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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

The use of *Lycopersicum esculentum* Mill. leaves extract against the survival of fungi

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In this study, the antifungal fraction of *Lycopersicum esculentum* Mill. leaves extract was evaluated for its effect against biomass and cell viability of three strains of *Aspergillus* (*Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus nidulans*). The results obtained show a reduction of the RNA concentration representing the biomass with the increase of the antifungal fraction content in the medium. Indeed, the mean of the RNA concentrations in the medium without antifungal fraction were about 113, 83 and 69 times those observed in the medium at 1% of antifungal fraction, respectively for *A. nidulans*, *A. fumigatus* and *A. flavus*. This reduction of the biomass was related to a reduction of the surviving cells with the increase of the antifungal fraction content in the medium. Indeed, the percentage in reduction of Alamar blue indicating cell viability which was 100% in the medium without antifungal fraction for the three strains tested decreased to reach the values of 2.40, 2.67 and 3.52% in the medium at 1%, respectively, for *A. nidulans, A. fumigatus* and *A. flavus* after five days of incubation. Thus, *L. esculentum* leaves extract exhibit a real inhibitory effect against fungi with an ability of killing them.

Key words: *Lycopersicum esculentum*, antifungal fraction, *Aspergillus*, biomass, cell viability.

INTRODUCTION

Fungi are major spoilage agents of crops, foods and feed-stuffs. Indeed, according to Akande et al. (2006), energy, crude protein and crude fat contents of moldy maize may reduce up to 5, 7 and 63%, respectively. Raju and Rao (2004) have reported previously that mold growth reduces all amino acids in diet, particularly lysine and arginine. In addition to this degradation of the nutritional quality of the products infected, some fungi species are capable of producing mycotoxins. These mycotoxins are secondary metabolites produced by fungi which mostly belong to the *Aspergillus, Penicillium* and *Fusarium* genera found in both animal feedstuffs and human foods (Steyn, 1995; Binder et al., 2007). These naturally occurring poisons can have acute or chronic effects on humans and animals and they were recently defined as a major food safety concern (Kuiper-Goodman, 2004). In order to protect health of consumers from mycotoxins ingestion, 77 countries have currently imposed regulatory limits for mycotoxins. This can results in undue economic burden on growers. Thus, in addition to this threat to human health, mycotoxins can cause great economic loss.

The Food and Agriculture Organization of the United Nations (FAO) has estimated a worldwide loss of about one billion metric tons of foodstuff per year as a result of

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mycotoxins (Choudhary and Kumari, 2010). Innovative technologies are urgently needed to reduce the risks of mycotoxin in food and feed. For many years now, it has been clear that the most effective means to prevent contamination of food by mycotoxins is to avoid growth of mycotoxigenic fungi (Bullerman, 1977).

The primary method of control is the use of chemical fungicides. However, they have become less favored by regulators due to the toxicological risks (Directive 91/414/CEE of the EU, 1991). Also, some of these chemical fungicides do not kill the fungi. They simply inhibit growth for a period of days or weeks (Rouabhi, 2010). Furthermore, the general public demands a reduced use of chemical preservatives and additives in food and feed (Brul, and Coote, 1999). Therefore, the use of natural substances capable of inhibiting fungi development and killing them is of a great importance. Indeed, several plants are known to possess antimicrobial activities (Gould, 1996; Friedman et al., 2002).

Among these plants, there is *Lycopersicum esculentum* Mill. (tomato), the most important *Solanaceae* crop grown throughout the world and the second most important vegetable crop in the world in terms of consumption per capita and recognized as a highly valuable and nutritious food (Rick, 1980). Its leaves contain the steroidal glycoalkaloid (solanine) known to possess antimicrobial properties (Hui et al., 2001). Its concentration is about 1 mM (Figen, 2006). In folk medicine, the leaves extract is used to treat mycosis. No toxicity of this glycoalkaloid (solanine) by contact and inhalation was shown up to now. The toxicity noted was by ingestion in opposite to many chemical fungicides such as pyraclo-strobin and methyl bromide used in agriculture which cause irritation and other toxicological effects by contact and by inhalation even at low concentrations (Iowa Depart-ment of Public Health, 2008). The toxicity by ingestion of the solanine contained in *L. esculentum* leaves is observed at concentrations above 200 mg/kg of leaves. In plants, the glycoalkaloids serve as phytoanticipins, providing the plant with a preexisting chemical barrier against a broad range of pathogens (Sandrock and Vanetten, 1998; Hoagland, 2009).

Thus, this study was carried out to evaluate the effect of *L. esculentum* leaves extract on the survival of fungi in order to contribute to the search for alternative natural fungicides in modern fungicides which inhibit fungi without killing them and which cause many toxicological effects on health and environment.

MATERIALS AND METHODS

Materials

In this study, *L. esculentum* Mill. leaves were used. The culture medium used was the Czapeck Yeast Extract (CY). Three *Aspergillus* strains (*Aspergillus flavus* K220fl, *Aspergillus nidulans* K217ni and *Aspergillus fumigatus* K320fu from the laboratory of Mycology of Pasteur Institute of Cocody-Abidjan (Ivory Coast) were also used.

