

Anti-Inflammatory Activity of Peptic–Tryptic Cowpea (*Vigna Unguiculata*) B-Vignin Hydrolysate in In Vitro Membrane Stabilization and Formaldehyde-Induced Inflammation Models

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<https://doi.org/10.56201/ijhpr.vol.11.no8.2026.pg57.76>

Abstract

Inflammation is a protective physiological response to tissue injury, infection, and other pathological stimuli, but persistent or excessive inflammation may contribute to tissue damage and chronic disease. Food-derived peptides have attracted interest as potential sources of biologically active compounds with safe, therapeutic anti-inflammatory properties. This study investigated the anti-inflammatory potential of peptic–tryptic hydrolysates derived from cowpea β -vignin using in vitro erythrocyte membrane stabilization assays and an in vivo formaldehyde-induced inflammation model in Wistar rats. Cowpea β -vignin was extracted from *V. unguiculata* var. Ifebrown via isoelectric precipitation and subjected sequentially to pepsin and trypsin hydrolysis. In vitro anti-inflammatory activity was evaluated using heat-induced and hypotonicity-induced haemolysis of human red blood cells (HRBC), with diclofenac sodium as the reference drug. The hydrolysate inhibited heat-induced erythrocyte haemolysis by 15.23 ± 0.41 – $37.86 \pm 1.09\%$ at 50–400 $\mu\text{g/mL}$, while diclofenac sodium produced 23.45 ± 0.71 – $45.26 \pm 1.09\%$ inhibition. In the hypotonicity-induced haemolysis assay, hydrolysate activity ranged from 18.53 ± 0.06 to $24.08 \pm 0.08\%$ at 100–400 $\mu\text{g/mL}$, increasing to $25.87 \pm 0.08\%$ at 500 $\mu\text{g/mL}$; at 500 $\mu\text{g/mL}$, this performance was not significantly different from diclofenac sodium ($25.43 \pm 0.07\%$). In vivo activity was assessed in thirty male Wistar rats divided into six groups: normal control, inflammation control, diclofenac sodium (10 mg/kg), and cowpea hydrolysate at 100, 300, and 500 mg/kg body weight (b.w.). Inflammation was induced by administering 0.02 mL of 2% (v/v) formaldehyde into the left hind paw on days 1 and 3, and paw oedema was monitored over seven days. The hydrolysate produced dose-dependent inhibition of paw oedema of 15.44, 25.32, and 36.81% at 100, 300, and 500 mg/kg, respectively, compared with 23.33% for diclofenac sodium. Treatment with 300 and 500 mg/kg hydrolysate significantly reduced plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity relative to the untreated inflamed group, showing strong tissue-stabilizing and hepatoprotective properties. Haematological analysis demonstrated treatment-associated modulation of white blood cell (WBC), lymphocyte, granulocyte, EMB, and platelet counts, while RBC, haematocrit, and haemoglobin profiles remained within standard parameters. Acute oral administration of the hydrolysate up to 5000 mg/kg produced no mortality or observable signs of toxicity during 14 days of observation ($\text{LD}_{50} > 5000 \text{ mg/kg b.w.}$). These findings demonstrate that peptic–tryptic cowpea β -vignin hydrolysate possesses measurable in vitro membrane-stabilizing and in vivo anti-

inflammatory activity, with additional evidence of treatment-associated modulation of haematological indices and plasma transaminases.

Keywords: Cowpea; *Vigna unguiculata*; β -vignin; Bioactive peptides; Anti-inflammatory activity; Erythrocyte membrane stabilization; Paw oedema; Formaldehyde.

Introduction

Inflammation is a complex biological response to tissue injury, infection, and other harmful stimuli and involves coordinated changes in vascular permeability, cellular recruitment, mediator release, and tissue repair (Okoli et al., 2008). Although inflammation is essential for host defence and restoration of tissue integrity, uncontrolled or persistent inflammatory responses can contribute to tissue injury and the progression of chronic pathological conditions (Anyasor et al., 2019). While synthetic therapeutics such as non-steroidal anti-inflammatory drugs (NSAIDs) are highly effective in mitigating acute inflammatory triggers, repeated long-term use remains a serious clinical concern due to adverse secondary profiles (Singh et al., 2021). These compounds frequently cause negative side effects, including mucosal gastrointestinal ulceration, acute kidney injury, cardiovascular strain, and compounding long-term health care costs (Girgih et al., 2014). As a result of this, there is an urgent need for holistic therapies through safe lifestyle choices, diet-related interventions, and natural prophylactic options (Gunathilake et al., 2018). The search for new anti-inflammatory agents from dietary and natural sources has therefore received considerable attention (Chakrabarti et al., 2018). Food proteins are increasingly recognized as precursors of physiologically active peptides (Chakrabarti et al., 2018). These specific sequences, typically 2 to 20 amino acid residues in length, remain latent within the parent protein structure but exert systemic health benefits once released (Kitts & Weiler, 2003). Peptides may be released from intact proteins during gastrointestinal digestion, fermentation, food processing, or controlled enzymatic hydrolysis (Abdel-Hamid et al., 2017). Their biological properties depend on structural factors including amino acid composition, peptide sequence, molecular size, hydrophobicity, charge, and the specific cleavage patterns of the enzymes used during hydrolysis (Mojica & de Mejía, 2016). Enzymatic hydrolysis is preferred over microbial fermentation because it requires a shorter reaction time, provides better control over the molecular weight profile, and ensures structural predictability and easy quantifiability (Agyei & Danquah, 2011). Low-molecular-weight peptides (<3 kDa) routinely display superior mass-transfer kinetics, cellular accessibility, and tissue affinity compared to their high-molecular-weight counterparts (Ketnawa et al., 2018; Wattanasiritham et al., 2016). Controlled proteolysis has been successfully employed to generate peptide mixtures with documented antioxidant, antihypertensive, antimicrobial, and anti-inflammatory properties (Daliri et al., 2017). Several plant-derived proteins have been investigated as sources of anti-inflammatory peptides (de Medeiros et al., 2022). Protein hydrolysates and individual peptides may influence inflammatory processes through membrane stabilization, modulation of inflammatory mediators, reduction of oxidative stress, and regulation of cellular signaling pathways (Zhu et al., 2018). Human erythrocyte membrane stabilization assays are frequently used as a preliminary *in vitro* model because erythrocyte membranes share structural characteristics with lysosomal membranes (Shenoy et al., 2010). Stabilization of erythrocyte membranes against hypotonicity- or heat-induced haemolysis is therefore interpreted as an indication of potential membrane-protective activity, preventing the extracellular release of lysosomal constituents that cause tissue destruction (Shinde et al., 1999).

