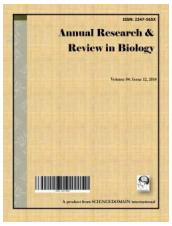

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Antibacterial Effect of Haemolymph Extracts of Edible Snail on Multi-drug Resistant Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ESD, FD, IAB and GF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ESD and FD managed the analyses of the study. Authors ESD and FD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate antibacterial effect of haemolymph in edible snail against multi-drug resistant bacterial isolates.
Study Design: This was an experimental study involving susceptibility testing of bacterial isolates to haemolymph extracts of edible snail.
Place and Duration of Study: The experiments were carried out at the School of Biomedical and Allied Health Sciences of the University of Ghana from February to June, 2014.
Methods: Haemolymph was extracted from two *Achatina achatina* snails (haemolymph extract from one of the snails was labelled "Haemolymph A" and the other "Haemolymph B"). Both haemolymph extracts were tested against 15 multi-drug resistant isolates each of *Staphlyococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* by the agar well diffusion method.

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Results: Overall, the proportion of isolates inhibited by Haemolymph A were 20% (3/15) for *S. aureus*, 20% (3/15) for *E. coli* and 13.3% (2/15) for *P. aeruginosa*; the proportion of isolates inhibited by Haemolymph B were 33.7% (5/15) for *E. coli*, 26.7% (4/15) for *S. aureus* and 13.3% (2/15) for *P. aeruginosa*. For both Haemolymphs A and B extracts, *S. aureus* had the largest mean diameter zone of inhibition of 19.00±3.61mm and 22.25±2.63 respectively. *E. coli* had the smallest mean diameter for Haemolymph A (13.67±3.22mm) while *P. aeruginosa* had smallest mean diameter for Haemolymph B (16.00±5.66mm). For each of the three bacterial pathogens, there was no significant difference in the proportion of isolates inhibited by Haemolymph A and Haemolymph B or the mean zone sizes of inhibition ($p > 0.5$).
Conclusion: Haemolymph of *Achatina achatina* exhibits antibacterial activity against multi-drug resistant isolates of *S. aureus, P. aeruginosa* and *E. coli.* However, there is a high tendency for multi-drug resistant bacterial isolates to be haemolymph-resistant. The antibacterial effect of haemolymph extracts from *Achatina achatina* snails appear to be consistent.

Keywords: Haemolymph; multi-drug resistance; Staphylococcus aureus; Pseudomonas aeruginosa.

1. INTRODUCTION

Bacterial pathogens are implicated in a wide range of human diseases and exert an enormous impact on public health. Although antibiotics have reduced the burden of several bacterial diseases, pathogens are becoming resistant to such drugs at an alarming rate in recent times [1-3]. Antibiotic resistance is due to the misuse and overuse of antibacterial agents, which put selective pressure on bacterial organisms leading to the emergence of resistant strains [3]. Sometimes a bacterium may be resistant to various antibacterial agents simultaneously, a phenomenon referred to as multidrug resistance [2,3]. Multi-drug resistance of bacterial pathogens constitutes a major threat to human health, as it limits treatment options, and enhances morbidity and mortality of superbugs [4-6]. Epidemiological data indicate that infections caused by multi-drug resistant bacterial pathogens constitute an important economic burden, estimated at over 20 billion dollars per year in the United States only [5]. Generally, multi-drug resistance occur in bacteria as a result of accumulation of multiple resistance genes on mobile genetic elements such as plasmids or increased expression of genes that encode multi-drug efflux pumps [4,6].

The increasing burden of antibiotic resistance coupled with the failure of antibiotic discovery in the last few decades has necessitated the search for antimicrobial agents from a wide variety of sources. Haemolymph is a fluid in the circulatory system of arthropods and molluscs, and is analogous to the fluid and cells making up blood and interstitial fluid in mammals [7]. Several studies have already documented antimicrobial activity of haemolymph of several invertebrate organisms [8-12]. However, it is important to obtain further evidence of this by testing a wide range of bacterial pathogens from diverse geographical locations. Additionally, it may be worthwhile to obtain information on susceptibility of resistant bacterial strains to haemolymph, an area that has so far not been addressed by previous studies. The aim of this study was to evaluate antibacterial effect of haemolymph in edible snail against multi-drug resistant isolates of *Staphlyococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* isolated from patients in Ghana.