Methods

Leaves extract preparation

L. esculentum leaves were dried in the shelter of the sun. These dried leaves were grinded and 30 g of the obtained homogenate were added to 150 mL of 100% ethanol. The mixture was boiled in water bath at 80°C for 1 h under gentle stirring. The resulting mixture was centrifuged at 2000 rpm for 10 min. The supernatant was then filtered through Whatman paper (Kouadio et al., 2011). The resulting solution was evaporated to dryness under Fume Hood. The residue obtained was dissolved into 15 mL of boiled distilled water and shaken until total dissolution. In order to purify the homogenate obtained and used the fraction containing the antifungal compounds, the method of purification by ethyl acetate was used. This purification of the extract was made by adding to the homogenate obtained, 15 mL of ethyl acetate. The resulting mixture was shaken for 1 min and centrifuged at 2000 rpm for 10 min. Aqueous and ethyl acetate phases were obtained. The ethyl acetate phase was recovered into a new tube. To the remaining aqueous phase, 15 mL of ethyl acetate were added again, shaken and centrifuged as described above. This purification was done three times. The three ethyl acetate phases were put into the same tube and the aqueous phase into another tube and then, these two solutions obtained were dried under Fume Hood. The residues of the aqueous and ethyl acetate phases were dissolved respectively into 15 mL of distilled water and 15 ml of ethyl acetate.

The resulting solutions were then filtrated separately onto 0.20 µm cutoff membranes to eliminate residues which were not dissolved and eventual contaminants. These aqueous and ethyl acetate fractions were evaluated for their antifungal activities. The fraction containing the antifungal compounds was used to evaluate its effect on proliferation and fungi survival.

Evaluation of the antifungal activities of the fractions obtained after purification of the leaves extract by the ethyl acetate method

Each *Aspergillus* suspension of 10^6 spores/mL was sprayed onto the Czapeck Yeast Extract Agar (CYA) medium by inundation. A disc of 1 cm of diameter was impregnated with 100 µl of each frac-tion of the extract and put onto the medium inoculated. Each medium with impregnated disc was incubated at 30°C for *A. flavus* and 37°C for *A. fumigatus* and *A. nidulans*. The disc around which any fungal growth was observed was identified as the disc impregnated with the fraction containing the antifungal compounds.

Preparation of the tested strains

The *Aspergillus* strains were sprayed onto the Czapeck Yeast Extract Agar (CYA) for 3 days. The different suspensions of spores were then prepared by scraping the conidiospores into 10 mL of sterilized distillated water and filtered onto sterilized Mira cloth.

The conidia concentration of each strain was determined by counting them in a hemacytometer and appropriate dilution was made to obtain a concentration of 10^6 spores /mL. A quantity of 1 mL of this spore's suspension at 10^6 pores /mL was inoculated into liquid medium of Czapeck yeast extract (CY) of 150 ml for 18 h at 37°C for *A. fumigatus* and *A. nidulans* and 30°C for *A. flavus* under shaking at 250 rpm to obtain the microbial ball. This microbial ball was re-suspended into a new liquid medium of CY of 50 mL. Then, 2 mL of the ball suspension was put into different tubes aseptically (CLSI, 1999). Then, into each tube, the antifungal fraction was added to obtain concentrations of 0.05, 0.1, 0.5 and 1%. Medium without antifungal fraction was used as control. For each concentration, 3 tubes were used. Then, all the tubes were incubated also at 37°C for *A. fumigatus* and *A. nidulans* and 30°C for *A. flavus* under shaking

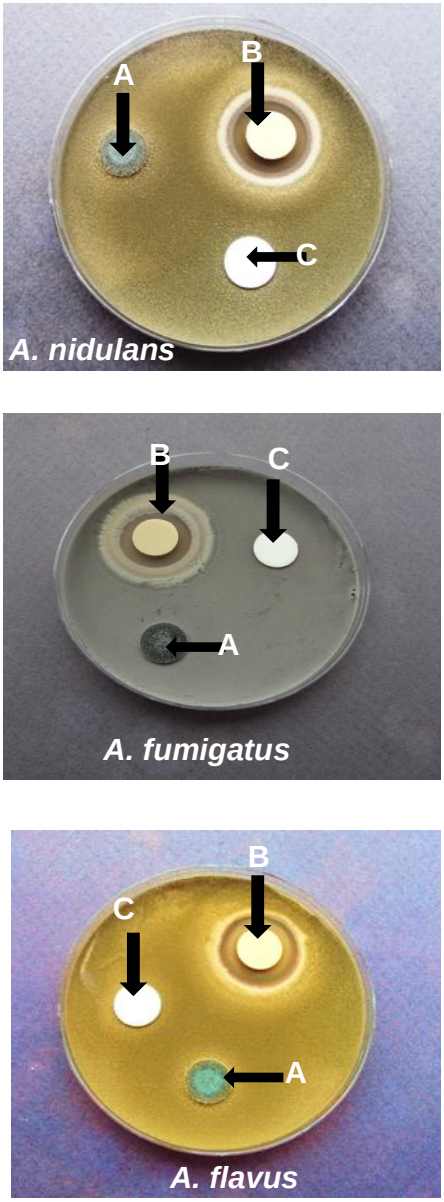

Figure 1. Inhibitory effect of (A): ethyl acetate fraction, (B): aqueous fraction of *L. esculentum* leaves extract and (C): ethyl acetate on fungi growth.

at 250 rpm. The microbial ball obtained after the incubation time was used for the RNA analysis and for the bioassay analysis (test for the determination of the percentage in reduction of Alamar blue).