Cowpea (*Vigna unguiculata*) is an important food legume widely cultivated and consumed in tropical and subtropical regions, including West Africa (Boukar et al., 2015). Produced in abundance and at low prices relative to other protein sources, it is culturally referred to as "poor man's meat" in Nigeria, where its local names include "ewa" (Yoruba), "agwa" (Igbo), and "wake" (Hausa) (Nigerian Foodstuff Market, 2020). In addition to its nutritional importance, cowpea contains storage proteins that may serve as precursors for bioactive peptides (Jayathilake et al., 2018). The total protein content of cowpea seeds ranges between 20% and 34%, heavily dominated by globulins, glutelins, and prolamins (Gupta et al., 2010). β -Vignin is the major vicilin-type 7S storage globulin of cowpea (Ferreira et al., 2018). It has attracted academic interest due to its balanced amino acid composition, showing high concentrations of aspartic and glutamic acids, leucine, arginine, lysine, and isoleucine (Ilesanmi & Gungula, 2016). Intact cowpea seed extracts have been investigated for antioxidant, antibacterial, and hypoglycemic activities, confirming the crop's viability as an excellent candidate for the generation of functional biopeptides (Sombié et al., 2018; Osman et al., 2021). Despite growing evidence for biological activities associated with plant-derived protein hydrolysates, the anti-inflammatory properties of peptides generated specifically from cowpea β -vignin through sequential pepsin–trypsin hydrolysis have received comparatively limited attention. In particular, evidence integrating *in vitro* erythrocyte membrane stabilization with an animal inflammation model and accompanying haematological and biochemical responses remains limited. The present study therefore evaluated the anti-inflammatory potential of peptic–tryptic cowpea β -vignin hydrolysate using heat-induced and hypotonicity-induced erythrocyte haemolysis assays and a formaldehyde-induced paw-oedema model in rats. The effects of treatment on selected haematological indices and plasma ALT and AST activities were also evaluated to provide additional information on systemic responses associated with the hydrolysate.

Materials and Methods

Sample Collection and Pretreatment

Dry seeds of *Vigna unguiculata* var. Ifebrown were obtained and identified at the Institute of Agricultural Research and Training (IAR&T), Ibadan, Oyo State, Nigeria. Seeds were manually sorted to remove stones, sand, chaff, damaged seeds, and other insect-infested grains. The sorted cowpea seeds were thoroughly washed with distilled water, soaked for 5 min at room temperature (25 °C), and manually dehulled. The dehulled seeds were oven-dried at 40 °C for 36 h, milled into a fine flour using a laboratory mill, and passed through a nylon mesh filter to ensure uniform particle size.

Defatting of Cowpea Flour

The uniform cowpea flour was defatted by soaking it in n-hexane at a ratio of 1:5 (w/v) for 18 h with intermittent shaking. Following extraction, the hexane was decanted, and the mixture was filtered through Whatman No. 1 filter paper. The defatted flour cake was air-dried inside a chemical fume hood until the residual solvent had evaporated completely. The dried, defatted flour was pulverized and stored in an airtight container at room temperature until protein extraction.

Isolation of Cowpea β -Vignin (7S Globulin)

The extraction and isolation of the major storage protein fraction (β -vignin) was carried out according to the method of Frota et al. (2018) via alkaline solubilization followed by acid isoelectric precipitation. The defatted cowpea flour was stirred in a low-ionic-strength extraction

buffer consisting of 50 mM Tris-HCl and 200 mM NaCl (pH 8.5) at a ratio of 1:10 (w/v) for 2 h at room temperature to solubilize the protein. This slurry was filtered through multi-layered muslin cloth, and the coarse residue was discarded. The filtrate was centrifuged at $10,000 \times g$ for 20 min at 4 °C; the supernatant was removed, and the pellet was discarded.

The pH of the supernatant was adjusted to 4.5 (the vicilin isoelectric point) by the dropwise addition of 1 M HCl to facilitate acid-induced protein precipitation. The mixture was refrigerated for 12 h at 4 °C to allow the protein to precipitate completely. After this incubation, the suspension was centrifuged at $10,000 \times g$ for 20 min at 4 °C. The precipitated protein fraction was collected as the cowpea protein isolate, lyophilized, and stored at -20 °C until enzymatic hydrolysis.

Sequential Enzymatic Hydrolysis (Pepsin-Trypsin Digestion)

The isolated β -vignin protein was hydrolysed using the method described by Udenigwe et al. (2009) with slight modifications. Conditions for hydrolysis were optimized for each enzyme to ensure maximum catalytic activity. The lyophilized protein isolate was dissolved in phosphate buffer at a final substrate concentration of 5% (w/v) based on the crude protein weight, and adjusted to pH 2.2 for pepsin. Pepsin was added to the slurry at an enzyme-to-substrate (E:S) ratio of 2:100 (w/w). Digestion was performed at 37 °C for 4 h with continuous stirring. The peptic reaction was terminated by boiling the reaction mixture in a water bath at 95–100 °C for 15 min. After cooling to room temperature, the pH of the mixture was adjusted to 8.0 using 2 M NaOH. Trypsin was then introduced into the slurry at an E:S ratio of 2:100 (w/w), and the mixture was incubated at 37 °C for an additional 4 h under continuous stirring. Trypsin was subsequently inactivated by heating the reaction assembly at 95–100 °C for 15 min in a water bath.

Filtration and Partial Purification of Cowpea Hydrolysate

The crude peptic-tryptic digest was passed through a Merck Whatman Nylon Welded Membrane Syringe Filter (25 mm diameter, pore size 0.22 μ m, Germany) to remove coarse particulates and isolate a clarified, low-molecular-weight peptide pool. The sample was loaded into a sterile syringe, attached securely to the membrane filter via a twisting motion, and the syringe plunger was pressed gradually to harvest the clarified cowpea protein hydrolysate. Total protein concentration was determined using a modified Biuret method with bovine serum albumin (BSA) as the standard.

Thin Layer Chromatography (TLC) Profiling

Thin Layer Chromatography (TLC) was carried out on commercial silica gel plates (Merck, Germany) to visualize cowpea β -vignin after extraction, trace its progressive breakdown at different levels of digestion, and confirm the clearing efficacy of the 0.22 μ m syringe filter. A starting origin line was drawn 1 cm from the bottom of the TLC plate. Samples of undigested β -vignin, the intermediate peptic digest, the crude pepsin-trypsin mixture, and the final filtered hydrolysate were spotted onto the origin using a thin capillary tube.

The plate was placed in a developing chamber containing a mobile phase of n-butanol, acetic acid, and water (3:1:1 v/v/v). Once the solvent front migrated to 1 cm from the top, the plate was removed and air-dried. Peptide bands were detected by spraying with a ninhydrin reagent (1 g ninhydrin in 95 mL pyrimidine and 5 mL glacial acetic acid) and visualized by heating the plate at 100 °C for 5 min in a laboratory oven.

