



## *Escherichia coli* endotoxin modulates haemolymph-mediated immune system of *Achatina fulica* (Bowdich 1822)

M. O. Salawu\*<sup>1</sup>, L. B. Yusuf<sup>1</sup>, A. O. Amuzat<sup>2</sup>, and H. O. B. Oloyede<sup>1</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria

<sup>2</sup>Department of Biochemistry, Ibrahim Badamasi Babangida University, Lapai, Niger State, Nigeria

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\*Corresponding author:  
[salawu.mo@unilorin.edu.ng](mailto:salawu.mo@unilorin.edu.ng)  
Tel: +2348056168553

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### ABSTRACT

*Achatina fulica* (Bowdich, 1822), is a unique species due to its ability to maintain three different life cycle stages, demonstrating exceptional capability to survive in its environment, despite microbial challenges, for millions of years. The survival of the snail is important for the ecology and should be studied. There is however a dearth of empirical information about the effects of endotoxin on the immune system of *Achatina fulica* in particular and to what extent the endotoxin can induce the production of immune-related molecules, of diverse significance (when explored) from *A. fulica*. This research was aimed at investigating the phenoloxidase activity and *Escherichia coli* endotoxin-induced hemolymph coagulation in the *A. fulica*. Fractions of haemolymph from twelve *A. fulica* snails were exposed to endotoxin while the control was made endotoxin-free (0.002 EU/ml). Haemolymph:plasma (2:3 v/v) was exposed to different concentrations of endotoxin. The concentration of coagulated protein and activity of phenoloxidase were then determined. In the results, significantly higher endotoxin-induced protein coagulation was observed in the haemolymph fractions ( $p < 0.05$ ). Maximum protein coagulation in the mixture of HL/plasma 2:3 was recorded. Exposure of the HL/plasma at optimal ratio to varied endotoxin caused linear protein coagulation up to 2.5 EU/ml, beyond which it remained unresponsive to further increase in endotoxin doses. The plasma exhibited the highest phenoloxidase activity up till 10 EU/ml. There was endotoxin-induced protein coagulation and phenoloxidase activity which were endotoxin-concentration-dependent. This study has therefore highlighted the roles of coagulation and phenoloxidase reactions in *A. fulica* immunity and the potential of *A. fulica* hemolymph as a model for innate immune studies and bioassay development.

### 1.0 Introduction

Immunity denotes the ability of the body to defend itself against pathogenic microorganisms or viruses, either by blocking their entry or by neutralizing their damaging effects on the normal physiological functions of the host. This defense mechanism encompasses both innate and adaptive immunity. In innate immunity, the diversity of molecules that

recognize foreign agents is encoded in the germline, while in adaptive immunity, this diversity arises from somatic diversification and the selective expression of specific clones [1]. Invertebrates lack a vertebral column derived from a notochord and their immune systems rely on a variety of complex processes, such as phagocytosis, melanization (the production and secretion of melanin around pathogens), the production of extracellular matrix, cell adhesion,

recognition molecules, reactive oxygen and nitrogen intermediates, antimicrobial peptides and proapoptotic and pro-inflammatory molecules [2-4]. Among these diverse immune responses, arthropods, in particular, utilize key mechanisms such as protein coagulation, melanogenesis (leading to melanization), and clotting [5-7].

The innate immune system of invertebrates consists of a wide range of specific and non-specific responses triggered by the presence of foreign invaders. A key component of these responses is the enzyme, phenoloxidase (PO), which generates indole groups that polymerize to form melanin through a process called melanogenesis. This melanization process plays a critical role in encapsulating multicellular pathogens, aiding tissue repair, and providing defense against various pathogens [4, 8, 9]. The enzymatic reactions involved also produce intermediate compounds such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates that are essential for combating pathogens. Studies have highlighted the significance of PO activity as a vital defense mechanism against numerous pathogens [10, 11].

Antimicrobial peptides (AMPs), also referred to as host defense peptides (HDPs), play a vital role in the innate immune system and are present in all forms of life. These peptides have demonstrated the ability to effectively target and destroy gram-negative and gram-positive bacteria, enveloped viruses, fungi, and even cancerous or abnormal cells [12].

