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

Abstract

Background: Inflammation is seen as a defense mechanism used to get rid of dangerous stimuli like pathogens, damaged cells, or irritants and start the body's healing process.The ever increasing discovery of large number medicinal plants and the screening of their bioactivity to provide data that will help physicians and patients make wise decision before using them.

Aims: The study evaluated 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 determined 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 Science and Technology Agbani Nigeria, between March 2021 and August 2021.

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

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

Introduction

Inflammation is seen as a defense mechanism used to get rid of dangerous stimuli like pathogens, damaged cells, or irritants and start the body's healing process.[1] Reactive oxygen species (ROS) generation and the enlistment of inflammatory mediators at the site of injury define the early stages of inflammation. Oxidative stress is inevitable when the generation of ROS exceeds the ability of antioxidants to counteract it.[2].Inflammatory processes are linked to oxidative stress. Chronic inflammatory illnesses can result from the protracted release of inflammatory mediators, which can cause oxidative stress. Pro-inflammatory mediators are expressed by gene activation in response to oxidative stress, whereas anti-proteinases are oxidatively inactivated. Previous research has demonstrated that medicinal herbs with anti-inflammatory properties can reduce oxidative stress and enhance immunological performance.[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 without doubt responsible for the medicinal use of the plant in the management of inflammation. For example, several studies have shown that alkaloids decrease lymphocyte proliferation triggered 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 stop 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]. Interestingly, safer and more effective drugs have been developed from medicinal plants on the basis of their ethnomedicinal importance.[11] .Inflammation continues to be an area of great interest for research, probably due to the non-availability 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 collected was gotten within the school premises of the Enugu State University of Science and Technology in March, 13, 2021 at the time this research was carried out. 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 utilized 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, cut into small sizes and shade-dried. The dried leaves was pulverized into the powdered form using a mechanical grinder. A weighed quantity (1000 g) was macerated in 3.5 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. Whatman No 1 filter paper was used for further filtration. A rotary evaporator was used to concentrate the filtrate 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 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 evaluated using method of Vane [12] with modifications by Enechi et al. [13]. Fresh human blood samples collected from healthy individuals were centrifuged at 3000 rpm for 10 min afterwards; 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 gotten 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 varying concentrations. The highest percentage of inhibition of the extract, 67.67%, was observed at the highest concentration 1.0 mg/ml. The standard drug, indometacin followed a similar trend as the plant extract.

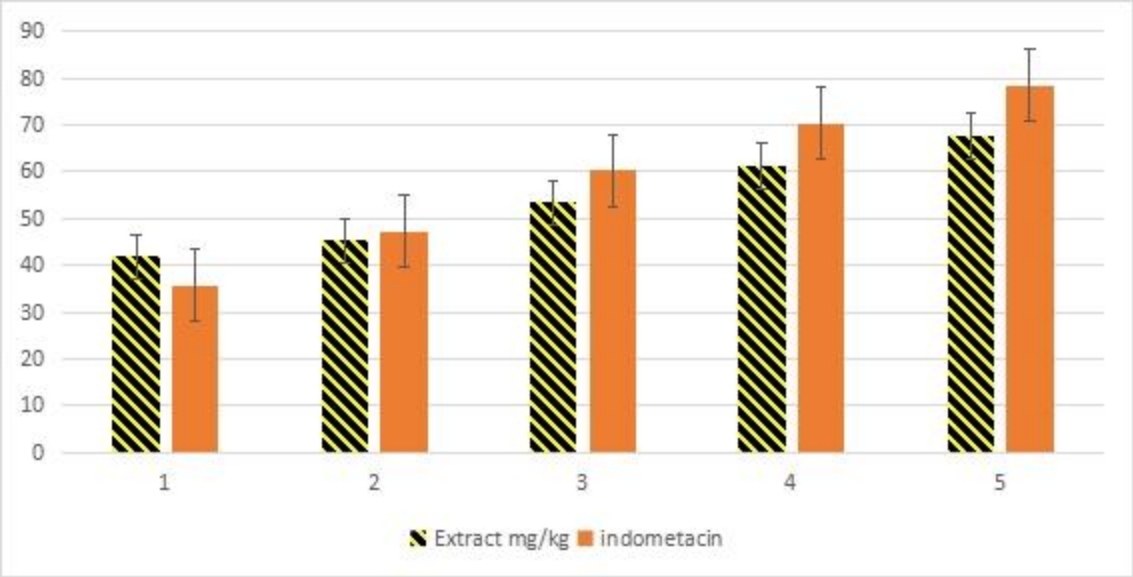

Figure 1: Effect of Methanol Extract of *Rauwolfia vomitoria* on PLA2

The result in fig2 shows the effect of 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. The percentage inhibition increases with increase in concentrations of the plant extract. This is comparable to the standard drug (indometacin) used at concentrations of 1.0 mg/ml.