2. MATERIALS AND METHODS

2.1 Study Design

This was an experimental study involving susceptibility testing of bacterial isolates to haemolymph extracts of edible snail. The experiments were carried out at the School of Biomedical and Allied Health Sciences of the University of Ghana from February to June, 2014.

2.2 Bacterial Isolates

Fifteen multi-drug resistant isolates each of *S. aureus*, *P. aeruginosa* and *E. coli* were used for the study. The isolates were clinical isolates obtained from the Central Laboratory of the Korle-Bu Teaching Hospital in Accra. The bacterial isolates were purified on nutrient agar and their identification was confirmed by colonial morphology, Gram stain and standard biochemical tests. The isolates were confirmed as multi-drug resistant by the Kirby Bauer method and the antibiotics tested included Penicillin, Ampicillin, Cotrimoxazole, Gentamicin, Cefuroxime, Azithromycin, Cefoxitin, Amoxiclav,

Ceftriaxone, Cefotaxime, Ceftazidine, Meropenem, Erythromycin, Amikacin and Ciprofloxacin.

2.3 Extraction of Haemolymph

Ten edible snails were purchased from a market in Accra and identified as *Achatina achatina* by a zoologist at the Department of Animal Biology and Conservation Sciences, University of Ghana.

Two of the *Achatina achatina* snails were randomly selected and haemolymph was extracted as follows. The snails were gently washed in clean saline. A piece of cotton wool was dipped in 70% ethanol and the shell of the snail was thoroughly cleaned. The snail was carefully and gently immobilised using one hand. A pair of sterile scissors was flamed and the shell of the snail was meticulously removed to expose the haemolymph (blue fluid). A sterile syringe was then used to siphon the fluid by pulling the plunger. The collected haemolymph was then transferred into sterile universal bottles. The bottles were tightly capped and stored in a refrigerator at 4℃ to prevent the proteins in the haemolymph from disintegrating. The extraction process was undertaken separately for each snail and was performed aseptically. Haemolymph extract from one of the snails was labelled "Haemolymph A" and the other "Haemolymph B".

2.4 Antimicrobial Susceptibility Testing of Bacterial Isolates to Haemolymph

Antimicrobial susceptibility testing of bacterial isolates to haemolymph was done by the agar well diffusion method [12,13]. The multi-drug resistant isolates were separately inoculated into peptone water to prepare suspensions of the same turbidity as 0.5 McFarland's standard. Using sterile swab sticks, bacterial suspension of the test organism was gently streaked on a Mueller Hinton agar. Five wells, each with a depth of 4mm were made in the inoculated agar using a sterile cork borer. One hundred microliters each of the two haemolymph extracts were pipetted into separate wells; two wells for Haemolymph A and two wells for Haemolymph B. One hundred microliters of gentamicin (20 µg/ml) was pipetted into the fifth well, to serve as a control. This procedure was repeated separately for all the test organisms and the plates were incubated at 35-37℃ for 24 hours. After incubation zones of inhibition were noted and measured.

2.5 Data Analysis

The data were analysed using SPSS version 20.0. Descriptive analysis including means and standard deviations were calculated for zones of inhibition as well as frequencies and proportion of inhibited isolates. An unpaired (independent) student T- test was used to compare mean zone sizes between the different haemolymph extracts and among the different bacterial pathogens. The Chi-square test was used to compare frequencies and proportions of inhibited isolates of the different haemolymph extracts. Generally, p values <0.05 were considered to be significant in the various tests of significance.

3. RESULTS

Antibiograms of the 45 isolates of *S. aureus*, *P. aeruginosa* and *E. coli* used in the study are shown in Table 1. Each of the isolates was resistant to at least three different classes of antibiotics, indicating they were multi-drug resistant. Generally, isolates of each of the three bacterial pathogens showed considerable variations in susceptibility or resistance to a particular antibiotic and multi-drug resistance involved many different combinations of antibiotic resistance (inferred from Table 1).