*Achatina fulica* (Bodwich 1822) commonly known as the Giant African land snail, is a member of the Achatinidae family [13]. It is one of the largest and most widespread land snails, often regarded as a significant agricultural and horticultural pest due to its rapid growth and high reproductive rate. This species is particularly unique because it can sustain three distinct life cycle stages, showcasing its remarkable adaptability and resilience to environmental and microbial challenges over millions of years. As a result, *A. fulica* is often used as a bio-indicator of ecosystem health. It is one of the largest land snails globally, with individuals growing up to 19 cm in length [14]. Its polyphagous feeding habits have contributed to its reputation as one of the most destructive pests worldwide, earning it a place on the Global Invasive Species Database as one of the "100 of the world's worst invasive alien species" [15-17].

*Achatina fulica* shares the name "giant African snail" with other species like *A. achatina* and *Archachatina marginata*. Functional hemocyanin has been known to be found in the hemolymph of these snails [18]. Unlike vertebrates, insect "blood" typically lacks hemoglobin, although hemoglobin may exist in the tracheal system and contributes to respiration [19]. Hemolymph is a fluid similar to

vertebrate blood that circulates within the bodies of arthropods, directly interacting with their tissues. It consists of fluid plasma containing suspended hemocytes (hemolymph cells) and various chemicals. It is a key component of the open circulatory system found in arthropods, including arachnids, crustaceans, and insects [20]. Additionally, some non-arthropods, such as molluscs, also possess a hemolymph-based circulatory system. While oxygen transport systems were once thought unnecessary in insects, ancestral and functional hemocyanin has been discovered in their hemolymph [18].

*Escherichia coli* is a facultative anaerobic, gram-negative, rod-shaped bacterium commonly found in animal faeces, the lower intestines of mammals, and even near hot springs. It thrives at 37°C and is a non-spore-forming organism, making it easy to eliminate through boiling or basic sterilization. *Escherichia coli* can be categorized into hundreds of strains based on serotypes. For example, *E. coli* O157:H7 is a well-known strain that produces Shiga-like toxins, causing severe illness when contaminated meat or cheese is consumed [21].

Endotoxins are components of the outer membrane of gram-negative bacterial cell walls. They are always associated with gram-negative bacteria, regardless of whether the bacteria are pathogenic. Although the term "endotoxin" is sometimes used loosely to describe any cell-associated bacterial toxin, it specifically refers to the lipopolysaccharide (LPS) complex found in the outer membrane of gram-negative pathogens like *Escherichia coli*. The cell wall of gram-negative bacteria is an intricate structure comprising the cytoplasmic membrane, periplasm, peptidoglycan layer, outer membrane, and often additional features like capsules, extracellular polysaccharides, fimbriae, and flagella. Endotoxin LPS is located exclusively in the outer leaflet of the outer membrane.

Lipopolysaccharides, the key molecular components of endotoxins, are found in the outermost layer of the Gram-negative bacteria. Examples of such bacteria include *E. coli*; Enterobacteriaceae like *Shigella* spp, Enterobacteriaceae, *Proteus* spp, and pathogens such as *Vibrio cholerae*, *Yersinia pestis*, and *Brucella abortus*. A single *E. coli* cell contains approximately 2 million LPS molecules, equivalent to about 20 femtograms [22].

Research on the immune systems of invertebrates, including mollusks, has shown that these organisms possess peptides with antimicrobial properties. Invertebrates have developed unique mechanisms to detect and respond to microbial surface antigens, such as the case of LPS found in the bacterial endotoxins. As a result, mollusks, like many other invertebrates, represent a valuable source of pharmacologically significant molecules that could be explored for

diagnosing, managing, and treating diseases caused by pathogenic microorganisms. Given this background, this study was aimed at investigating the effects of *E. coli* endotoxin on haemolymph coagulation and phenoloxidase activity, which are critical components of the immune response in *A. fulica*.

## 2.0 Materials and Methods

### 2.1 Snail Collection and Identification

*Achatina fulica* was obtained from Alanamu Market in Ilorin, Kwara State, Nigeria and identified at the Zoology Department, University of Ilorin, Ilorin, Nigeria.

### 2.2 Ethical Approval

This work was approved by the Postgraduate Committee of the Department of Biochemistry and Faculty of Life Sciences and the research was carried out according to the ethical guidelines of the University of Ilorin Research Ethics Committee.

