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EVALUATION OF BACTERIA BINDING POTENTIAL OF HAEMOLYMPH FROM TWO SPECIES OF GIANT AFRICAN LAND SNAILS (*ARCHACHATINA MARGINATA* AND *ACHATINA ACHATINA*)*

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Abstract

A study was conducted to evaluate the bacteria binding potential of haemolymph of two species of giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four species of bacteria isolates (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella gallinarum*). Three liveweight groups (< 100 g, 101–150 g and > 150 g) were used for this study. Haemolymph aseptically collected from each liveweight group were incubated at 37ºC for one hour after which samples were examined under microscope for binding. Result obtained showed that binding pattern differ across the three liveweight groups with highest bacteria binding recorded at > 150 g. It can be concluded that lectin-like substance which agglutinate bacteria are present in the haemolymph of both species and that *Archachatina marginata* showed better binding potential considering livewieght group > 150 g. It can be concluded that substance (s) which agglutinate bacteria are present in the haemolymph of both specie.

Introduction

Giant African land snails are known to be found in the forest and moist area especially in plantations like cocoa, cola and plantain where they could obtain shade and moisture. Molluscs generally possess a natural

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immunity and anatomical structure that protect their soft tissue, body fluid losses and infections of pathogenic microorganisms and parasites (GLINSKI and JAROSZ 1997). As the snails go about their normal activity in their environment, there are tendencies of been constantly challenged by different types of microbial population which may hamper their normal body mechanism if not checked. The big question is 'how do they survive'? It means this animal must have a mechanism which enables them to get rid or control the invaders. DWEK et. al. (2001) revealed that this animal has agglutinin (Lectin) which has binding ability to sugar (oligosaccharides) and as such has been used as prognostic indicator for some cancers. ABIONA et. al. (2009) in his preliminary study identified the presence of agglutinins in the haemolymph of Giant African Land snails (*A. marginata* and *A. achatina*). Haemagglutination potential in both species to four different erythrocyte sources was also carried out to further evaluate their binding abilities (ABIONA et. al. 2014).

The haemocyte content of the haemolymph of invertebrates have been reported to play key roles in both cellular and humoral immune reactions by phagocytosis or delivering immune factors such as lectin and anti-microbial peptides (KANG et. al. 2006) It was further verified that bacterial infection causes changes in components such as lectins, anti-bacterial peptides and lysosomal enzymes of plasma or haemolymph in molluscs (KANG et. al. 2006). However, these component found in haemolymph have been reported to have therapeutic potentials (YANG et. al. 2008). The ability to produce copious amount of these component will determine to larger extent the survivability of this animal in their environment. The two most common species found in Western part of Nigeria are *Archachatina marginata* and *Achatina achatina.* However, their survivability differs especially to different environmental challenges. Other factor such as temperature may also be implicated for this reason but ability to constantly clear opportunistic infection in their system will make them survive better. CHRISTOPER (1980) reported that bacterial (*Pseudomonas aeruginosa*) which can induce elevated immunity in the foreign species of snail (Helix) is agglutinated by snail plasma. In this respect the animal will survive from enormous population of such bacterial. It therefore becomes important to evaluate the binding potential of the two common giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four bacterial species to confirm presence of agglutinins and the quantity produced by white blood cells analoque known as hemocytes and other cellular components of the immune system in controlling the internal environment of the animal. This evaluation will further validate the presence of therapeutic agent in this animal and will also justify the usage of this animal for medical pur-

pose. Prompt recommendation could also be made to guide farmers who want to go into production with the sole aim of harvesting haemolymph/plasma for medicinal purpose on which species that will give the maximum yield at a particular stage of their growth.

Materials and Methods

Sample collection

About 5 ml of sterile snail haemolyph was aseptically collected from apex of three liveweight groups of giant African land snails (*Archachatina marginata* and *Achatina achatina*) after washing with distilled water and cleaning with methanol into a sterile universal bottle. Also, different clinical isolates of pure overnight broth suspension of *Eschericia coli*, *Salmonella gallinarum*, *Pseudomonas aeruginosa* and *Staphylococus aureus* were obtained from different veterinary diseased condition.