Susceptibility testing of the 45 isolates against the two haemolymph extracts resulted in zones of inhibition for 12 isolates which included 4 isolates of *S. aureus*, 3 isolates of *P. aeruginosa* and 5 isolates of *E. coli* (Table 2). The four *S. aureus* isolates showed inhibition to Haemolymph B with zone diameters of 20-25mm, while 3 of the isolates showed inhibition to Haemolymph A with zone diameters of 15-22 mm. Of the 3 isolates of *P. aeruginosa* inhibited by haemolymph, one was inhibited by both Haemolymphs A and B with zone diameters of 15 mm and 12 mm respectively. One of the other two isolates of *P. aeruginosa* was inhibited by only Haemolymph A (zone diameters = 14 mm) while the other was inhibited by only Haemolymph B (zone diameters = 20 mm). Of the 5 *E. coli* isolates inhibited by haemolymph, 3 were inhibited by only Haemolymph A with zone diameters ranging from 10-16 mm, while 5 were inhibited by Haemolymph B and the zone diameters were 12-24 mm. Thus 3 of the *E. coli* isolates were inhibited by both Haemolymphs A and B. Overall, the proportion of isolates inhibited by Haemolymph A were 20% (3/15) for *S. aureus*, 20% (3/15) for *E. coli* and 13.3% (2/15) for *P.*

aeruginosa; the proportion of isolates inhibited by Haemolymph B were 33.7% (5/15) for *E. coli*, 26.7% (4/15) for *S. aureus* and 13.3% (2/15) for *P. aeruginosa*. For each of three pathogens, there was no significant difference in the proportion of isolates inhibited by Haemolymphs A and Haemolymph B (p> 0.5). There were two cases where highly resistant bacterial isolates (ie isolates resistant to six or more antibiotics) were inhibited by haemolymph. The first case was an isolate of *S. aureus* (2682) which was resistant to Gentamicin, Amoxiclav, Ampicillin, Erythromycin, Penicillin and Cloxacillin. This isolate was also resistant to the control antibiotic (zone size= 0mm) and was inhibited by Haemolymph B but not Haemolymph A. The second case was an *E. coli* isolate (6355) which was resistant to Cefuroxime, Nitrofurantoin, Ciprofloxacin, Amikacin, Cefotaxime, Ampicillin and Cotrimoxazole; this isolate was inhibited by both Haemolymphs A and B.