Extraction and determination of RNA concentration

After 24 h of incubation, the RNA was extracted. For this RNA extraction, the method of Sánchez-Rodríguez et al. (2008) was used. The culture was put onto a sterilized Mira cloth filter Buchner funnel to separate the medium. The ball was removed from the Mira cloth and put into a tube. In each tube, 250 µl of 0.5 mm Zirconium/Silica beads and 1 ml of Trizol reagent were added. The resulting mixture obtained was homogenized into a Mini-Beadbeater at 4°C at maximum speed for 2.5 min.

It was then incubated at room temperature for 20 min. A quantity of 200 µl of chloroform was added to the mixture obtained, shaken vigorously for 20 s and centrifuged at 11600 rpm for 10 min at 4°C. The supernatant obtained was transferred into new microfuge tube. To this supernatant, 500 µl of isopropanol were added and mixed by inversion. It was incubated at room temperature for 10 min and centrifuged at 11600 rpm for 10 min at 4°C. Then, the supernatant obtained was removed and the pellet was washed with 1 mL of 70% ethanol made with DEPC-treated water. The mixture obtained was centrifuged again at 11600 rpm for 5 min at 4°C and the supernatant was removed. The resulting pellet was dried at room temperature for 10 min, re-suspended into 50 µl DEPC-water. After extraction according to the method described above, the purified RNA obtained was quickly put onto ice. The RNA concentration was determined by spectrophotometry at a wavelength of 600 nm. The electrophoresis of the RNA was then done on Formaldehyde Agarose Gel to show the RNA bands.

Bioassay analysis

The experiment was conducted over a span of 5 days. After each 24 h of incubation, 700 µl of liquid medium of CY and 300 µl of Alamar blue reagent were added into each tube. The final concentration of the Alamar blue reagent into each test-tube was 10%. Then, the microbial ball with the Alamar blue reagent was incubated at 37°C for 4 h. A liquid medium of CY without the microbial ball but containing Alamar blue reagent was also incubated.

After this incubation time, 100 µl of each suspension was put into separate wells of a micro-plate and the absorbance was monitored at 570 nm using 600 nm as a reference wavelength in an apparatus Bio-Teck ELISA.

Statistical analysis

The statistical analysis of data was done by Analysis of Variance (ANOVA) using 5% level of significance. The statistical package used is IBM SPSS Statistics version 20. Tukey's Multiple Comparison test was used to identify these differences.

RESULTS

The results show that the antifungal compounds derived from *L. esculentum* Mill. leaves extract are water-soluble compounds. Indeed, no fungal growth was observed around the discs impregnated with the aqueous fraction (Figure 1). With the increases in this antifungal fraction content in the medium, a decrease of RNA concentration representing the biomass in a dose-dependent manner was noted. Indeed, the RNA concentrations which were 3709.50, 38.74.067 and 4758.27 µg/mL in the medium without antifungal fraction, respectively, for *A. nidulans*, *A. fumigatus* and *A. flavus* decreased to reach respectively, the values of 32.80, 46.867 and 69.07 µg/mL in the medium at 1% of antifungal fraction (Figure 2). It was noted that the reduction of the biomass was influenced significantly by antifungal fraction content in the medium (Table 1). The electrophoresis of the RNA showed an absence of RNA bands in the medium at 0.5 and 1% of