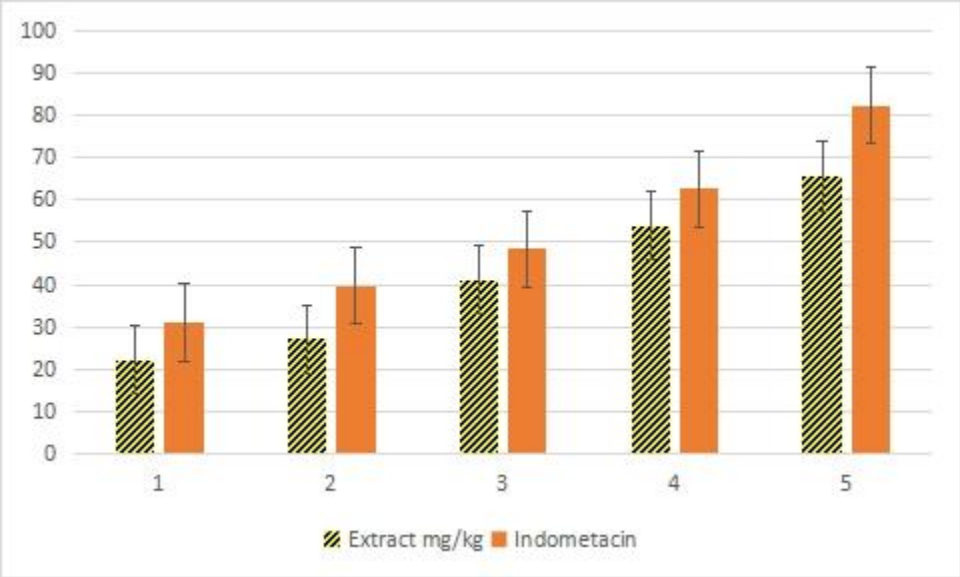

Figure 2 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Membrane Stabilization

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. Different concentrations of the extract exhibited significant ($P<0.05$) percentage inhibition. The highest percentage of inhibition (62.06%) was observed at the highest concentration of the MERVL . These results were comparable to the standard drug (indometacin) used as it also exhibited concentration dependent inhibition of albumin denaturation.

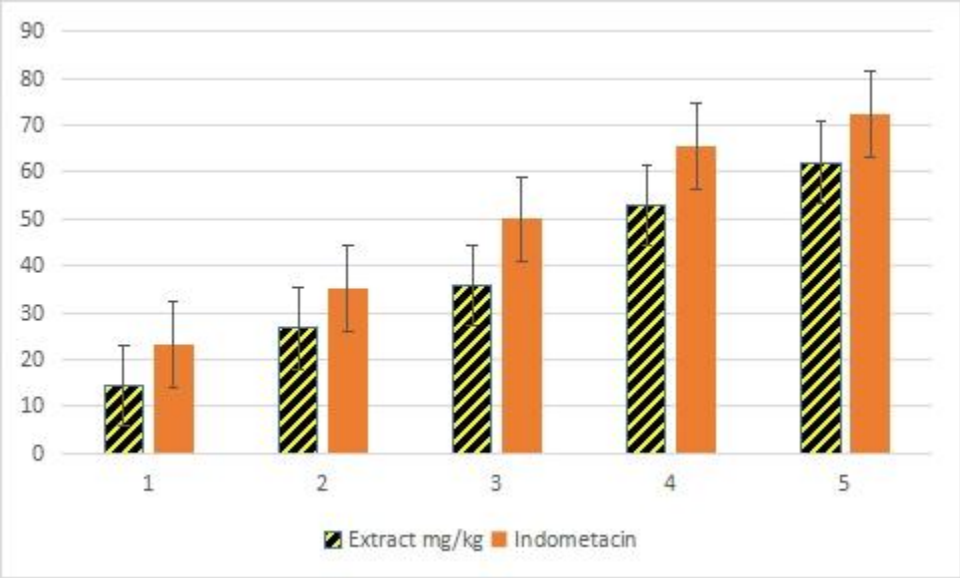

Figure 3 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Albumin Denaturation

The resultin 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). When compared to the other concentrations, the maximum enzyme activity was observed at 1.0mg/ml with a corresponding percentage inhibition of 53.98%. 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.