Table 1. Antibiogram of isolates tested against haemolymph extracts

Specimen ID	Isolate	Sensitivity
2553	*S. aureus*	GEN + ERY - COT + CXM + AMC+ AMP - PEN - CXC + MEM -
2563	*S. aureus*	CXM+ AMK + FOX- GEN+ PEN - AMP - CXC+
2575	*S. aureus*	ERY+ PEN- AMC + GEN+ CXM - FOX- CXC+CAZ-
2592	*S. aureus*	CXM +GEN- COT- AMP- ERY+ AMC- PEN- CXC-
6545	*S. aureus*	FOX- CXC- PEN- COT- CXM- NIT+ CIP+ GEN +
2691	*S. aureus*	CXM+ GEN- AMC- PEN- COT+ ERY+ AMP- CXC-
2682	*S. aureus*	CXM+ GEN- FOX+ AMC- AMP- ERY- PEN- CXC-
6344	*S. aureus*	PEN- AMK+ TET- MEM+ VA+ ERY- GEN -
6610	*S. aureus*	CXC- AMP- NIT+ MEM+ VA- AMP- OXA- GEN+
2442	*S. aureus*	CHL- AMK+ NIT- MEM- PEN-GEN+ CIP+ CAZ+ VA-
6295	*P. aeruginosa*	GEN- CAZ- CIP- AMK- MEM- TZP-
6635	*P. aeruginosa*	GEN+ AMK- CAZ- TZP+ MEM- CIP+
2548	*P. aeruginosa*	GEN- AMK+ TZP- CAZ+ MEM-
6011	*P. aeruginosa*	CAZ- CIP- GEN + TZP - AMK+ MEM-
2550	*P. aeruginosa*	AK+ GEN- CAZ+ TZP- CIP+ MEM-
2548	*P. aeruginosa*	GEN- AMK- CAZ- MEM- AMC- LEV+
6259	*P. aeruginosa*	CAZ+ MEM- GEN+ TZP+ AMK+ CIP- ERY -
6295	*P. aeruginosa*	GEN- CAZ- CIP- CXC- CTX- LEV+
6011	*P. aeruginosa*	CAZ- CIP- GEN+ TZP- AMK+ MEM-
2315a	*P. aeruginosa*	AMK+ MEM- TZP- GEN- CAZ- CIP+
6284	*P. aeruginosa*	CIP- CAZ+ MEM- GEN- TZP+ AMK+
6551	*P. aeruginosa*	CAZ- MEM- GEN- TZP- AMK- CIP-
6280	*E. coli*	NIT+ CXM- CIP+CAZ- COT- AMK+ AMC- GEN+ AMP-
2669	*E. coli*	AMK+ AMC- CXM- CTX- GEN+ CIP- AMP- MEM+
6044	*E. coli*	CAZ- AMC- GEN+ AMP- NIT+ CXM- COT- CIP- AMK+
2315b	*E. coli*	CTX+ AMC- CXM+ AMP- CIP- AMK- GEN-
6291	*E. coli*	COT- AMP- CAZ- CIP- GEN+ CXM- AMK- NIT+ MEM+
2683	*E. coli*	AMP- CXM+ AMK+ NIT+ AMC+ GEN+ CIP+ COT- CTX-
6355	*E. coli*	CXM- NIT- CIP- AMK- CTX- AMP- GEN+ COT- MEM+
6299	*E. coli*	AMC- AMP- GEN- NIT + CIP- CTX+ CXM- CTX- AMK+ COT- MEM+
2550	*E. coli*	AMP- CXM- AMC-AMK+ GEN+ NIT+ COT- CIP+ CAZ+
6402	*E. coli*	AMC- COT- CXM+ GEN+ AMP- NIT- CIP+ AMK+ CAZ+
2593	*E. coli*	CXM- COT- GEN+ CIP+ AMP- CTX- AMK+ AMC +
6401	*E. coli*	AMK+ GEN- AMC- COT- MEM+ NIT+ CXM- CAZ- CIP-

PEN (Penicillin) *CAZ (Ceftazidine)* *CHL (Chloramphenicol)* *LEV (Levofloxacin)*
AMP (Ampicillin) *FOX (Cefoxitin)* *MEM (Meropenem)* *TZP (Piperacillin/Tazobactam)*
COT (Cotrimoxazole) *AMC (Amoxiclav)* *ERY (Erythromycin)*
GEN (Gentamicin) *AMK (Amikacin)* *NIT (Nitrofurantoin)*
CXC (Cloxacillin) *CXM (Cefuroxime)* *VA (Vancomycin)*
CIP (Ciprofloxacin) *CTX (Cefotaxime)* *OXA (Oxacillin)*

Table 2. Zones of inhibition of haemolymph extracts against bacterial isolates

Bacteria	Antibiogram	Zone of inhibition (mm)		Control
		Haemolymph A	Haemolymph B	
S. aureus				
2575	ERY+ PEN- AMC + GEN+ CXM - FOX- CXC+ CAZ-	22	24	35
2563	CXM+ AMK+ FOX- GEN+ PEN- AMP- CXC+	15	20	35
2682	CXM+ GEN- FOX+ AMC- AMP- ERY- PEN- CXC-	0	20	0
2553	GEN+ ERY- COT+ CXM+ AMC+ AMP- PEN- CXC+ MEM-	20	25	30
P. aeruginosa				
6259	CAZ+ MEM- GEN+ TZP+ AMK+ CIP- ERY-	15	12	20
6011	CAZ- CIP- GEN + TZP - AMK+ MEM-	0	20	24
6635	GEN+ AMK- CAZ- TZP+ MEM- CIP+	14	0	24
E. coli				
2593	CXM- COT- GEN+ CIP+ AMP- CTX- AMK+ AMC+	0	24	>30
2683	AMP- CXM+ AMK+ NIT+ AMC+ GEN+ CIP+ COT- CTX-	15	14	35
2669	AMK+ AMC- CXM- CTX- GEN+ CIP- AMP- MEM+	10	12	24
6280	NIT+ CXM- CIP+ CAZ- COT- AMK+ AMC- GEN+ AMP-	0	20	30
6355	CXM- NIT- CIP- AMK- CTX- AMP- GEN+ COT- MEM+	16	14	35