### 2.3 Chemicals and Reagent

Endotoxin, *E. coli*, L-3,4-dihydroxyphenylalanine (L-DOPA), dextrose monohydrate powder, ethylene diamine tetraacetic acid (EDTA), phosphate buffered saline (PBS), NaCl, CaCl<sub>2</sub>, and all other chemicals used in the preparation of reagents were of analytical grade (Sigma-Aldrich).

### 2.4 Extraction of Hemolymph

Four snails were cleaned by washing them in distilled water to remove dirt from their shells and foot, gently rubbing by hand. Hemolymph was collected using the apical cracking method [23]. Each snail yielded approximately 20-25 ml of fresh hemolymph. An equal volume of 10 mM of EDTA in PBS of pH 7.0 (containing 10 mM phosphate buffer, 0.142 M NaCl and 0.18 M glucose), was added to the hemolymph; the resulting mixture was centrifuged at 1,000 x g for 20 minutes at 28°C ± 3°C (Kopacek *et al.*, 1993). The supernatant was separated and collected as *A. fulica* plasma. The pellets were then washed in 10 mM phosphate buffer (pH 7.0) containing 0.1 M CaCl<sub>2</sub> and centrifuged at 1,000 x g for 5 minutes. After discarding the supernatants from this second centrifugation, the pellets, representing hemocytes, were homogenized in 10 mM phosphate buffer (pH 7.0) containing 10 mM EDTA. The homogenate was suspended in the same buffer and adjusted to the original volume of the processed hemolymph. This homogenate was designated hemocyte lysate (HL).

A 10 ml aliquot of the HL was centrifuged at 1,000 x g for 20 minutes, and the resulting supernatant was collected as the HL supernatant (HLS). The pellet, containing cytosol-free hemocyte lysate debris (HLD) and hemocyte membranes, was washed twice with 1 ml of 10 mM phosphate buffer (pH 7.0) and re-suspended in the same buffer, and adjusted to the initial hemolymph volume that produced the HLD. All these were collected, labeled accordingly and stored frozen for further analysis.

### 2.5 Coagulation Reactions in the Haemolymph Fractions

#### 2.5.1 Experimental Setup and Endotoxin-Induced Coagulation

Borosilicate glass bottles and pipettes, depyrogenated by dry heating at 250°C for 30 minutes (Radhakrishna, 2010) prior to the commencement of the experiment, were used for the study. To initiate the reaction, 0.1 Endotoxin Units (EU) of *E. coli* endotoxin in 0.1 ml of distilled water was added to 0.8 ml of each of the hemolymph fractions and 0.1 ml of 0.1M CaCl<sub>2</sub>: hemocyte lysate (HL), HL supernatant (HLS), hemocyte lysate debris (HLD), and plasma in six replicates. Endotoxin-free water (< 0.025 EU/ml) was prepared in replicates and used as control for each of the fractions. The reaction mixtures were incubated at 37°C for 1 hour. Coagulants formed in each bottle were observed and then fixed to the bottom of the bottles by centrifugation at 800 x g for 5 minutes [26], after which the supernatants were discarded. The pellets were dissolved in 0.9% (w/v) NaCl solution, and the coagulated protein content was determined.

#### 2.5.2 Determination of Protein Content in Coagulants

The total protein content of each fraction and its corresponding control was determined using the biuret reaction reagent [27]. A 0.5 ml of the dissolved pellet was mixed with 4.5 ml of Biuret reagent, incubated at 37°C for 15 minutes, and the absorbance was read on a Spectrophotometer at 550 nm. The samples were prepared in duplicates. A blank was prepared with 0.5 ml of 0.9% (w/v) physiological saline to which 4.5 ml of biuret solution was added and treated like the sample as earlier described. A standard solution of protein, 1 mg/ml was equally treated with biuret reagent after which the absorbance was also read accordingly. The concentration of coagulated protein was calculated using the following expression:

$$A_{\text{sample}}/A_{\text{standard}} \times \text{Concentration of Standard}$$

Where, A is the Absorbance at 550 nm.