Qualitative bacteria agglutination

One milliliter (1 ml) each of overnight broth suspension of *E. coli*, *S. gallinarum*, *P. aeruginosa* and *S. aureus* (10^6 cfu/ml) was added to 1 ml of haemolymph collected aseptically from apex of three liveweight groups of giant African land snails (*A. marginata* and *A. achatina*) after washing with distilled water and cleaning with methanol. All metallic instruments used were gently heated and cooled before and after collection. Both haemolymph and bacteria samples were thoroughly mixed and incubated at 37°C for one hour in LTE incubator (Model UM040, LTE Scientific Ltd, UK). After incubation, the samples were microscopically examined for bound organisms and scored. This was then centrifuged to obtain the supernatant which was microscopically examined for unbound organisms using Centurion Scientific bucket centrifuge (Model M19, UK). The degree of aggregation/agglutination was estimated visually as follows:

- 4+: 80–100% of cells clumped (under a microscope);
- 3+: 60–70% of cells clumped (under a microscope);
- 2+: 30–40% of cells clumped (under a microscope);
- 1+: 10–20% of cells clumped (under a microscope).

Examination of bound and unbound organisms was carried out with three replicates of each of the sample used before arriving at the mean values used for qualitative agglutination count

Quantitative bacteria agglutination study

Supernatants obtained were serially diluted in phosphate buffered saline at ratio 1 : 10.1 ml of diluted sample at dilution of 10^6 bacteria was spread on Nutrient agar (Oxoid, UK) and incubated at 37°C overnight. Discrete colonies seen on the plates were counted. Total viable count of unbound bacteria cells in supernatants were calculated as described by MILES and MISTRAL (1938).

Total viable bacteria cell (unbound) in supernatant = $B \cdot 1 \cdot 1 \cdot 10^6$ cfu/ml.

Total count of bounded bacterial cells = 10^6 cfu/ml of broth – total viable count (unbound) in the supernatant.

Bacteria agglutination with lectin

Equal volume of 2 ml of 10^6cfu/ml bacteria isolates (*E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *S. gallinarum*) was added to 2 ml of various concentrations of standard lectin (*Canavalia ensiformis*) (1000 µg/ml, 800 µg/ml, 600 µg/ml and 400 µg/ml). They were thoroughly mixed, and incubated for 60 minutes at 37°C using LTE incubator (LTE Scientific UM040, UK). Each mixture was centrifuged at 3,500 rpm for 5 minutes using bucket centrifuge, and supernatant was aseptically removed. The supernatant was serially diluted and 1ml of $1/10^6$ dilution was spread on a well-dried Nutrient agar plate and allowed to set. The plates were incubated at 37°C for 18–24 hrs in a well aerated LTE incubator (LTE Scientific UM040, UK). Bacteria colony seen on the plates were counted using Biocote Colony Counter (Barloword Scientific, UK). The total viable count of each sample of the supernatant was calculated as follows:

Total viable count of unbound isolates obtained in the supernatant = $K \cdot 1 \cdot 10^6$.

Total viable count of the bound isolates = 10^6 cfu/ml – total viable count of unbound isolates obtained in the supernatant.

Results

Binding activity of haemolymph from three liveweight groups of two species of giant African land snails (*A. marginata* and *A. achatina*) is presented in Table 1. The large liveweight group (> 150 g) of *A. marginata* shown a very strong agglutination with *Staphylococcus aureus,* while *Escherichia coli* did not agglutinate with the haemolymph of medium liveweight group. Haemolymph from both species showed fair binding for *Pseudomonas aeruginosa* while *Salmonella gallinarum* was loosely agglutinated in both species. Table 2 shows binding activity of commercially prepared Lectin (Concanavalia A) with different clinical isolates. *Escherichia coli*, *Salmonella gallinarum*, *Staphylococcus aureus* had very strong binding with lectin at concentrations of 1000 µg/ml and 800 µg/ml before centrifugation (whole) while while *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed loose binding at this concentration of 800 µg/ml in supernatant. The result of bacteria agglutination quantification is presented in Table 3. It was clear that at 400, 600, 800 and 1000 µg/ml, quantitative estimation of cell deposit after centrifugation increased with increase in lectin concentration, compared to cell number in the supernatant of the four bacteria isolates used except for *Escherichia coli* and *Pseudomonas aeruginosa* whose supernatant cell numbers were higher at 400 µg/ml (0.631 vs 0.369 cfu/ml and 0.51 vs 0.49 cfu/ml).