PEN (Penicillin) *CAZ (Ceftazidine)* *NIT (Nitrofurantoin)*
AMP (Ampicillin) *FOX (Cefoxitin)* *MEM (Meropenem)*
COT (Cotrimoxazole) *AMC (Amoxiclav)* *ERY (Erythromycin)*
GEN (Gentamicin) *AMK (Amikacin)*
CXC (Cloxacillin) *CXM (Cefuroxime)*
CIP (Ciprofloxacin) *CTX (Cefotaxime)*

Table 3. Mean zones of inhibition of haemolymph extracts against isolates of *S. aureus*, *P. aeruginosa* and *E. coli*

Test organism	Zone of inhibition (mm)		P- value
	Haemolymph A	Haemolymph B	
S. aureus	19.00±3.61	22.25±2.63	0.223
P. aeruginosa	14.50±0.71	16.00±5.66	0.746
E. coli	13.67±3.22	16.80±5.02	0.212

The mean zone diameters of inhibition of *S. aureus*, *P. aeruginosa* and *E. coli* to the haemolymph extracts are shown in Table 3. For both Haemolymphs A and B extracts, *S. aureus* had the largest mean zone diameters of 19.00±3.61 mm and 22.25±2.63 respectively. *E. coli* had the smallest mean zone diameter for Haemolymph A (13.67±3.22mm) while *P. aeruginosa* had the smallest mean zone diameter for Haemolymph B (16.00±5.66 mm). For each of the three bacterial pathogens, there was no significant difference in the mean zone diameters of inhibition for Haemolymphs A and B extracts. A representative agar plate showing inhibition zones of Haemolymph A and Haemolymph B are shown in Fig. 1.

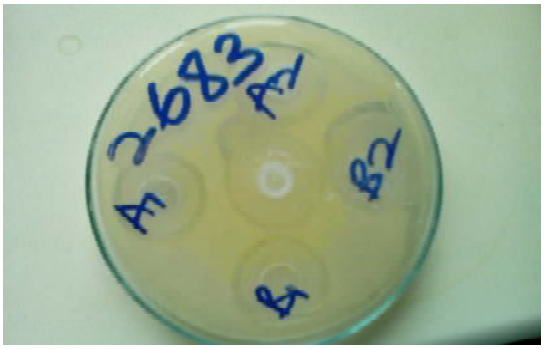

Fig. 1. Inhibition of *E. coli* by haemolymph extracts

A_1 and A_2 are zones of inhibition of Haemolymph A; B_1 and B_2 are zones of inhibition of Haemolymph B; zone in the centre of the plate is the zone of inhibition of the control antibiotic; 2683 is the specimen ID of the isolate tested

4. DISCUSSION

In this study, haemolymph of *Achatina achatina* was tested against multi-drug resistant isolates of *S. aureus*, *P. aeruginosa* and *E. coli*. These bacterial pathogens are among the leading causes of infections reported to hospitals and clinics in Ghana [1,14]. *S. aureus* is responsible for a wide range of invasive infections including meningitis, septicaemia, pneumonia, endocarditis and osteomyelitis [15,16]. *E. coli* is the leading cause of urinary tract infections [17], and is also implicated in several other diseases particularly diarrhea [18]. Some of the highly virulent strains of *E. coli* such as *E. coli* O157:H7 have been reported in Ghana [19]. *P. aeruginosa* is known to cause several types of infections particularly in immune-compromised individuals [20,21]. These infections include malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia [20,21]. It is important to note that multi-drug resistance observed among the bacterial pathogens involved many different combinations of antibiotic resistance including resistance to last resort antibiotics such as carbapenems (Meropenem). This reflects a complex situation of bacterial resistance, which could pose a major challenge to controlling antibiotic resistance of these pathogens in Ghana.