### 2.5.3 Determination of the Optimal HL:Plasma Ratio for Endotoxin-Induced Coagulation

Hemocyte lysate and plasma mixtures were prepared in various ratios and exposed to endotoxin of a final endotoxin concentration of 0.1 endotoxin unit per milliliter (EU/ml). Thereafter, a 0.1 ml of 0.1 M CaCl<sub>2</sub> was added. The resulting solution was then made up to a total reaction mixture volume of 1.0 ml. A control was prepared for each reaction mixture in quadruplicates. The samples were incubated at 37°C for 1 hour, followed by centrifugation at 800 × g for 5 minutes to fix the coagulants to the bottom of the test tubes. The supernatants were discarded, and the coagulants were dissolved in 1 ml of 0.9% (w/v) NaCl. The protein content was then determined using the biuret reagent [27].

### 2.5.4 Determination of Concentration Dependence of Endotoxin-induced Protein Coagulation Using HL:Plasma Mixture

The concentration dependence of endotoxin-induced protein coagulation in *A. fulica* hemolymph was computed using the optimal HL:plasma ratio [28]. The reaction mixtures contained equal volumes of HL and plasma, 0.1 ml of 0.1M CaCl<sub>2</sub>, and 0.1 ml of varying endotoxin concentrations (0.0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 EU/ml). A blank was also prepared using endotoxin-free water (< 0.025 EU/ml). Quadruplicate reaction mixtures were incubated for 60 minutes at 37°C, centrifuged at 800 × g for 5 minutes, after which the supernatants were discarded. The coagulants were dissolved in 0.9% (w/v) NaCl, and the protein content was determined using the biuret reagent [27]. Values were corrected using blanks prepared with endotoxin-free water incubated with each HL:plasma mixture.

### 2.5.5 Assay of Specific Activity of Phenoloxidase Determination of Endotoxin-induced Specific Phenoloxidase Activity

The effect of endotoxin LPS on PO activity in *A. fulica* hemolymph fractions was analyzed using the method described by Mason [29], as modified by Sung *et al.* [30], Liu *et al.* [31] and Salawu and Oloyede [32]. This method measures the synthesis of dopachrome, with an absorbance peak at ≈460 nm at pH 8.0, from L-3,4-dihydroxyphenylalanine (L-DOPA). For the assay, 0.1 ml of plasma was added to 200 μl of 5 mM L-DOPA, 1.7 ml of carbonate buffer (pH 8.0), and 0.1 ml of LPS solution (1.0 EU/ml). Reaction blanks were prepared simultaneously using LPS-free water instead of LPS. All the samples were prepared as 4 replicates (n = 4) and incubated at 37°C. Absorbance at 460 nm was read immediately after the

addition of LPS (time 0) and then, after 10 minutes, allowing for the determination of the conversion of L-DOPA to dopachrome (a dark green product). The change in absorbance (ΔA460 nm/10 minutes) was calculated to reflect PO activity. Using the protein content of the aliquots (plasma = 5.92 mg, HL = 2.81 mg), the specific activity of PO was calculated using the expression:

$$\text{Specific activity of PO (nmol/min/mg protein)} = \Delta A_{460} \text{ nm}/10 \text{ min/mg protein}$$

### 2.5.6 Determination of Concentration-dependence of Endotoxin on Phenoloxidase Activity

The effect of varying the concentrations of LPS on PO activity in *A. fulica* hemolymph fractions was determined using the same procedure highlighted in Section 2.5.5. In this instance, 0.1 ml of plasma was added to 0.2 ml of 5 mM L-DOPA, 1.7 ml of bicarbonate buffer (pH 8.0), and the different concentrations of LPS solution. Blanks were prepared using LPS-free water (WLPS). All the samples were prepared as 4 replicates (n = 4) and incubated at 37°C. Absorbance at 460 nm was read at time 0 and after 10 minutes to determine the conversion of L-DOPA to dopachrome. The change in absorbance (ΔA460 nm/10 minutes) was used to calculate the activity of PO.