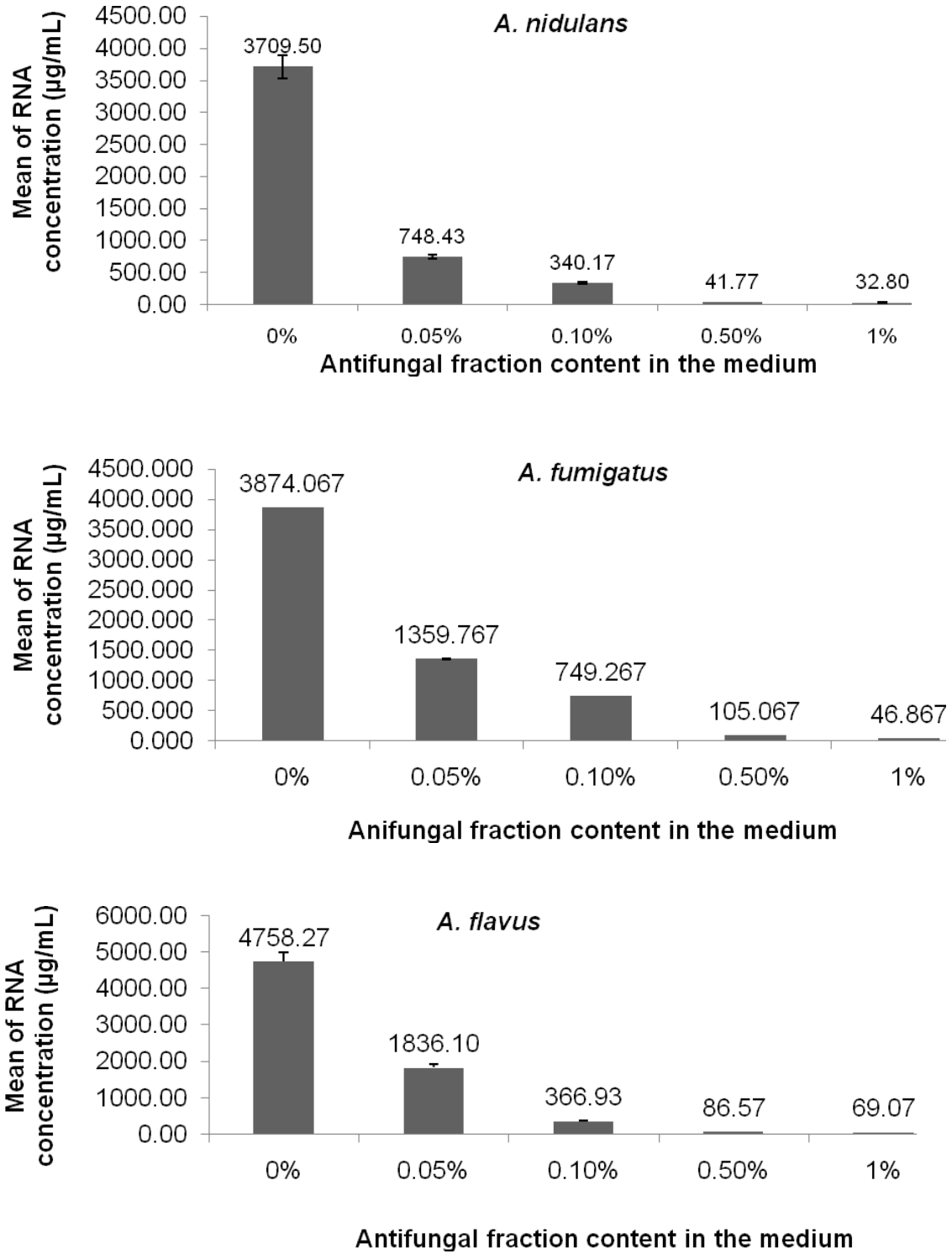

Figure 2. Effect of the antifungal fraction of *L. esculentum* leaves extract on RNA concentration of *A. fumigatus*, *A. nidulans* and *A. flavus*.

the antifungal fraction for *A. nidulans*, while for *A. fumugatus* and *A. flavus*, this absence of RNA bands was observed in the medium at 1% of the antifungal fraction (Figure 3). The reduction of the biomass when the antifungal fraction content in the medium increased was related to a decrease of the percentage of reduced

Table 1. Dose-dependent effect of the antifungal fraction of *Lycopersicum esculentum* leaves extract on biomass of *A. nidulans*, *A. fumigatus* and *A. flavus*.

Fungi	Antifungal fraction content in the medium	N	RNA concentration (µg/mL)				
			Subset for alpha = 0.05				
			1	2	3	4	5
Aspergillus nidulans	Medium at 1% of antifungal fraction	3	32.800				
	Medium at 0.5% of antifungal fraction	3		41.767			
	Medium at 0.1% of antifungal fraction	3			340.167		
	Medium at 0.05% of antifungal fraction	3				748.433	
	Medium without antifungal fraction	3					3709.500
	Significance		1.000	1.000	1.000	1.000	1.000
Aspergillus fumigatus	Medium at 1% of antifungal fraction	3	46.867				
	Medium at 0.5% of antifungal fraction	3		105.067			
	Medium at 0.1% of antifungal fraction	3			749.267		
	Medium at 0.05% of antifungal fraction	3				1359.767	
	Medium without antifungal fraction	3					3874.067
	Significance		1.000	1.000	1.000	1.000	1.000
Aspergillus flavus	Medium at 1% of antifungal fraction	3	69.067				
	Medium at 0.5% of antifungal fraction	3		86.567			
	Medium at 0.1% of antifungal fraction	3			366.933		
	Medium at 0.05% of antifungal fraction	3				1836.100	
	Medium without antifungal fraction	3					4758.267
	Significance		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Used harmonic mean sample size = 3.000. Homogeneous subsets, Tukey HSD

Alamar blue indicating the decreasing of surviving cells for the three strains tested. Indeed, the more the Alamar blue reagent was reduced, the more the percentage of surviving cells was high. The percentage in reduction of Alamar blue which was 100% in the medium without antifungal fraction for the three strains tested was decreased to reach the values of 5.76, 6.16 and 6.73% in the medium at 1% of the antifungal fraction after one day of incubation, respectively for *A. nidulans*, *A. fumigatus* and *A. flavus* (Figure 4).

It was noted that the reduction of the surviving cells was influenced significantly by the antifungal fraction content in medium (Table 2). This reduction of the surviving cells was also influenced significantly by the incubation time (Table 3A, B, C). Indeed, from the first day to the fifth day of incubation, the percentage in reduction of Alamar blue indicating surviving cells, decreased to reach the values of 2.409, 2.678 and 3.52% in the medium at 1% of antifungal fraction, respectively for *A. nidulans*, *A. fumigatus* and *A. flavus* (Table 3A, B, C). However, from the fourth to the fifth day of incubation (Table 3A, B, C), no significance difference was observed between the percentages in reduction of Alamar blue whatever the *Aspergillus* species tested.

DISCUSSION

In this study, the effect of the antifungal fraction of *L. esculentum* leaves extract on the biomass and the survival of *A. nidulans*, *A. fumigatus* and *A. flavus* was recorded. A significant reduction of the biomass of the three strains of *Aspergillus* tested was noted with the increase of the antifungal fraction content in the medium. This antifungal fraction exhibited a significant inhibition on proliferation of the three *Aspergillus* strains with a dose-dependent manner. The highest reduction of the biomass was observed in the medium containing 1% of the antifungal fraction which was the highest antifungal fraction content in the medium tested. These results confirm those obtained previously by Hui et al. (2001) which showed that the glycoalkaloid contained in *L. esculentum* leaves possess antifungal properties. Other previous studies showed also the antifungal activities of the glycoalkaloids.