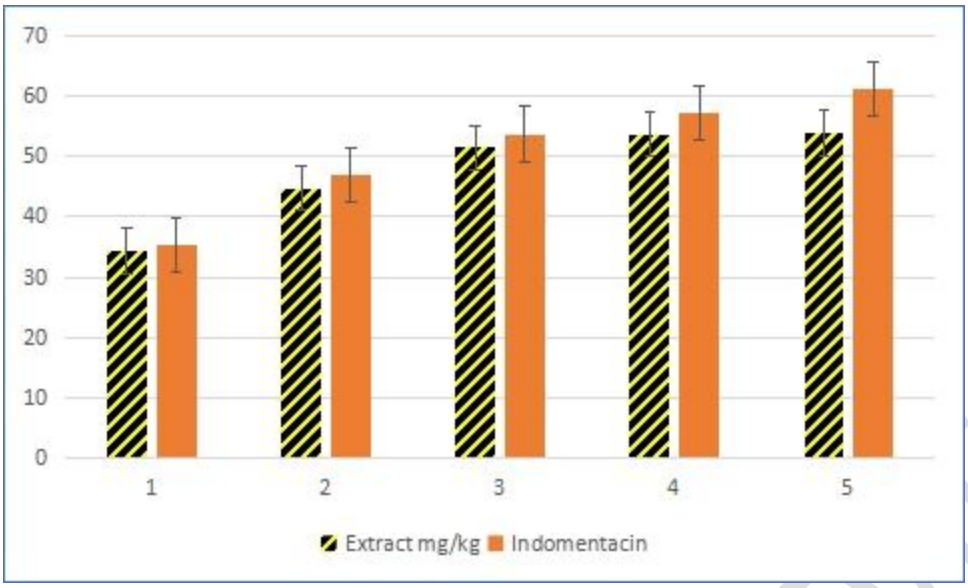

figure 4: Effect of Methanol Extract of *Rauwolfia vomitoria* on Protease Inhibition

The resultin 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.

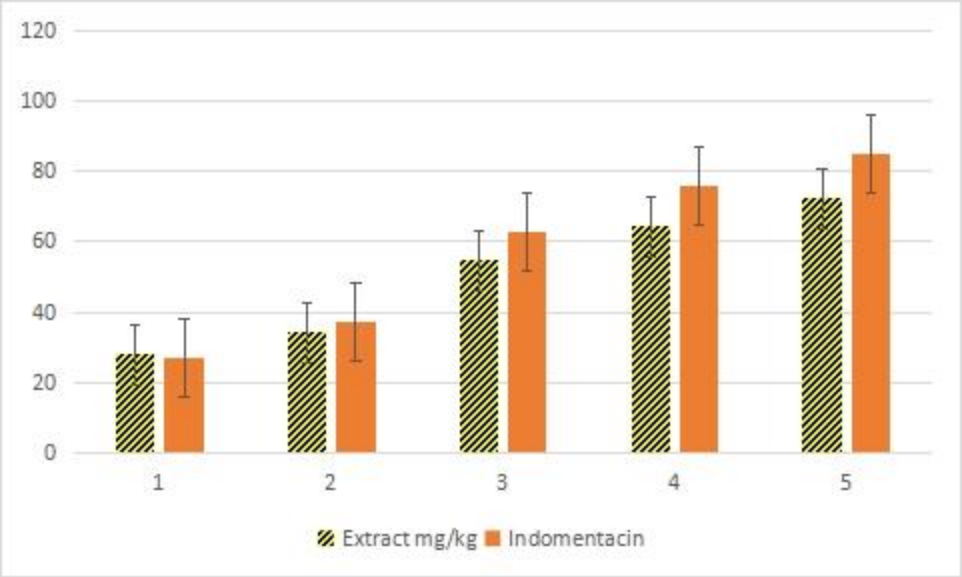

figure 5 : Effect of Methanol Extract of *Rauwolfia vomitoria* on Platelet Aggregation

RESULTS AND DISCUSION.

Results and Discussion 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 therefore shows that MERVL has modulatory effect on the vascular changes that occur during inflammation. The results indicate that the plant can be a potential source of anti-inflammatory agents if exploit.

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

REFERENCES

1. Celestine, U O, Jude A. I., Keneolisa, A. F., Chigozie, u-ugo J., Chijioke, O. R., Maduabuchi, O.R., Ifeanyi, U.F., Ogbonna, O.R.,& Assumpta, A. C.(2023). A nti-inflammatory Assessment of Methanol Extract of Acalypha ciliata leaves and its Leucocyte Mobilization in Adult Wistar Rats. European Journal of Medicinal Plants, 34(4), 22-29.

2. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked?. Free Rad Biol Med. 2010; 49(11):1603-1616.

3. Ricordi C, Garcia-Contreras M, Farnetti S, Diet and inflammation: possible effects on immunity, chronic diseases, and life span. J Am Coll Nutr. 2015; 34:10–13.