Table 1

Binding activity of haemolymph of three liveweight groups of giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four species of bacteria

Specification	Snail species									
	marginata				PBS (-ve)	achatina				PBS (-ve)
	Bacteria isolates									
Liveweight group	ST	EC	PS	SM	–	ST	EC	PS	SM	–
Small	–	–	–	+	–	++	–	+	++	–
Medium	+	–	+	++	–	+	–	+	++	–
Large	+++	–	++	+	–	+	+	++	+	–

Key: ST – *Staphylococcus aureus;* EC – *Escherichia coli;* PS – *Pseudomonas aeruginosa;* SM – *Salmonella gallinarum;* PBS – *Phosphate buffer saline;* -ve – negative

+++*:* strong; ++: fair; +: loose

small: < 100 g; medium: 101–150 g; large: > 150 g

Table 2

Lectin (*Concanavalia A*) binding activity with different clinical isolates

Isolate	Whole				Supernatant				Lectin	+ haemolymph
									whole	supernatant
	lectin concentration [μg/ml]				lectin concentration [μg/ml]				[μg/ml]	
	1000	800	600	400	1000	800	600	400	800	800
ST	++++	++++	+++	+++	++	+	+	+	+++	++
EC	++++	+++	+++	++	+++	++	++	+	+++	++
SM	++++	+++	+++	++	++	++	+	+	+++	+
PS	+++	+++	++	++	+	+	+	+	+++	+

Key: ST – *Staphylococcus aureus*; EC – *Escherichia coli*; PS – *Pseudomonas aeruginosa*; SM – *Salmonella gallinarum*
++++: very strong; +++: strong; ++: fair; +: loose

Table 3

Lectin – bacteria agglutination quantification

Bacteria isolate	Lectin concentration [μg/ml]							
	400		600		800		1000	
	supernatant	deposit	supernatant	deposit	supernatant	deposit	supernatant	deposit
	($\cdot 10^6$ cfu/ml) (SEM)							
Staphylococcus aureus	0.412±0.001	0.588±0.001	0.233±0.0012	0.767±0.002	0.103±0.001	0.897±0.001	0.03±0.001	0.967±0.003
Eschericia coli	0.631±0.001	0.369±0.001	0.380±0.011	0.620±0.011	0.235±0.003	0.765±0.001	0.065±0.001	0.935±0.003
Pseudomonas aeruginosa	0.51±0.0115	0.49±0.0173	0.340±0.011	0.660±0.011	0.231±0.001	0.769±0.001	0.019±0.001	0.981±0.001
Salmonella gallinarum	0.48±0.0115	0.52±0.0115	0.280±0.023	0.720±0.006	0.106±0.001	0.894±0.001	0.035±0.003	0.965±0.001

Discussion

The agglutination potential of the agglutinins present in the haemolymph of different snail species (*Archachatina marginata* and *Achatina achatina)* against clinical bacteria isolates was demonstrated in this study. ABIONA et. al. (2014) had earlier reported the presence of agglutinins in these two snail species. For the small liveweight group (< 100 g), *Archachatina marginata* which showed no binding with *Staphylococcus aureus*, *Eschericia coli* and *Pseudomonas aeruginosa* may be as a result of lower proportion of lectins produced in this group which is in contrast with those