In Vitro Anti-Inflammatory Assays

Preparation of Erythrocyte Suspension: Whole blood sample (5 mL) was obtained from a healthy human volunteer by venepuncture using a syringe and immediately transferred into an ethylenediaminetetra-acetic acid (EDTA) bottle. The human red blood cell (HRBC) sample was centrifuged for 10 min at 3000 rpm. The supernatant plasma layer was carefully removed, and the packed red blood cells (RBC) were washed three times in freshly prepared isosaline solution (0.85% NaCl) by repeated centrifugation until the supernatant became clear. A stock red blood cell suspension (10% v/v) was prepared in isosaline and used for membrane stabilization assays.

Heat-Induced Haemolysis: The effect of the extract on membrane stabilization under thermal stress was assessed according to the method of Sakat et al. (2010). Various concentrations of the hydrolysate (50–400 µg) were dissolved in isotonic phosphate-buffered saline (PBS, pH 7.4) and mixed with 0.1 mL of the 10% HRBC suspension. The resulting mixture was rocked gently and incubated in a controlled water bath at 56 °C for 30 min. After incubation, the tubes were cooled under running tap water, centrifuged at 2000 rpm for 10 min, and the absorbance of the supernatant was estimated at 560 nm. Diclofenac sodium served as the positive standard drug. The percentage of protection against heat induced hemolysis was calculated by using the following equation:

$$\% \text{ inhibition of haemolysis} = (1 - (OD1 - OD2/OD1)) \times 100$$

Where OD1 is the absorbance of the sample and OD2 is the absorbance of the control.

Hypotonicity-Induced Haemolysis: The assay was conducted as described by Shinde et al. (1999). 1 mL of phosphate buffer (pH 7.4), 2 mL of hyposaline solution (0.36% NaCl), and 0.5 mL of the HRBC suspension were added to test tubes containing 1 mL of different concentrations of the hydrolysate (100–500 µg). The mixture was incubated at 37 °C for 30 min and then centrifuged at 3000 rpm for 5 min. The absorbance of the released hemoglobin was measured at 560 nm. Diclofenac sodium served as the standard drug. The percentage of HRBC membrane stabilization was calculated using the formula:

$$\% \text{ stabilization} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

where A1 = absorption of the control, and A2 = absorption of test sample mixture.

Laboratory Animals and Ethical Clearances

Thirty healthy male Wistar albino rats weighing 100 to 150 g were purchased from the Animal Facility of Babcock University, Ilisan-Remo, Ogun State, Nigeria. The rats were acclimatized under laboratory conditions for 2 weeks, housed in metal cages at room temperature following a natural light-dark circadian cycle, and allowed free access to water and standard rat chow. All procedures were conducted in strict accordance with the ethical standards established by the Babcock University Health Research and Ethics Committee (BUHREC) under certificate reference BUHREC536/20.

Acute Oral Toxicity Testing

The median lethal dose (LD₅₀) was determined using the limit dose test of the up-and-down procedure according to the Organization for Economic Co-operation and Development (OECD) Test Guideline 425. A single dose of the cowpea hydrolysate was administered via oral gavage (between 2000 mg/kg and 5000 mg/kg b.w.) to 5 rats sequentially at 48 h intervals. Animals were fasted overnight prior to dosing, and their fasted body weights were determined. Following

administration, the rats were observed continuously for 14 days for signs of toxic symptoms (weakness, loss of appetite, difficulty in movement, reaction to noise) and mortality.

In Vivo Formaldehyde-Induced Inflammation Model

The in vivo anti-inflammatory evaluation was carried out following the method described by Agnel & Shobana (2012), with modifications by Anyasor et al. (2019). Thirty rats were randomly divided into six groups of five animals each and treated orally for 7 days according to the following design:

- Group I (Normal Control): Administered 0.2 mL of 0.9% NaCl saline solution.
- Group II (Inflamed Control): Administered 0.2 mL of 0.9% NaCl saline solution + sub-plantar formalin induction.
- Group III (Standard Group): Administered 10 mg/kg b.w. diclofenac sodium + sub-plantar formalin induction.
- Group IV (Low-Dose Test Group): Administered 100 mg/kg b.w. cowpea hydrolysate + sub-plantar formalin induction.
- Group V (Mid-Dose Test Group): Administered 300 mg/kg b.w. cowpea hydrolysate + sub-plantar formalin induction.
- Group VI (High-Dose Test Group): Administered 500 mg/kg b.w. cowpea hydrolysate + sub-plantar formalin induction.

On the first and third days of treatment, 0.02 mL of 2% (v/v) formaldehyde was injected into the sub-plantar region of the left hind paw of the rats to induce localized arthritis/inflammation. Linear paw thickness was measured using a micrometer screw gauge 30 min before formal induction and every 24 h for 7 consecutive days. The percentage inhibition of inflammation was calculated as stated below:

$$\% \text{ Inhibition of inflammation} = \left(1 - \frac{V_t}{V_c}\right) \times 100$$

Where V_t is the mean paw edema in the treated groups while V_c is the mean paw edema in the control group

Haematological and Liver Transaminase Assays

On day 8, blood samples were collected via cardiac puncture using 5 mL hypodermal syringes and transferred into EDTA and heparinized tubes. Whole blood samples collected in EDTA tubes were immediately analyzed using an automated hematology analyzer (Swelab Alfa 3-Part Analyzer, Boule Medicals, Sweden) to quantify White Blood Cells (WBC), lymphocyte percentages (Lymph%), granulocyte percentages (Gran%), Eosinophil-Monocyte-Basophil (EMB) fractions, and platelet (PLT) counts. Blood samples in heparinized tubes were centrifuged at 3500 rpm for 5 min to separate plasma. Plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured spectrophotometrically using commercial diagnostic kits (Randox Laboratories, United Kingdom) to monitor liver membrane stability.

Statistical Analysis

Data analysis was performed using GraphPad Prism (Standard Version 6.0). Inter-group variations were evaluated using One-Way Analysis of Variance (ANOVA), followed by post-hoc comparisons to separate distinct means. Results are expressed as the mean of replicates $\pm$ standard error of the mean (SEM), with statistical significance defined at $p < 0.05$.

Results and Discussion

Acute Toxicity Study

Single oral administration of the cowpea hydrolysate at doses ranging from 2000 to 5000 mg/kg body weight produced no mortality during the 14-day observation period. No obvious signs of toxicity, including weakness, loss of appetite, difficulty in movement, or other abnormal behavioural responses, were observed. The estimated oral LD₅₀ was therefore greater than 5000 mg/kg body weight under the conditions of the study.

Thin Layer Chromatography

TLC screening verified the progressive degradation of cowpea β -vignin. Intact β -vignin (Lane A) showed minimal migration, remaining concentrated near the baseline origin because of its long chain structure and high molecular weight. The peptic intermediate (Lane B) demonstrated enhanced upward migration, indicating initial breakdown. The final peptic-tryptic digest (Lane C) left no residual protein footprint at the origin line, confirming effective cleavage into smaller peptide fragments. Furthermore, subsequent TLC evaluation of the filtrate (Lane D) confirmed the successful removal of large macromolecular contaminants following passage through the 0.22 μ m membrane filter, yielding a clarified pool of mobile, low-molecular-weight peptides.