Based on the protein content in the sample (plasma = 1.366 mg), the specific activity of PO was calculated using the following expression:

$$\text{Specific activity of PO (nmol/min/mg protein)} = \Delta A_{460} \text{ nm}/10 \text{ min/mg protein}$$

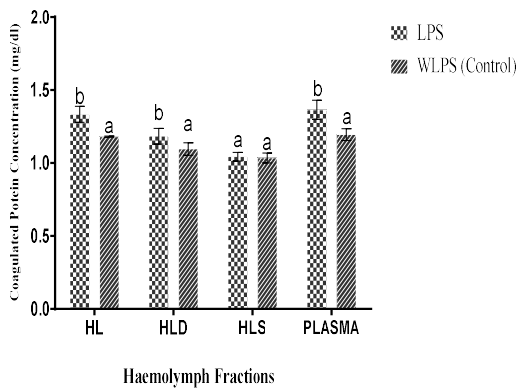
## 2.6 Statistical Analyses

Values were expressed as the mean ± Standard Error of Mean of 4 replicates. The results were analyzed using a one-way analysis of variance (ANOVA) followed by a paired student's t-test as *post hoc* using a Prism Software package (GraphPad, San Diego, CA). Values were considered significantly different at  $p < 0.05$ .

## 3.0 Results

Figure 1 shows the concentrations of coagulated protein obtained from various hemolymph fractions of *A. fulica* in the presence and absence of *E. coli* endotoxin. The concentrations of the coagulated protein in the plasma, HL, and HLD fractions were significantly higher ( $p < 0.05$ ) in the presence of endotoxin when compared to their corresponding controls (without endotoxin). In contrast, the coagulated protein concentration in the HLS fraction

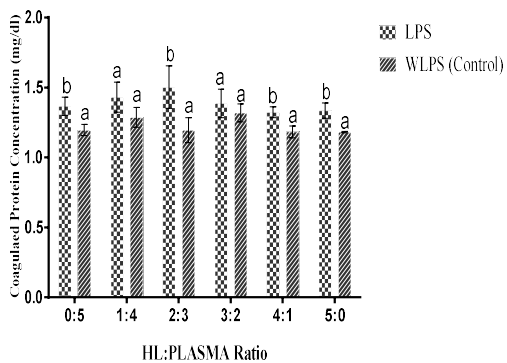
was not significantly ( $p > 0.05$ ) different from the endotoxin-treated samples and the controls. Notably, the control and test values for HLS were lower than those for the other fractions but without any significant difference ( $p > 0.05$ ).



**Figure 1: Endotoxin-induced protein coagulation in the haemolymph fraction of *Achatina fulica***

Different alphabets for each fraction are significantly different ( $p < 0.05$ ). HL: Haemolysate; HLD: Haemolysate debris; HLS: Haemolysate supernatant; LPS: Lipopolysaccharide (endotoxin); WLPS: without lipopolysaccharide

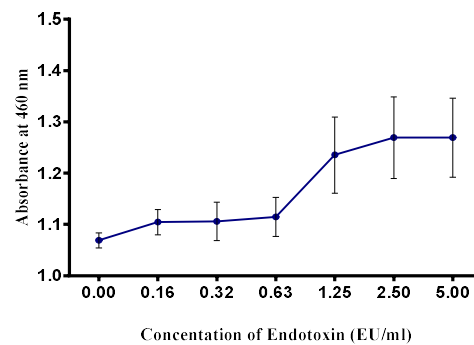
The coagulated protein concentrations were significantly higher ( $p < 0.05$ ) in the presence of endotoxin compared to their respective controls at HL:plasma ratios of 0:5, 2:3, 4:1, and 5:0 (Figure 2). Among these, the highest concentration of coagulated protein was recorded at the HL:plasma of 2:3 in the presence of endotoxin, which was significantly higher ( $p < 0.05$ ) than the concentrations obtained at other ratios.



**Figure 2: Coagulated protein concentrations of HL:Plasma from *Achatina fulica***

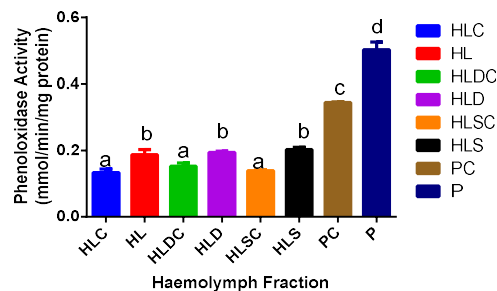
Different alphabets for each fraction are significantly different ( $p < 0.05$ ). HL: Haemolysate; LPS: Lipopolysaccharide (endotoxin); WLPS: without lipopolysaccharide