4. Gill, L.S. (1992). Ethnomedicinal Uses of Plants in Nigeria. Uniben Press, Benin City,

Nigeria. P. 276

6.Gill, L.S. (1992). Ethnomedicinal Uses of Plants in Nigeria. Uniben Press, Benin City,

Nigeria. P. 276

7. Akpanabiatu, M., Umoh I. B., Eyong, E. U and Edet, E. U. F. (2006). Influence of R. vomitoria root bark extract on cardiac enzymes of normal Wistar albino rats. *Journal of Medicinal Plants*: **14**: 273–278

.

8. José, M., Barbosa-Filho Márcia, R., Piuvezam Marcelo, D., Moura, M. S., Silva, K. V., Batista, L., Emídio, V., Leitão da-Cunha, I. M. and Fechine, O. S. M. (2006). Anti-Inflammatory Activity of Alkaloids: A Twenty Century Review. *Revista Brasileira de Farmacognosia*. **16** (1) :109-139.

.

9. José, M., Barbosa-Filho Márcia, R., Piuvezam Marcelo, D., Moura, M. S., Silva, K. V., Batista, L., Emídio, V., Leitão da-Cunha, I. M. and Fechine, O. S. M. (2006). Anti-Inflammatory Activity of Alkaloids: A Twenty Century Review. *Revista Brasileira de Farmacognosia*. **16** (1) :109-139.

10. Das B, Ferdous T, Mahmood QA, Hannan JM, Bhattacharjee R, Das BK. Antinociceptive and antiinflammatory activity of the bark extract of Plumeria rubra on laboratory animals. Eur J Med Chem. 2013: 3: 114-126.

11. Vane J. R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature: New Biology, 231 (25), 232–235.

12. Enechi O. C., Okeke E. S., Awoh O. E., Okoye C. O. and Odo C. K. (2021) Inhibition of phospholipase A2 , platelet aggregation and egg albumin induced rat paw oedema as antiinflammatory effect of Peltophorunpterocarpus stem-bark. Clinical Phytoscience, 7, 75.

13. Manaharan T, Teng LL, Appleton D, Ming CH, Masilamani T, Palanisamy UD. Antioxidant and antiglycemic potential of Peltophorum pterocarpum plant parts. Food Chem. 2011; 129(4):1355-1361.

14. Murugan R and Parimelazhagan T. Comparative evaluation of different extraction methods for antioxidant and antiinflammatory properties from Osbeckia parvifolia Arn.–An in vitro approach. J King Saud Univ Sci. 2014; 26(4):267- 275.

15. Mizushima Y and Kobayashi M. Interaction of anti- inflammatory drugs with serum proteins, especially with some biologically active proteins. J Pharm Pharmacol.1968; 20(3):169-173.

16. Oyedapo OO and Famurewa AJ. Antiprotease and membrane stabilizing activities of extracts of Fagara zanthoxyloides, Olax subscorpioides and Tetrapleura tetraptera. Int J Pharmacol. 1995; 33(1):65-69.

17. Sakat S, Juvekar AR, Gambhire MN. In vitro antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata Linn. Int J Pharm Pharm Sci. 2010; 2(1):146-155.

18. Sun J, Chu YF, Wu X, Liu RH. Antioxidant and antiproliferative activities of common fruits. J Agric Food Chem. 2002; 50(25):7449-7454.

19. Divya N, Nagamani JE, Prabhu S. Antioxidant and antihemolytic activities of Bombax Ceiba pentandra spike and fruit extracts. Int J Pharm Pharm Sci. 2012; 4(5):311- 315.

20. Divya N, Nagamani JE, Prabhu S. Antioxidant and antihemolytic activities of Bombax Ceiba pentandra spike and fruit extracts. Int J Pharm Pharm Sci. 2012; 4(5):311- 315.

21. Zohra M and Fawzia A. Hemolytic activity of different herbal extracts used in Algeria. Int J Pharm Sci Res. 2014; 5:8495-8494.

22. Anosike CA, Obidoa O, Ezeanyika LU. The anti— inflammatory activity of garden egg (Solanum aethiopicum) on egg albumin—induced oedema and granuloma tissue formation in rats. Asian Pac J Trop Med. 2012; 5(1):62-66.

23. Cunha TM, Verri Jr WA, Poole S, Parada CA, Cunha FQ, Ferreira SH. Pain facilitation by proinflammatory cytokine actions at peripheral nerve terminals. Immun Glial Reg Pain. 2007; 67:83.

24. Berbert AA, Kondo CR, Almendra CL, Matsuo T, Dichi I. Supplementation of fish oil and olive oil in patients with rheumatoid arthritis. Nutr. 2005; 21(2):131-136.

25. Kajay TD, Shanthi P, Sachdanandam P, Protein denaturation and white blood cell migration during acute inflammation. J Med Plants Res. 2006; 4:789-795