Figure 1: Thin layer chromatography showing the hydrolytic effect of pepsin and trypsin on β -vignin. Legend: **A** – undigested β -vignin;
B – peptic β -vignin digest;
C – peptic-tryptic β -vignin digest.



Figure 2: Thin layer chromatography showing the efficacy of syringe filter in the partial purification of peptic-tryptic digest of β -vignin. Legend: **B** – peptic β -vignin digest;
C – crude peptic-tryptic β -vignin digest;
D – partially purified β -vignin.

Heat-induced hemolysis

The cowpea hydrolysate demonstrated concentration-associated membrane-stabilizing activity in the heat-induced haemolysis assay. The percentage inhibition of erythrocyte haemolysis ranged from $15.23 \pm 0.41\%$ to $37.86 \pm 1.09\%$ over the tested concentration range of 50–400 $\mu\text{g/mL}$. Diclofenac sodium produced inhibition ranging from $23.45 \pm 0.71\%$ to $45.26 \pm 1.09\%$. Diclofenac sodium showed significantly greater inhibition than the hydrolysate at the tested concentrations ($p < 0.05$).

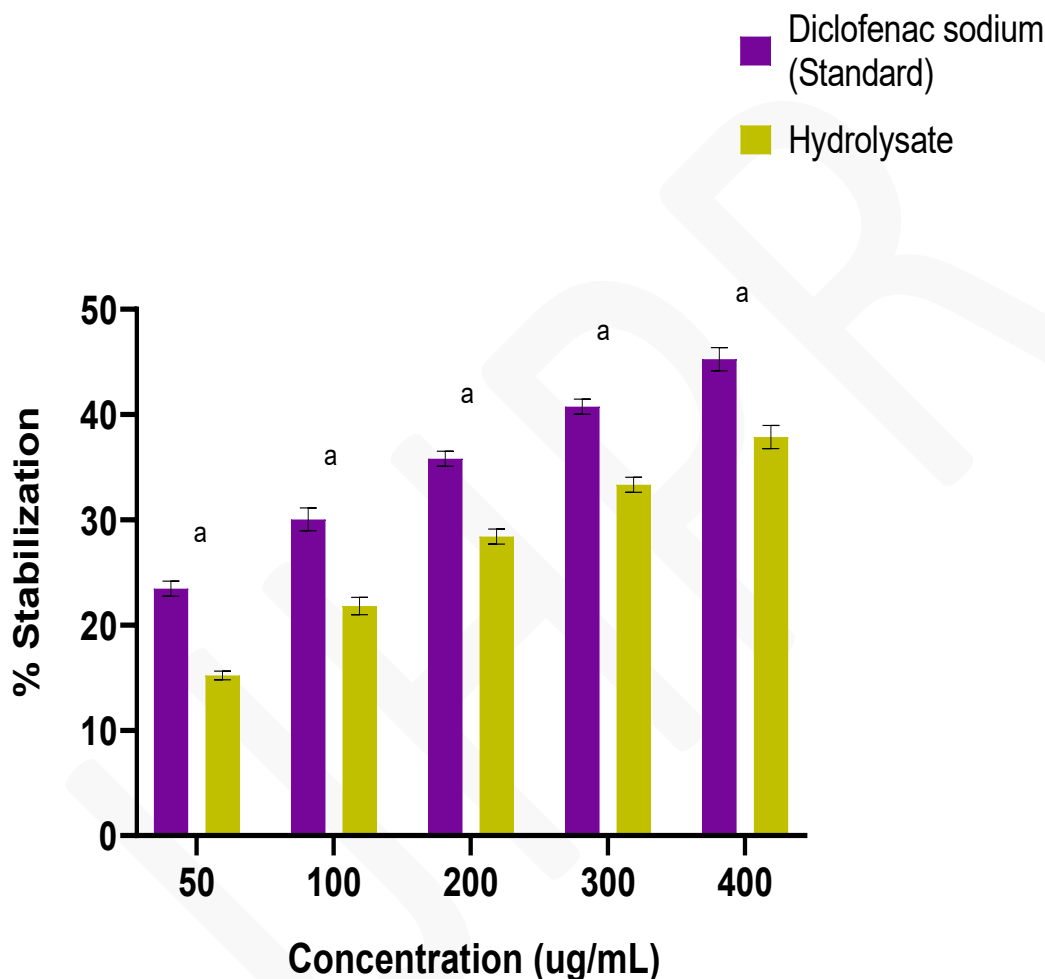


Figure 2: Effect of peptic–tryptic cowpea β -vignin hydrolysate and diclofenac sodium on heat-induced haemolysis of human erythrocytes at different

Hypotonicity-induced haemolysis

The hydrolysate exhibited concentration-dependent membrane-stabilizing activity in the hypotonicity-induced haemolysis assay. Between 100 and 400 $\mu\text{g/mL}$, membrane stabilization ranged from $18.53 \pm 0.06\%$ to $24.08 \pm 0.08\%$, compared with $22.44 \pm 0.082\%$ to $25.17 \pm 0.11\%$ for diclofenac sodium. At 500 $\mu\text{g/mL}$, the hydrolysate produced $25.87 \pm 0.08\%$ membrane stabilization, while diclofenac sodium produced $25.43 \pm 0.07\%$. The difference between the two treatments at 500 $\mu\text{g/mL}$ was not statistically significant.

