the extract showed a significant (p <0.05) increase in the percentage inhibition on the Protease activity (1.0mg/ml)., However, the maximum enzyme activity was observed at 1.0mg/ml with a corresponding percentage inhibition of 53.98% when compared to the other concentrations. As the concentration increases from 0.2mg/ml to 1.0mg/ml, the percentage enzyme activity increases with a corresponding increase of percentage inhibition. This is in comparable with standard drug (indometacin) used

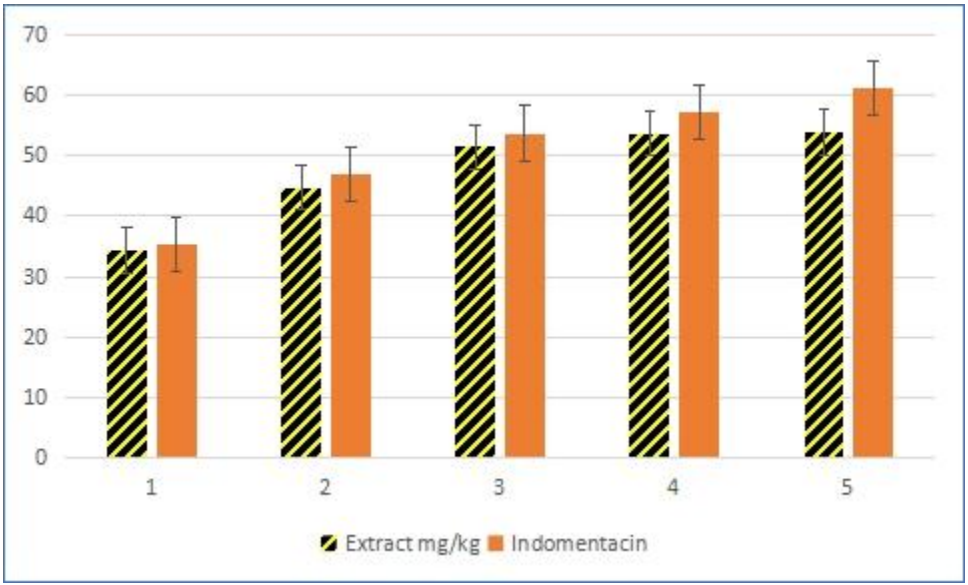

figure 4: Effect of Methanol Extract of *Rauwolfia vomitoria* on Protease Inhibition

The result in fig 5 shows the effect of MERVL on Platelet Aggregation. The concentration of the extract showed a significant ($p < 0.05$) increase in the percentage inhibition on the Platelet Aggregation (1.0mg/ml). When compared to the other concentrations, the maximum enzyme activity was observed at 1.0mg/ml with a corresponding percentage inhibition of 75.93%. As the concentration increases from 0.2mg/ml to 1.0mg/ml, the percentage enzyme activity increases with a corresponding increase of percentage inhibition. The standard drug used (indometacin) followed a similar trend when compared to the extract.

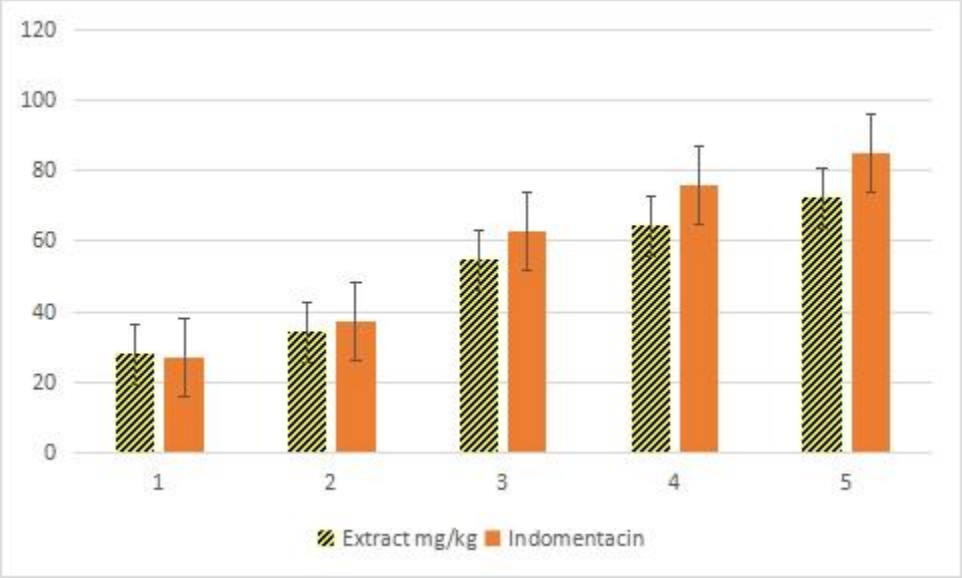

figure 5 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Platelet Aggregation

RESULTS AND DISCUSION.