Figure 3 shows the concentration of coagulated protein in *A. fulica* HL:plasma mixture (2:3). Increasing the endotoxin concentration significantly ( $p < 0.05$ ) and correspondingly increased the concentration of coagulated protein, up to a certain point. Specifically, the concentration of coagulated protein at the optimal HL:plasma ratio (2:3) increased with endotoxin concentrations from 0.00 to 2.50 EU/ml. However, within the concentrations studied, further increase in the endotoxin concentration from 2.50 to 5.00 EU/ml did not produce any additional protein coagulation. Thus, the concentration of coagulated protein in the optimal HL:plasma ratio (2:3) is dependent on *E. coli* endotoxin concentrations between 0.00 EU/ml and 2.50 EU/ml.



**Figure 3: Coagulated protein concentrations of HL:Plasma mixture of *Achatina fulica***

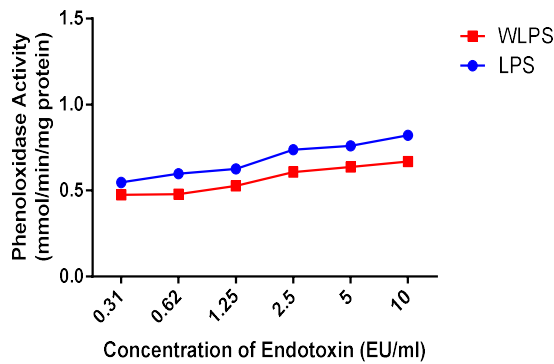
The specific activity of PO in the hemolymph fractions (HL, HLD, HLS, and plasma) of *A. fulica* is illustrated in Figures 4 (test and control of hemolymph fractions) and 5 (test minus control of hemolymph fractions) respectively. The plasma fraction exhibited the highest PO activity. The specific activity of phenoloxidase in the plasma fraction increased with rising concentrations of *E. coli* endotoxin, ranging from 0.31 EU/ml to 10 EU/ml.



**Figure 4: Specific activity of phenoloxidase in hemolymph fractions of *Achatina fulica***

Different alphabets for each fraction are significantly different ( $p < 0.05$ ). HL: Haemolysate; HLC: Hemolysate Control; HLD:

Hemolysate debris; **HLDC**: Hemolysate Debris Control; **HLS**: Haemolysate supernatant; **HLSC**: Hemolysate Supernatant Control; **P**: Plasma; **PC** Plasma Control



**Figure 5: Effect of endotoxin lipopolysaccharide on the phenoloxidase activity in the plasma fraction of hemolymph from *Achatina fulica***

Different alphabets for each fraction are significantly different ( $p < 0.05$ ). **LPS**: Lipopolysaccharide (endotoxin); **WLPS**: Without Lipopolysaccharide

#### 4.0 Discussion

Protein coagulation is a critical defense mechanism in invertebrates, protecting them from injury and pathogenic organisms. For example, in crustaceans like crabs and crayfish, coagulogen and transglutaminases (coagulating enzymes) exist as zymogens and participate in cascade reactions that lead to protein coagulation. These processes prevent excessive blood loss during injury and infection [28, 29]. In the present study, *A. fulica* hemolymph fractions (HL {hemocyte lysate}, HLS {hemocyte lysate supernatant}, HLD {hemocyte lysate debris}, and plasma, were exposed to *E. coli* endotoxin and distilled water (control). The differential protein coagulated in the hemolymph fractions suggests that LPS-binding proteins are likely to be present in the plasma, HL, and HLD fractions. This finding is consistent with that of Salawu et al. [32], who attributed increased protein coagulation in hemolymph fractions to the presence of LPS-binding proteins.

The observed concentration dependent hemolymph coagulation in the fractions indicates a quantitative response to *E. coli* endotoxin in *A. fulica* hemolymph. It also suggests the ability of the snail to defend itself against Gram-negative pathogens based on the microbial load during the exposure.

Previous research on *A. fulica* suggests that the coagulated proteins from the plasma may include proteins such as achatinin-H and achatinin, both being lectins which have been isolated and characterized from the hemolymph of *A. fulica* [32, 33]. These proteins bind to *E. coli* LPS and mediate agglutination, highlighting their role in the innate

immunity of snails. Lipopolysaccharide binding proteins have been reported also in *Archachatina marginata* plasma [33]. The LBP is capable of binding to endotoxin, also known as lipopolysaccharide and thus facilitate its removal from the snail circulation after being exposed to endotoxin.