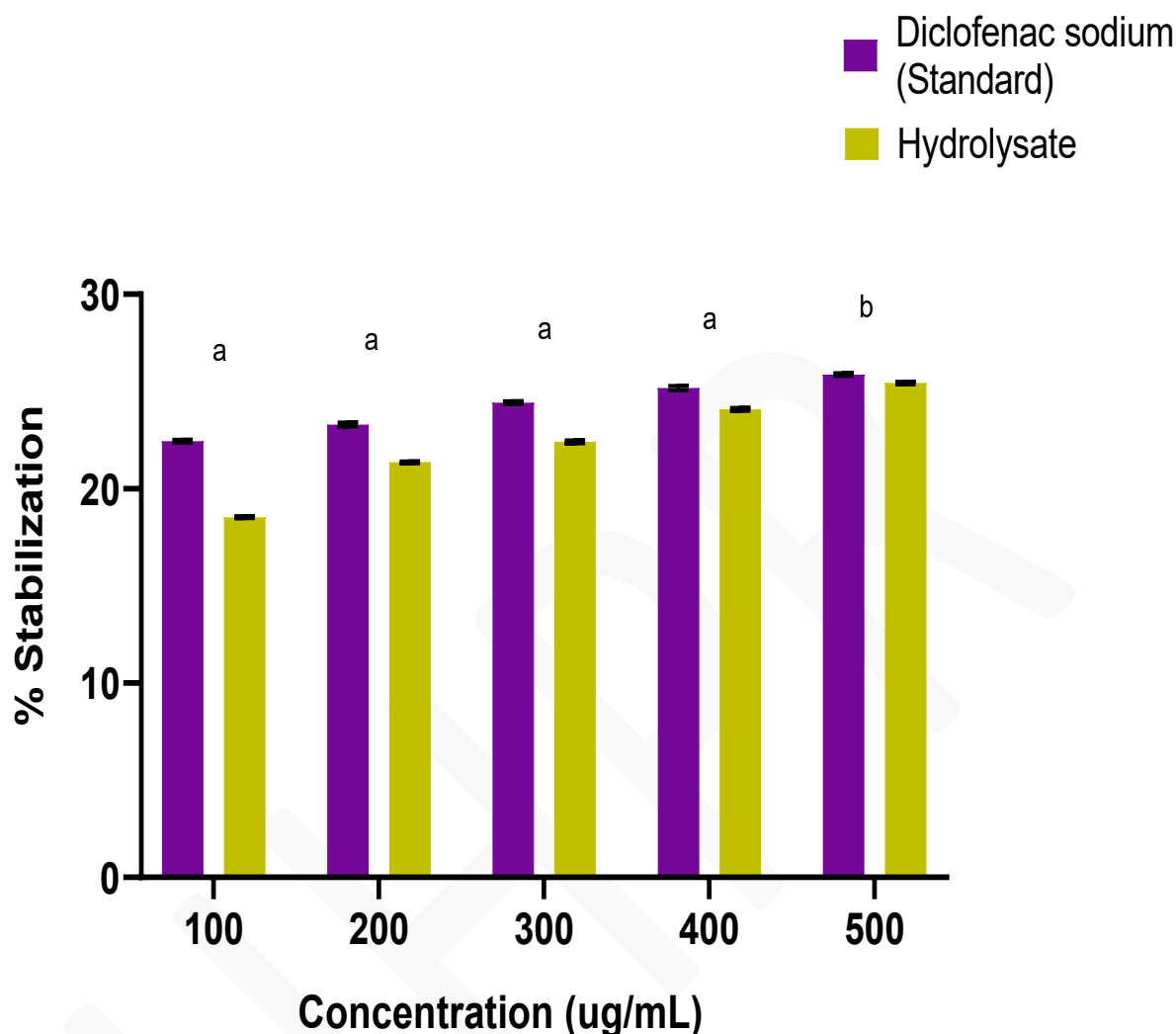


Figure 3: Effect of peptic-tryptic cowpea β -vignin hydrolysate and diclofenac sodium on hypotonicity-induced haemolysis of human erythrocytes

Formaldehyde-Induced Paw Oedema

Oral administration of cowpea hydrolysate at 100, 300, and 500 mg/kg significantly inhibited formaldehyde-induced paw oedema compared with the untreated inflamed control group ($p < 0.05$). The effect increased with dose.

At 100 mg/kg, the hydrolysate produced 15.44% inhibition, corresponding to a paw-oedema value of 1.33 ± 0.26 mm. At 300 mg/kg, inhibition increased to 25.32%, with paw oedema of 0.67 ± 0.25 mm. At 500 mg/kg, the highest inhibition was observed at 36.81%, with paw oedema of 0.29 ± 0.03 mm. Diclofenac sodium at 10 mg/kg produced 23.33% inhibition, with a paw-oedema value of 0.95 ± 0.35 mm.

Thus, the 500 mg/kg hydrolysate produced a greater percentage inhibition of paw oedema than the diclofenac-treated group in the reported endpoint.

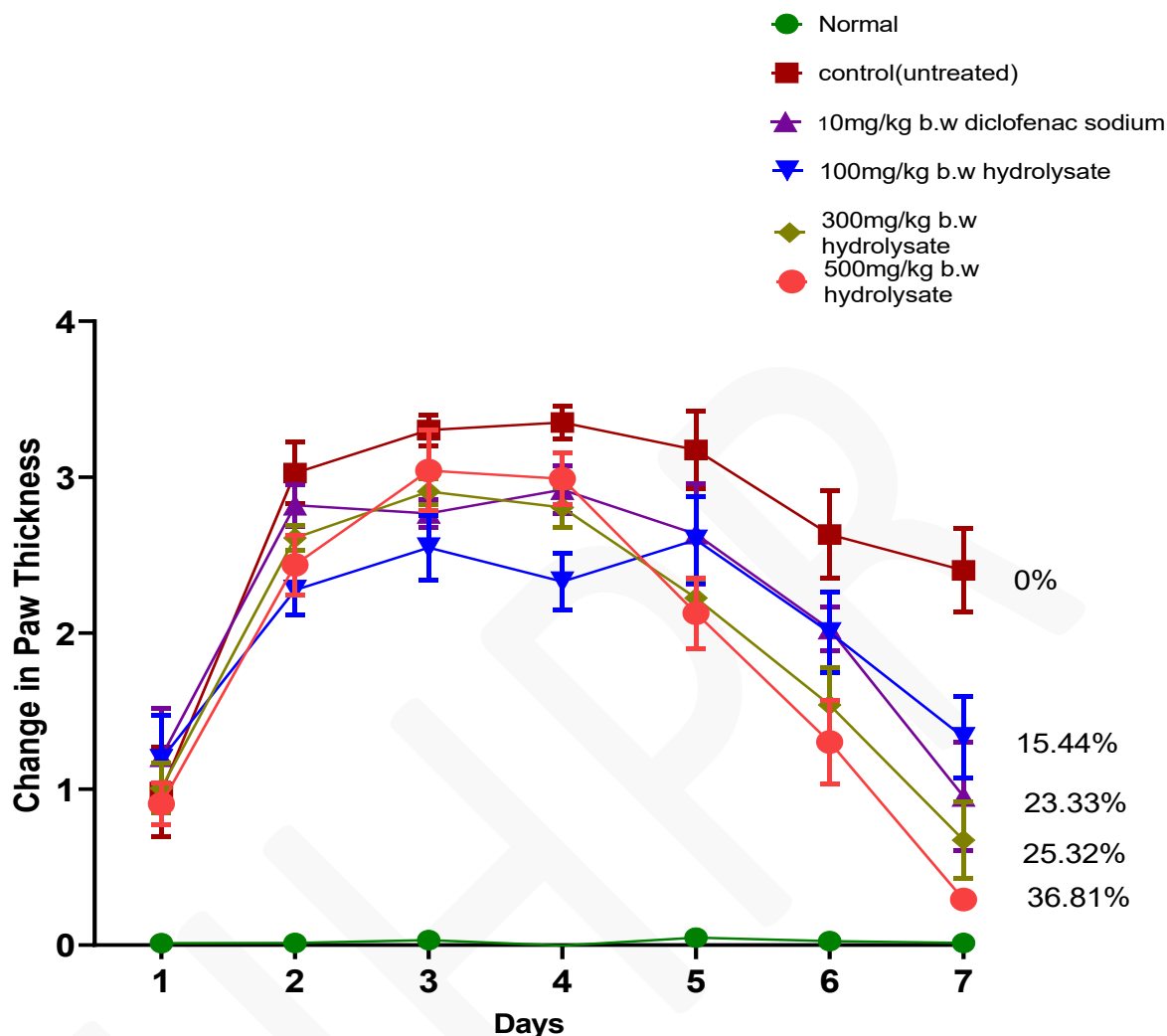


Figure 4: Change in paw edema by different doses of peptic-tryptic β -vignin hydrolysate in formaldehyde inflamed rats