In figure1, the methanol extract of Rauwofia vomitera leaf (MERVL) and indomethacin in a concentration dependent manner significantly ($p < 0.05$) inhibited the lysis of the human erythrocyte membrane which was induced by hypotonic solution when compared to the control. The methanol extract inhibited hypotonic solution induced lyses of HRBCs membrane. The supremacy of polyunsaturated fatty acids (PUFAs) within the RBCs membrane makes the cells intensely prone to oxidative harm Divya, (20) leading to hemolysis through which hemoglobin and different internal cellular components are released. Injurious agents such as hypotonic solution, heat, etc can lead to lyses of the RBC membrane. The MERV leaf at various concentrations significantly ($p < 0.05$) prevented the lysis of the HRBC membrane. The results indicated the capacity of MERVL to inhibit haemolysis. The inhibition of RBC membrane lyses is a measure of the anti-inflammatory activity. Divya, 2012, Zohra, 2014[20-21] .The ability of the MERV leaf to stabilize the erythrocyte membranes implies that it might stabilize lysosomal membranes as well. Stabilizing the lysosomal membrane is very paramout in regulating inflammatory responses which help to prevent the release of lysosomal constituents of activated leukocytes (such as bactericidal enzymes and proteases) which upon extracellular release lead to further tissue inflammation and consequently leads to tissue damage. Anosike, 2012, Cunha, 2007[22-23] .The result thus provides evidence for membrane stabilization as a potential mechanism of the anti-inflammatory effect of the methanol extract of *Rawuofia vomitera* leaf. Figure 2 shows the effect of the methanol extract of *Rawuofia vomitera* leaf on albumin denaturation. The MERV was effective in inhibiting albumin denaturation. Varying concentrations of the plant extract significantly ($P < 0.05$) inhibited the denaturation of albumin which indometacin showed a similar trend. The inhibition by the extract is concentration dependent with 0.2 µg/mL having an inhibition of 14.47% and 1.0 µg/mL with the highest inhibition of 62.06%. Protein denaturation is a process in which proteins lose their structure as a results of external stress or chemicals that is seen as a marker for inflammatory and arthritic diseases. Berbert, 2005[24] The ability of the methanol extract of *Rawuofia vomitera* to inhibit protein denaturation also give credence to its anti-inflammatory activity. The methanol extract was effective in inhibiting albumin denaturation. Figure 3 shows the protease activity of the methanol extract of *Rawuofia vomitera* leaf. The leaf inhibited significantly ($P < 0.05$) protease activity at different concentrations which standard drug showed a similar trend. The standard drug showed similar results (Figure 3). Protease inhibitors play important role for the better interpretation of basic principle of protein interaction. Proteolytic enzymes such as bromelain, papain, pancreatin, trypsin, chymotrypsin and rutin are essential regulators and modulators of inflammatory responses. Neutrophils are known to be a rich source of serine protease and are localized at lysosomes. It has been previously reported that leukocytes protease plays important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by protease inhibitors. Kajay, 2006[25]. Different concentrations of extract significantly ($p < 0.05$) inhibited protease activity at different concentrations. This could be as a result of its high flavonoid content. This assay provides another evidence for its promising anti-inflammatory properties.

Conclusion

Findings from the study reveal that the MERVL possesses remarkable anti-inflammatory activities. This study however shows that MERVL has modulatory effect on the vascular changes that come off during inflammation. The results indicate that the plant can be a potential source of anti-inflammatory agents if exploit.

Authors Contributions

This work was carried out in collaboration among all authors. Authors UOC and ACA designed the study, performed the statistical analysis, wrote the protocol. Author UOC, AIJ and UJC wrote the first draft of the manuscript. Authors ORC and ORM review the final drafted manuscript. Authors FKA and ACL managed the analyses of the study. Author UFI and ACL managed the literature searches. All authors read and approved the final manuscript.

Data Availability The numerical data used to support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest The authors declare that they have no competing interest that could have appeared to influence the work reported in this paper.

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