produced in *Achatina achatina* which agglutinated all the bacteria with the exception of *Eschericia coli*. The agglutination ability demonstrated by *Achatina achatina* of this group may be as a result of their level of sensitivity which may also be supported by their older age as compared to *Archachatina marginata* with lesser age due to their faster growth rate for this live weight group. It is quite understandable, the reason while age is playing prominent role in facilitating higher agglutination potential, since it takes times three of the age of *marginata* to obtain equal age with that of *Achatina*. Bindings/agglutination of bacteria by haemolymph which is said to contain lectin/lectin-like substance is an indication that certain sugar moieties present on the bacteria surface are the means of attachment for the lectin (KANG et. al. 2006) Also sugar types present on the surface at certain time also determines different lectin types which can bind them since lectins are sugar specific for their binding actions (PISTOLE 1981, MUNOZ-CREGO et. al. 1999). Consequently, the potential binding capacity of haemolymph from the three liveweight groups (small, medium and large) showed that pathogen surfaces bear a large number of oligoglucides that may be bound by specific lectins that can modulate host infection. According to MELHEM and VERDE (1984), adherence is said to be an important factor in bacteria colonization which is known to be supported by sugar carbohydrates of various forms. The medium liveweight group (101–150 g) in both species showed similar binding pattern. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were loosely bound while *Salmonella* spp. was fairly bound. This may be an indication that lectin types which are specific for these bacteria types are present in both species at this liveweight. For *Escherichia coli* that were not bound by both species, it can be deduced that lectin or protein substance that can agglutinate the bacterium is not present in this group. For the large group (>150 g), *Archachatina marginata* haemolymph had a very strong binding with *Staphylococcus aureus* while loose binding was the case with *Achatina achatina*. This observation may be as a result of increased production of lectin specific type *for Staphylococcus aureus* or more protein substance which had the capacity to agglutinate this species of bacteria than *Achatina achatina*. *Pseudomonas aeruginosa* were fairly bound by both species. This is an indication that both species have the same binding ability for this bacterium. The same reason could be adduced for *Salmonella* spp. which was loosely bound by both *Archachatina marginata* and *Achatina achatina*. Loose binding demonstrated by the large group of *Achatina achatina* to *Eschericia coli* in this study is contrary to expectation, since *marginata* out-performed *Achatina* considering the four bacteria used in this study. This observation may be a pointer to the fact that few differences

occur in types of lectin produced. Several lectins have been shown to possess agglutination properties against bacteria strains (PISTOLE 1981, WATANABE et al. 2006, JIN et al. 2013, WANG et al. 2016, TEMPLIER et al. 2016, LV et al. 2016). However, where differences in sugars on the surface which act as attachment points for both micro-organism and lectin are noticed, binding will definitely be affected (SHERIFF 1994).

The results of lectin-binding pattern with the four species of bacteria at different concentrations showed that binding increase as the concentration increases for all the four bacteria strains. This observation is a further pointer to the fact that *Canavalia ensiformis* lectin (Con A) identified sugar moieties that it can bind on the surface of the bacteria. Con A is known to be mannose/glucose specific and as such aggregates a variety of gram-negative bacteria (LE MINOR 1973). Also, the sites of Con A binding are thought to be the exposed sugars of the bacteria lipopolysaccharide and the O-antigen factor 1 (LIENER 1976). So at higher lectin concentration more of lipopolysaccharide of the bacteria are exposed and occupied by available lectin and this explain the reason while binding increases with corresponding increase in lectin concentration. This observation is also in line with the report of MUNOZ-CREGO et. al. (1999) and SCHMID et. al. (2003) that pathogen surfaces bear a large number of oligoglucides that may be bound by specific lectins. The fact that Con A used in this study bind the four bacteria is a confirmation that this type of lectin is present in the haemolymph of giant African land snails (*Archachatina marginata* and *Achatina achatina*.

Considering the results of bacteria-agglutination quantification (Bacteria count), bacteria counted in coliform/ml were noted to increase as the concentration of the *Canavalia ensiformis* lectin increased (i.e 400, 600, 800 and 1000 µg/ml) across board for the four species of bacteria used. This progressive increase in binding was as a result of large amount of lectin available to occupy the binding sites available on the bacteria surfaces as the concentration increased. Also it could be inferred from this study that all the four bacteria have common sugars which are bound by this lectin type. According to AHMED (2005), *Canavalia ensiformis* lectin falls into D-mannose/D-glucose group of the plant lectin sources. This is a further confirmation that all the bacteria used have this sugar sequence on their surface which facilitated strong binding.

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