Plasma Alanine Aminotransferase (ALT) activity

Plasma ALT activity in the untreated inflamed group was 3.56 ± 0.07 U/L, compared with 2.904 ± 0.21 U/L in the normal control group. The 100 mg/kg hydrolysate-treated group had an ALT value of 3.72 ± 0.22 U/L, which did not differ significantly from the untreated inflamed group. Treatment with 300 and 500 mg/kg hydrolysate significantly reduced ALT activity to 2.65 ± 0.23 U/L and 2.43 ± 0.35 U/L, respectively, compared with the untreated inflamed control group ($p < 0.05$). The decrease occurred in a dose-dependent manner.

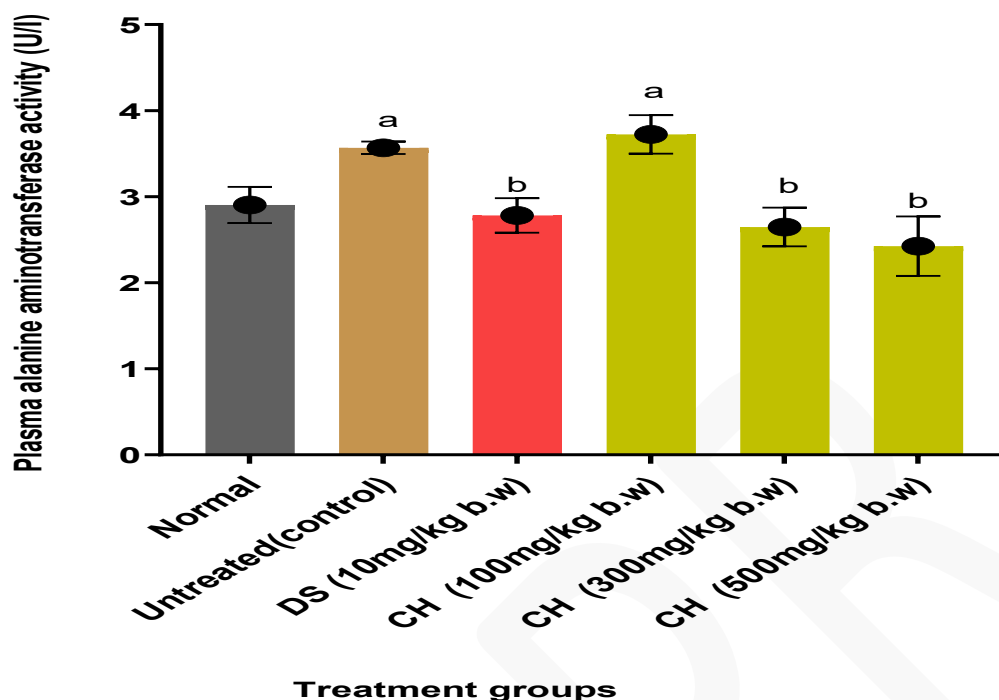


Figure 5: Effects of peptic–tryptic cowpea β -vignin hydrolysate on plasma ALT activity in normal and formaldehyde-inflamed rats

DS – diclofenac sodium

CH – cowpea hydrolysate

Plasma Aspartate Aminotransferase (AST) activity

AST activity increased from 0.99 ± 0.16 U/L in the normal control group to 1.30 ± 0.04 U/L in the untreated inflammation group. Treatment with the cowpea peptic–tryptic hydrolysate produced a dose-related reduction in AST activity. The values were 1.35 ± 0.05 U/L, 0.91 ± 0.04 U/L, and 0.69 ± 0.09 U/L at doses of 100, 300, and 500 mg/kg body weight, respectively. The diclofenac-treated group recorded 0.85 ± 0.10 U/L. The 300 and 500 mg/kg hydrolysate groups showed significantly lower AST activity compared with the untreated inflammatory group ($p < 0.05$), with