Indeed, they have shown that the glycoalkaloids inhibited conidia germination (Fewell and Roddick, 1997). In addition to molds, the inhibitory effect of the glycoalkaloids on yeasts has also been shown (Wang et al., 2000). This could explain why in plants, the glycoalkaloids serve

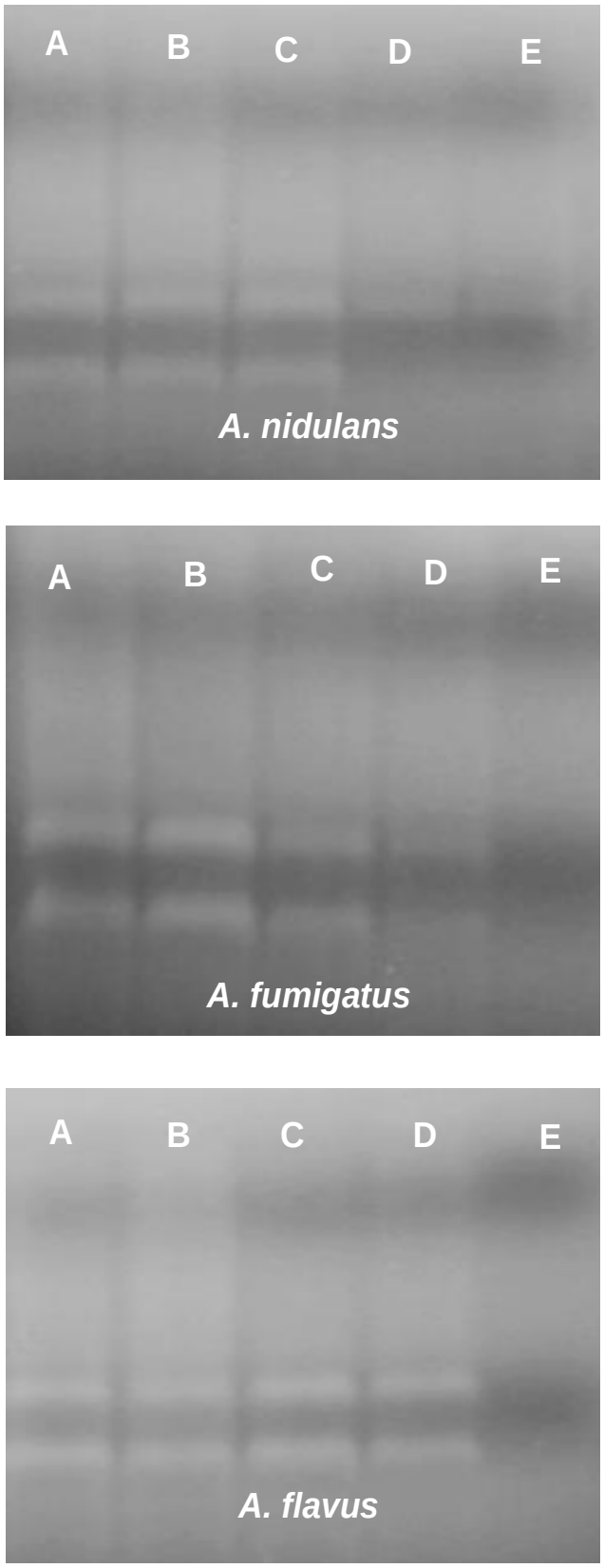

Figure 3. Electrophoresis of the RNA of *A. nidulans*, *A. fumigatus* and *A. flavus* grown in medium at different concentrations of antifungal fraction of *L. esculentum* leaves extract (A: 0%, B: 0.05%, C: 0.1%, D: 0.5%, E: 1%).

as phytoanticipins, providing the plant with a preexisting chemical barrier against a broad range of pathogens (Sandrock et al., 1998; Hoagland, 2009). At this concentration of 1% of antifungal fraction content in the medium, the RNA of the strains tested seems to be damaged as an absence of RNA bands was observed on the Formaldehyde Agarose Gel Electrophoresis. These results could confirm those obtained by Hoagland in 2009 which showed that the glycoalkaloids antifungal activity is believed to be their interaction with sterols in plant pathogen membranes, causing a loss of the membrane integrity and cell lysis. This loss of the membranes due to the glycoalkaloids effect was also shown by Steel and Drysdale (1988) and Keukens et al. (1995). Indeed, these authors have shown that the glycoalkaloids act via disruption of membranes, followed by the leakage of electrolytes and depolarization of the membrane potential. This reduction of the biomass when the extract content increased in the medium could be explained by the death of the fungi cells. Indeed, the more the antifungal fraction content in medium was high, the less the Alamar blue reagent was reduced. This less reduction of the Alamar blue reagent indicating a low rate of surviving cells was observed also in the medium at 1% of the antifungal fraction. This reduction of surviving cells decreased during the incubation time and became stable from the fourth day of incubation. The reduction of surviving cells was observed already in the medium at 0.05% of the antifungal fraction. This indicates that the minimum killing concentration could be at this value.