Haemolymph extracts of both snails demonstrated antibacterial activity against each of the three bacterial pathogens, though 13.3-33.7% of the multi-drug resistant bacteria could be inhibited. Our findings concur with several other studies that have reported inhibition of bacterial pathogens by haemolymph. Ravichandran et al. [8] reported that haemolymph extract of *Ocypode macrocera* crab inhibited *P. aeruginosa, Shigella flexineri*, *Vibrio cholerae* and *S. aureus.* Vizioli & Salzet [22] demonstrated inhibition of *B. subtilis, S. aureus, E. coli* and *P. aeruginosa* by larval haemolymph of the fly species; *Musca domestica linnaeus* and *Chrysomya megacephala fabricius*. A study by Mukherjee et al. [9] showed that *Listeria spp*. showed significant sensitivity, though varied, against the haemolymph of *Galleria mellonella.* Inhibition of some extremely highly antibiotic-resistant isolates by haemolymph in the current study is worth noting, and shows that haemolymph research could contribute to solving the problem of antibiotic resistance in this era of failing antibiotics.

The two haemolymph extracts exhibited similar levels of antibacterial activity. This is evident from the mean zone diameters of inhibition of the three bacterial pathogens that were tested as well as the numbers of isolates of these organisms that were inhibited. Further studies are needed to explain the mechanism of antibacterial action of haemolymph of *Achatina achatina*. Studies on the haemolymph of some molluscs, e.g. the *Carcinus meanas* crab, show they contain antimicrobial peptides and proteins, which target microbial macromolecules by disrupting the structure or function of microbial cell membrane [23]. This is similar to the mechanism of action of the antibiotic polymyxin B, which binds to the cell membrane of bacteria and alters the permeability leading to leakage of cellular contents and death [24].

Despite the fact that the haemolymph extracts demonstrated antibacterial activity against all three bacterial pathogens, it is important to note that majority of the isolates were haemolymph-resistant. This may be attributed to the highly resistant nature of the isolates that were tested. Previous studies that have documented antibacterial activity of haemolymph from various organisms were not based on multi-drug resistant isolates unlike the current study [8-11]. Our data suggests that antibiotic resistance in bacteria tends to limit the antibacterial activity of haemolymph. Further studies comparing haemolymph activity against resistant and non-resistant isolates can provide a better picture of this.

Interestingly, for both of the haemolymph extracts*, S. aureus* had significantly larger mean

zone diameters than *P. aeruginosa* and *E. coli*, which had similar zone diameters. This may be partly attributed to the nature of the cell wall of the Gram-positive and Gram-negative bacteria [25,26]. In Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, the presence of an outer membrane generally excludes antibacterial agents from penetrating the cell compared to Gram-positive bacteria such as *S. aureus* which lack this structure in the cell wall [25,26].

There are a few limitations of the study. Firstly, the zone diameters produced in the antibacterial susceptibility testing of bacterial isolates against haemolymph extracts could not be classified as sensitive or resistant as they are no protocols for doing that currently. Consequently, we were unable to make direct comparisons between antibacterial effect of haemolymph and conventional antibiotics. Secondly, the study may have been more informative if we had included more haemolymph samples, though antibacterial effect of the two haemolymph extracts tested appeared to be consistent. Thirdly, we did not determine concentration of the haemolymph extracts.

5. CONCLUSION

Haemolymph of *Achatina achatina* exhibit antibacterial activity against multi-drug resistant isolates of *S. aureus, P. aeruginosa* and *E. coli.* However, there is a high tendency for multi-drug resistant bacterial isolates to be haemolymph-resistant. The antibacterial effect of haemolymph extracts from *Achatina achatina* snails appear to be consistent.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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