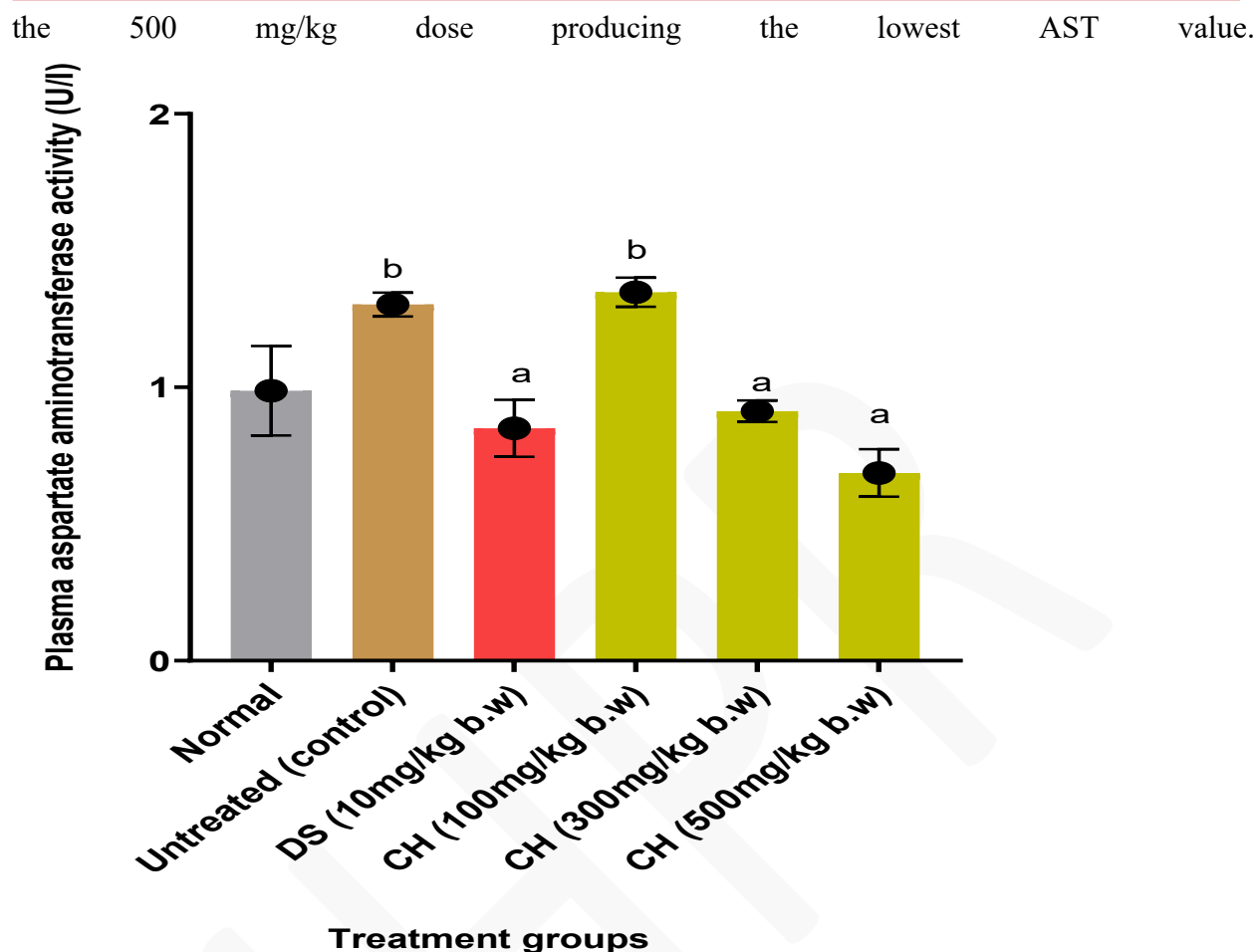


Figure 6: Effects of peptic–tryptic cowpea β -vignin hydrolysate on plasma AST activity in normal and formaldehyde-inflamed rats.

DS – diclofenac sodium

CH – cowpea hydrolysate

Haematological analysis

The result in Table 4.2 shows that 100 and 300mg/kg b.w cowpea peptide hydrolysate treated animals induced with formaldehyde had ($p < 0.05$) a significant difference in their WBC, lymph and gran counts while the 500mg/kg b.w cowpea peptide hydrolysate treated animals showed no significant difference when compared with the untreated control group. Furthermore, only the 100mg/kg b.w cowpea peptide hydrolysate treated animals had ($p < 0.05$) a significant difference in the EMB counts. 300mg/kg b.w cowpea peptide hydrolysate treated animals had ($p < 0.05$) a significant difference in the PLT counts.

However, at $p < 0.05$, there was a significant difference in the WBC and PLT counts and no difference in the lymph and gran counts between 10mg/kg b.w diclofenac sodium treated animals

and the untreated control group. RBC, HCT and HGB did not differ significantly from the untreated inflamed control.

Table 1: Effects of peptic-tryptic hydrolysate of β -vignin on the hematological parameters of rats subjected to formaldehyde-induced inflammation

Parameters	Normal	Untreated (control)	Diclofenac 10mg/kg	Hydrolysate 100mg/kg	Hydrolysate 300mg/kg	Hydrolysate 500mg/kg
WBC $10^3/\mu\text{L}$	7.16 $\pm$ 1.02	11.21 $\pm$ 2.05	8.275 $\pm$ 1.96	7 $\pm$ 1.54	8.367 $\pm$ 1.76	10 $\pm$ 2.14
Lymph %	33 $\pm$ 2.68	36.8 $\pm$ 3.74	39.75 $\pm$ 3.94	30.8 $\pm$ 1.59	23.33 $\pm$ 2.03	44.67 $\pm$ 8.41
Gran %	65.2 $\pm$ 3.01	59.8 $\pm$ 4.16	58.5 $\pm$ 3.61	67.6 $\pm$ 1.91	75 $\pm$ 3.22	53.67 $\pm$ 9.21
EMB ($10^9/\text{L}$)	2 $\pm$ 0.77	3.2 $\pm$ 0.73	1.75 $\pm$ 0.85	1.6 $\pm$ 0.51	2 $\pm$ 1.53	1.67 $\pm$ 0.88
RBC $10^{12}/\text{L}$	7.34 $\pm$ 0.24	7.13 $\pm$ 0.31	6.72 $\pm$ 0.61	7.696 $\pm$ 0.30	7.843 $\pm$ 0.32	6.99 $\pm$ 0.30
HCT %	39.52 $\pm$ 1.75	38.4 $\pm$ 0.75	36.825 $\pm$ 3.95	41.04 $\pm$ 1.72	41.067 $\pm$ 2.17	40.4 $\pm$ 3.07
HGB g/dL	12.96 $\pm$ 0.60	12.92 $\pm$ 0.26	12.2 $\pm$ 1.18	13.94 $\pm$ 0.77	14.07 $\pm$ 0.55	12.93 $\pm$ 0.84
PLT $10^9/\text{L}$	618.4 $\pm$ 58.29	337 $\pm$ 121.47	691.75 $\pm$ 208.20	454 $\pm$ 16.73	530.33 $\pm$ 96.88	482.33 $\pm$ 10.48

White blood cell (WBC), lymphocytes (Lymph%), granulocyte (Gran%), red blood cell (RBC), haematocrit (HCT %), haemoglobin (HGB), platelet (PLT) and Eosinophil, monocyte and basophil (EMB) The present study demonstrates that peptic–tryptic hydrolysis of cowpea β -vignin generated a hydrolysate with measurable anti-inflammatory activity in both *in vitro* and *in vivo* models. These findings are significant because this biological protection was demonstrated using complementary experimental approaches: the stabilization of human erythrocyte membranes under chemically or physically induced stress and the systematic suppression of formaldehyde-induced paw oedema in Wistar rats. Erythrocyte membrane stabilization provides a reliable preliminary indication of systemic membrane-protective activity (Shenoy et al., 2010). Exposure of erythrocytes to hypotonic conditions or elevated temperatures disrupts membrane structural integrity, altering ion channels and promoting cellular haemolysis (Sakat et al., 2010). Compounds capable of stabilizing the erythrocyte membrane interface can prevent this lysis, minimizing the release of intracellular components. This assay serves as an effective analogue for investigating lysosomal membrane stability *in vitro* (Shinde et al., 1999). Preventing the extracellular leakage of lysosomal constituents such as acid hydrolases and proteases from activated neutrophils is a key step in limiting tissue damage and the amplification of local inflammatory cascades (Shenoy et al., 2010). In the heat-induced haemolysis assay, the cowpea hydrolysate inhibited erythrocyte lysis by 15.23–37.86% across the tested concentration range. While this was lower than the protection shown by the reference drug, diclofenac sodium (23.45–45.26%), it confirms a clear, concentration-associated protective effect. The lower relative activity of the hydrolysate is expected; diclofenac sodium represents a pure, highly targeted pharmacologically active agent,

whereas the filtered hydrolysate is a complex mixture of diverse peptide chains with varying configurations (Chakrabarti et al., 2018).