Conclusion

Regarding the obtained results, we can conclude that the antifungal compounds of *L. esculentum* leaves could be proposed as an effective and powerful antifungal agent against fungi proliferation with an ability of killing them. It highlights the discovery of natural substances for the research in alternative chemical fungicides that inhibit fungal growth without killing them. The highest inhibition of fungi proliferation and the lowest percentages of reduced Alamar blue reagent indicating the lowest rates of viable cells were observed in the medium at 1% of antifungal fraction. At this concentration, the RNA of the strains tested was damaged. These results suggest the use of the antifungal fraction of *L. esculentum* leaves extract at the concentration of 1% or above as a natural fungicide in alternative chemical fungicides which cause environmental and health risks. This antifungal fraction of *L. esculentum* leaves extract could also be used for the prevention of the rottenness of crops.

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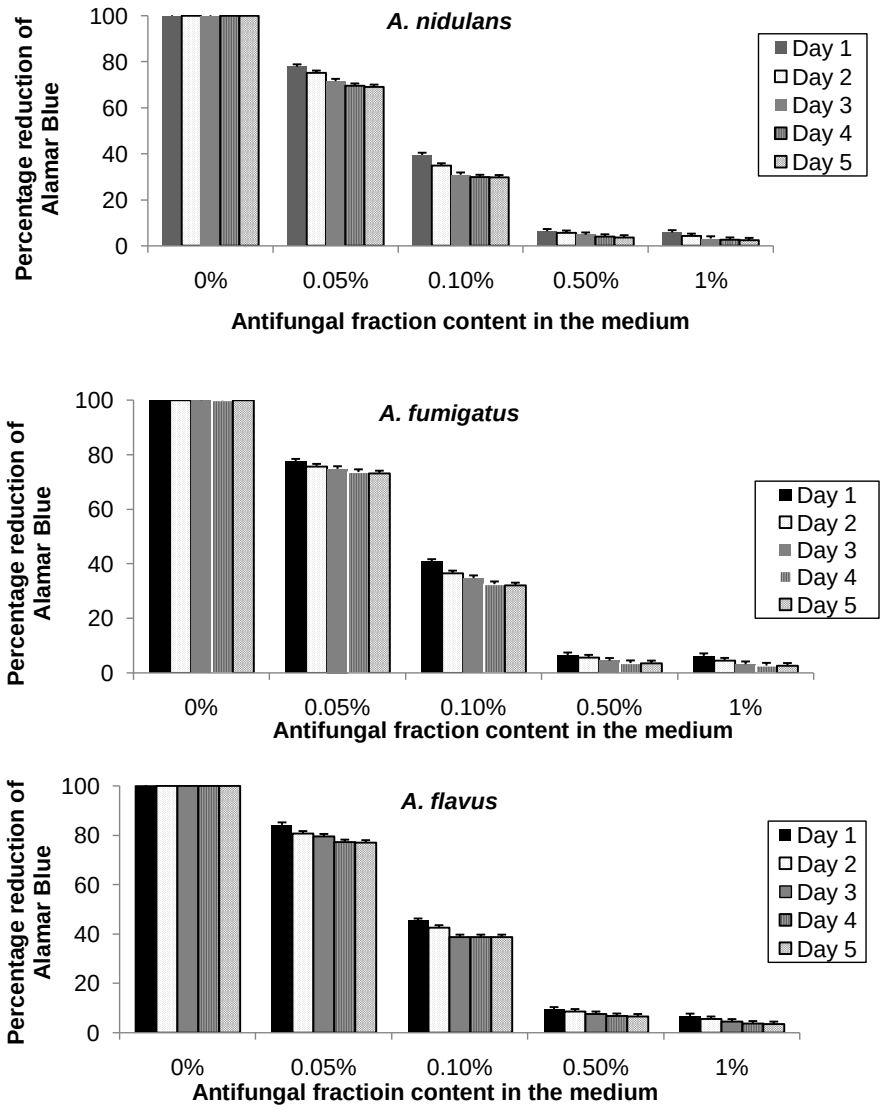

Figure 4. Effect of the antifungal fraction of *L. esculentum* leaves extract on percentage in reduction of Alamar blue of *A. nidulans*, *A. fumigatus* and *A. flavus*.

Table 2A. Dose-dependent effect of the antifungal fraction of *Lycopersicum esculentum* leaves extract on cell viability of *A. nidulans* during 5 days of incubation.

(I) Antifungal fraction content in the medium		Mean difference (I-J)	Multiple comparisons		95% Confidence interval	
			Std. Error	Significance	Lower bound	Upper bound
Medium without antifungal fraction VS	Medium at 0.05% antifungal fraction	21.4196* - 30.0948*	0.02044 - 0.2834	0.000	20.4868 - 30.0276	22.3524 - 30.1621
	Media at 0.1% antifungal fraction	59.8507* - 69.4374*	0.02044 - 0.2834	0.000	58.9180 - 69.3701	60.7835 - 69.5047
	Medium at 0.5% antifungal fraction	95.5928* - 93.0335*	0.02044 - 0.2834	0.000	92.1007 - 95.5255	93.9663 - 95.6601

Table 2A. Contd.