Phenoloxidase (PO) is a key enzyme in invertebrate immunity, catalyzing melanin synthesis through melanogenesis. The PO is typically present in hemolymph as an inactive zymogen (proPO) and is activated proteolytically by exogenous or endogenous triggers. Active PO, a copper-dependent enzyme, plays a crucial role in defense by producing melanin, which encapsulates pathogens and aids in wound healing [34-39]. Molluscs, encompassing diverse species such as bivalves, gastropods, and cephalopods, rely on innate immunity for defense against pathogens. A critical component of this system is the PO cascade, which mediates melanisation - a process essential for pathogen encapsulation and neutralization. This section outlines the role, activation, and regulation of PO in molluscs, and is supported by key research findings in the present study, *A. fulica* in this instance. The PO system is an evolutionarily conserved defense mechanism in invertebrates. In molluscs including *A. fulica* in the present study, the PO is primarily stored in hemocytes (immune cells) as an inactive zymogen, prophenoloxidase (proPO). Upon pathogen detection via pattern recognition receptors (PRRs), such as lectins or toll-like receptors, a serine protease cascade activates proPO into functional PO [39]. This activation is triggered by pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS) and  $\beta$ -1,3-glucans [40]. Once activated, PO catalyzes the oxidation of phenolic substrates (e.g., tyrosine, DOPA) to quinones, which polymerizes into melanin. Melanin deposition encapsulates pathogens, restricts nutrient uptake, and generates reactive oxygen species and cytotoxic intermediates, directly damaging microbial invaders [41], as previously reported for the mussel, *Mytilus galloprovincialis*, where PO activity in the hemocytes increased significantly upon bacterial challenge and thus correlated with pathogen clearance [41]. The hemocytes are central to PO activity, storing and releasing proPO into hemolymph during infection. Melanization synergizes with other immune mechanisms, such as phagocytosis and antimicrobial peptide production. A previous study on *Crassostrea gigas* revealed that the PO-derived melanin enhanced phagocytosis by hemocytes, suggesting a multifunctional role [40]. Uncontrolled PO activation can harm host tissues; thus, molluscs employ inhibitors like serpins (serine protease inhibitors) to regulate the cascade as is the case of *Biomphalaria glabrata*, where serpins modulated the proPO-

activating protease network, preventing excessive melanisation [42].

Knockdown experiments in molluscs highlight the necessity of PO. Suppressing PO activity in *Octopus vulgaris* resulted in higher susceptibility to *Vibrio* (a genus of gram-negative bacteria with characteristic curve shape that can cause foodborne infection) infections [43]. Phylogenetically, the mollusc PO system shares homology with arthropods but exhibits unique adaptations, such as lineage-specific protease regulators [39]. While the role of PO in bacterial defense is well-documented, its efficacy against fungi or parasites remains understudied. Additionally, the molecular components of the proPO cascade in cephalopods and gastropods require further characterization.

Furthermore, the observed endotoxin concentration-dependent increase in the activity of PO in the plasma in the present study also suggests that it as an important defence mechanism in snails. Phenoloxidase reactions lead to formation of dopachrome and eventually increased the level of melanin at the site of action. Melanin has been considered toxic to invading pathogen and help in the immunity of the invertebrate [38].

## 5.0 Conclusion

This study concluded that *E. coli* endotoxin enhanced the coagulation of protein and phenoloxidase activity in *A. fulica*. This thus highlights the importance of these processes in the mediation of immunity by the haemolymph and the potential of *A. fulica* hemolymph as a model for innate immune studies in the development of bioassay for detecting and quantifying endotoxin.

### Disclosure of conflict of interest

None

### Disclosure of source of funding/grant

None

### Authors Contributions

MOS - Conceptualization, Supervision and Final Draft Writing; LBY - Investigation, Data Curation and Initial Draft Writing; OAA - Resources Management, Initial Draft Writing and Editing; HOB - Supervision, Review and Editing. All authors approved the submission for publication in JOBAS.

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