A similar pattern was observed in the hypotonicity-induced haemolysis assay, where the hydrolysate produced 18.53–24.08% stabilization between 100 and 400 µg/mL, compared with 22.44–25.17% for the reference drug. Notably, at the highest tested concentration of 500 µg/mL, the hydrolysate achieved $25.87 \pm 0.08\%$ stabilization, matching the standard diclofenac sodium group ($25.43 \pm 0.07\%$). This lack of statistical variance indicates that increasing the concentration of the hydrolysate may effectively compensate for its lower relative activity observed at lower doses. The *in vivo* findings provide further evidence of anti-inflammatory efficacy within the 2021–2023 experimental window. Sub-plantar administration of 2% formaldehyde into the hind paw triggers a localized inflammatory response, resulting in severe paw oedema. Oral treatment with the cowpea hydrolysate at 100, 300, and 500 mg/kg b.w. significantly reduced paw swelling relative to the untreated inflamed control. The percentage inhibition increased progressively from 15.44% at 100 mg/kg to 25.32% at 300 mg/kg, and reached 36.81% at 500 mg/kg. This dose-related response confirms that the hydrolysate exerts a predictable, biologically meaningful effect rather than producing an isolated response at a single concentration.

At the 500 mg/kg b.w. threshold, the hydrolysate achieved a higher percentage inhibition of paw swelling (36.81%) than the 10 mg/kg diclofenac group (23.33%). However, this comparison must be interpreted cautiously. The two agents are chemically distinct and were administered at substantially different doses. The appropriate conclusion is that the cowpea hydrolysate demonstrates substantial anti-inflammatory efficacy in this experimental model, rather than claiming a higher absolute potency than diclofenac. The underlying molecular mechanism cannot be established conclusively from these experiments because the specific peptide sequences were not isolated. However, sequential enzymatic cleavage by pepsin and trypsin released specific amino acid sequences capable of interacting with biological targets involved in acute inflammation (de Medeiros et al., 2022). Trypsin preferentially cleaves peptide bonds at the carboxyl side of lysine and arginine residues, while pepsin targets hydrophobic or aromatic residues (Mojica & de Mejía, 2016). This combined cleavage pattern releases low-molecular-weight, amphipathic peptide fractions. Previous research indicates that such food-derived fragments can downregulate inflammatory pathways by modifying the production of nitric oxide, prostaglandins, cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines (de Medeiros et al., 2022). The biochemical markers provide additional insights into the systemic responses of the animal models. The untreated inflamed group showed an elevation in plasma ALT activity (3.56 ± 0.07 U/L) compared to the normal healthy baseline (2.904 ± 0.21 U/L), showing systemic stress from formaldehyde exposure. Oral administration of the hydrolysate at 300 and 500 mg/kg b.w. significantly reduced plasma ALT activity in a dose-dependent manner. Plasma AST levels followed a matching trend: the untreated inflamed group exhibited elevated activity (1.30 ± 0.04 U/L), which was systematically reduced in both the diclofenac sodium and 300 mg/kg hydrolysate groups. The AST value for the 500 mg/kg b.w. group was verified from the raw records; the true value is 0.69 ± 0.09 U/L, representing the lowest treatment value. This dose-dependent reduction in circulating transaminases indicates that the cowpea hydrolysate may stabilize structural tissue membranes *in vivo*, mitigating enzyme leakage into the plasma during an inflammatory challenge. However, because ALT and AST variations alone do not fully establish hepatoprotection, this interpretation remains provisional until validated by future histopathological tissue examination. The hematological profiles also support the presence of an active anti-inflammatory response. The untreated inflamed control group showed a sharp increase in total WBC counts, which is consistent

with the activation, proliferation, and recruitment of immune cells to sites of local tissue injury (Okoli et al., 2008). Daily oral administration of the cowpea hydrolysate lowered total WBC counts at 100 and 300 mg/kg b.w. These treatment groups also showed significant modulations in lymphocyte and granulocyte percentages, while the 100 mg/kg b.w. dose altered the EMB count. These findings demonstrate that the peptide hydrolysate can influence the systemic leukocyte response associated with formaldehyde-induced inflammation. The platelet parameters require a similarly cautious interpretation. The untreated inflamed group showed a lower platelet count than the normal group, while the 300 mg/kg b.w. hydrolysate group showed a significant difference. While platelets contribute to homeostasis, cellular signaling, and tissue repair, these experiments do not directly demonstrate a wound-healing effect. Thus, these variations are best reported as part of the overall hematological response rather than definitive proof of accelerated tissue regeneration. Importantly, red blood cell (RBC) counts, hematocrit (HCT), and hemoglobin (HGB) concentrations showed no significant differences from the untreated inflamed group. This preservation of erythrocyte indices suggests that the experimental peptide treatments did not produce adverse toxic effects on erythropoiesis during the study period.

This safety profile is supported by the acute oral toxicity study. A single oral dose up to 5000 mg/kg b.w. caused no mortality or visible signs of clinical toxicity, establishing that the calculated LD₅₀ is greater than 5000 mg/kg b.w. under these experimental conditions. While this confirms favorable short-term tolerability in rat models, it does not establish long-term safety profiles or clinical safety in humans. In conclusion, the *in vitro* membrane stabilization trends, dose-dependent inhibition of localized paw edema, modulation of leukocyte indices, and reduction of plasma transaminase activities indicate that peptic–tryptic cowpea β -vignin hydrolysate possesses clear anti-inflammatory activity. Future investigations utilizing downstream chromatographic fractionation, mass spectrometry sequencing, cytokine profiling, and tissue histology are recommended to fully resolve the specific molecular mechanisms of action.

Conclusion

Peptic-tryptic hydrolysis of cowpea β -vignin produced a hydrolysate with measurable anti-inflammatory activity *in vitro* and *in vivo*. The hydrolysate inhibited heat- and hypotonicity-induced erythrocyte haemolysis, indicating membrane-stabilizing activity, and significantly reduced formaldehyde-induced paw oedema in rats in a dose-dependent manner, with the highest inhibition of 36.81% observed at 500 mg/kg body weight. Treatment was also associated with reductions in plasma ALT at 300 and 500 mg/kg and changes in selected leukocyte and platelet indices, while RBC, HCT, and HGB remained relatively unaffected. Acute oral administration up to 5000 mg/kg produced no mortality or obvious signs of toxicity during the 14-day observation period. These findings support further investigation of cowpea β -vignin hydrolysate as a potential source of anti-inflammatory peptides. Future studies should isolate and characterize the active peptide fractions and determine their effects on specific inflammatory mediators and signalling pathways.

Ethical Statement

The animal experiments were conducted in accordance with the ethical standards established by the relevant Animal Research Ethics Committee and the Babcock University Health Research and Ethics Committee.

Conflict of Interest

The authors declare no conflict of interest.

Funding

Not applicable

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