	Medium at 1% antifungal fraction	93.5266* - 96.7568*	0.02044 - 0.2834	0.000	92.5939 - 96.6896	94.4594 - 96.8241
Medium at 0.05 % antifungal fraction VS	Medium at 0.1% antifungal fraction	38.4312*- 39.3425*	0.02044 - 0.2834	0.000	37.4984 - 39.2753	39.3639 - 39.4098
	Medium at 0.5% antifungal fraction	71.6100*- 65.4980*	0.02044 - 0.2834	0.000	70.6812 - 65.4307	72.5467 - 65.5653
	Mediium at 1% antifungal fraction	72.1071* - 66.6620*	0.02044 - 0.2834	0.000	71.1743 - 66.5947	73.0398 - 66.7293
Medium at 0.1 % antifungal fraction VS	Medium at 0.5% antifungal fraction	33.1828* - 26.1554*	0.02044 - 0.2834	0.000	32.2500 - 26.0881	34.1155 - 26.2227
	Medium at 1% antifungal fraction	33.6759* - 27.3194*	0.02044 - 0.2834	0.000	32.7431 - 27.2522	34.6087 - 27.3867
Media at 0.5 % antifungal fraction VS	Medium at 1% antifungal fraction	0.4931 - 1.1640*	0.02044 - 0.2834	0.454 - 0.000	- 0.4397 - 1.0967	1.4259 - 1.2313

*The mean difference is significant at the 0.05 level. Dependent Variable: Percentage in reduction of Alamar blue; Tukey HSD .

Table 2B. Dose-dependent effect of the antifungal fraction of *Lycopersicum esculentum* leaves extract on cell viability of *A. fumigatus* during 5 days of incubation.

(I) Antifungal fraction content in the medium		Mean difference (I-J)	Multiple comparisons		95% Confidence interval	
			Std. Error	Significance	Lower bound	Upper bound
Medium without antifungal fraction VS	Medium at 0.05% antifungal fraction	21.801* - 25.762*	0.2569 - 0.0500	0.000	22.6472 - 25.5981	22.6472 - 25.9276
	Media at 0.1% antifungal fraction	58.568* - 66.882*	0.2569 - 0.0500	0.000	57.7225 - 66.7174	59.4138 - 67.0469
	Medium at 0.5% antifungal fraction	92.793* - 95.444*	0.2569 - 0.0500	0.000	91.9470 - 95.2793	93.6384 - 95.6088
	Medium at 1% antifungal fraction	93.119* - 96.342*	0.2569 - 0.0500	0.000	92.2735 - 96.1774	93.96487 - 96.5069
Medium at 0.05% antifungal fraction VS	Medium at 0.1% antifungal fraction	36.766* - 41.119*	0.2569 - 0.0500	0.000	35.9209 - 40.9545	37.6123 - 41.2839
	Medium at 0.5% antifungal fraction	70.991* - 69.681*	0.2569 - 0.0500	0.000	70.14549 - 69.5164	71.83685 - 69.8459

Table 2B. Contd.

	Mediium at 1% antifungal fraction	71.317* - 70.579*	0.2569 - 0.0500	0.000	70.471930 - 70.4145	72.1633 - 70.7440
Medium at 0.1% antifungal fraction VS	Medium at 0.5% antifungal fraction	34.224* - 28.562*	0.2569 - 0.0500	0.000	33.3789 - 28.3972	35.07026 - 28.7267
	Medium at 1% antifungal fraction	34.551* - 29.460*	0.2569 - 0.0500	0.000	33.7053 - 29.2953	35.3967 - 29.6248
Media at 0.5% antifungal fraction VS	Medium at 1% antifungal fraction	0.326 - 0.898*	0.2569 - 0.0500	0.000	0. 5112 - 0.7333	1.1721 - 1.0628

*The mean difference is significant at the 0.05 level. Dependent Variable: Percentage in reduction of Alamar blue; Tukey HSD.

Table 2C. Dose-dependent effect of the antifungal fraction of *Lycopersicum esculentum* leaves extract on cell viability of *A. flavus* during five days of incubation.

(I) Antifungal fraction content in the medium		Mean difference (I-J)	Multiple comparisons		95% Confidence interval	
			Std. Error	Significance	Lower bound	Upper bound
Medium without antifungal fraction VS	Medium at 0.05% antifungal fraction	15.1988* - 22.1671*	0.18729 - 0.035066	0.000	14.5827 - 22.05168	15.8149 - 22.2825
	Media at 0.1% antifungal fraction	54.1416* -60.5126*	0.18729 - 0.035066	0.000	53.5255 - 60.3972	54.75781 - 60.62807
	Medium at 0.5% antifungal fraction	90.0539* - 92.6700*	0.18729 - 0.035066	0.000	89.4378 - 92.5581	90.67016 - 92.7888
	Medium at 1% antifungal fraction	92.6886* - 95.73*	0.18721953280	0.000	92.0724 - 95.614780	93.3047 - 95.8456
Medium at 0.05 % antifungal fraction VS	Medium at 0.1% antifungal fraction	38.9428* - 38.3456*	0.18729 - 0.035066	0.000	38.3270 - 38.2302	39.5589 - 38.4610
	Medium at 0.5% antifungal fraction	74.85513* - 70.5064*	0.18729 - 0.035066	0.000	74.2390 - 70.3910	75.4713 - 70.6218
	Mediium at 1% antifungal fraction	77.4897* - 73.5631*	0.18729 - 0.035066	0.000	76.8736 - 73.4480	78.1059 - 73.6785
Medium at 0.1 % antifungal fraction VS	Medium at 0.5% antifungal fraction	35.9123* - 32.1608*	0.18729 - 0.035066	0.000	35.2961 - 32.0454	36.5285 - 32.2762
	Medium at 1% antifungal fraction	38.5469* - 35.21752*	0.18729 - 0.035066	0.000	37.9308 - 35.1021	39.1630 - 35.3329

Table 2C. Contd.

Media at 0.5 % antifungal fraction VS	Medium at 1% antifungal fraction	2.6346* - 3.0567*	0.18729 - 0.035066	0.000	2.0185 - 2.9413	- 89.4378 - 3.1721

*The mean difference is significant at the 0.05 level.Dependent variable: Percentage in reduction of Alamar blue; Tukey HSD.

Table 3A. Effect of incubation time on cell viability of *A. nidulans* grown in the medium at 0, 0.05, 0.1, 0.5 and 1% of antifungal fraction of *Lycopersicum esculentum* leaves extract.

Antifungal fraction content in the medium	N	Percentage in reduction of Alamar blue				
		Subset for alpha = 0.05				
		1	2	3	4	5
Medium at 0.05% of antifungal fraction for day5	3	69.0717304233				
Medium at 0.05% of antifungal fraction for day4	3	69.5753138133				
Medium at 0.05% of antifungal fraction for day3	3		71.5617302400			
Medium at 0.05% of antifungal fraction for day2	3			75.1718948033		
Medium at 0.05% of antifungal fraction for day1	3				77.8694301567	
Significance		0.720	1.000	1.000	1.000	
Medium at 0.1 % of antifungal fraction for day5	3	29.7291762700				
Medium at 0.1 % of antifungal fraction for day4	3	29.8681066267				
Medium at 0.1% of antifungal fraction for day3	3		30.8487519167			
Medium at 0.1% of antifungal fraction for day2	3			34.8483130467		
Medium at 0.1% of antifungal fraction for day1	3				39.4382560667	
Significance		0.949	1.000	1.000	1.000	
Medium at 0.5% of antifungal fraction for day5	3	3.5737370427				
Medium at 0.5% of antifungal fraction for day4	3		3.9670709717			
Medium at 0.5% of antifungal fraction for day3	3			4.7637355548		
Medium at 0.5% of antifungal fraction for day2	3				5.5975273043	
Medium at 0.5% of antifungal fraction for day1	3					6.2554838797
Significance		1.000	1.000	1.000	1.000	1.000
Medium at 1% of antifungal fraction for day5	3	2.4097170990				
Medium at 1% of antifungal fraction for day4	3	2.6163437327				
Medium at 1% of antifungal fraction for day3	3		3.0996235989			
Medium at 1% of antifungal fraction for day2	3			4.2515685323		
Medium at 1% of antifungal fraction for day1	3				5.7623609830	
Significance		0.324	1.000	1.000	1.000	

Means for groups in homogeneous subsets are displayed. Used harmonic mean sample size = 3.000. Homogeneous subsets; Tukey HSD.

Table 3B. Effect of incubation time on cell viability of *A. fumigatus* grown in the medium at 0, 0.05, 0.1, 0.5 and 1% of antifungal fraction of *Lycopersicum esculentum* leaves extract.

Antifungal fraction content in the medium	N	Percentage in reduction of Alamar blue			
		Subset for alpha = 0.05			
		1	2	3	4
Medium at 0.05% of antifungal fraction for day 5	3	73.2573294133			
Medium at 0.05% of antifungal fraction for day 4	3	73.6647853533			
Medium at 0.05% of antifungal fraction for day 3	3		74.7965475767		
Medium at 0.05% of antifungal fraction for day 2	3			75.6569177933	
Medium at 0.05% of antifungal fraction for day 1	3				77.4828032867
Significance		0.209	1.000	1.000	1.000
Medium at 0.1% of antifungal fraction for day 5	3	32.1381074000			
Medium at 0.1% of antifungal fraction for day 4	3	32.5552233567			
Medium at 0.1% of antifungal fraction for day 3	3		34.7644540500		
Medium at 0.1% of antifungal fraction for day 2	3			36.5230586300	
Medium at 0.1% of antifungal fraction for day 1	3				40.7162083600
Significance		.108	1.000	1.000	1.000
Medium at 0.5% of antifungal fraction for day 5	3	3.5761280427			
Medium at 0.5% of antifungal fraction for day 4	3	3.5927265687			
Medium at 0.5% of antifungal fraction for day 3	3		4.4613932053		
Medium at 0.5% of antifungal fraction for day 2	3			5.6274128230	
Medium at 0.5% of antifungal fraction for day 1	3				6.4916272387
Significance		1.000	1.000	1.000	1.000
Medium at 1% of antifungal fraction for day 5	3	2.6780559420			
Medium at 1% of antifungal fraction for day 4	3	2.7287387120			
Medium at 1% of antifungal fraction for day 3	3	3.2176108570			
Medium at 1% of antifungal fraction for day 2	3		4.5155355080		
Medium at 1% of antifungal fraction for day 1	3			6.1651937720	
Significance		.314	1.000	1.000	

Means for groups in homogeneous subsets are displayed. Used harmonic mean sample size = 3.000. Homogeneous subsets; Tukey HSD.

Table 3C. Effect of incubation time on cell viability of *A. flavus* grown in the medium at 0, 0.05, 0.1, 0.5 and 1% of antifungal fraction of *Lycopersicum esculentum* leaves extract.

Antifungal fraction content in the medium	N	Percentage in reduction of Alamar blue			
		Subset for alpha = 0.05			
		1	2	3	4
Medium at 0.05% of antifungal fraction for day5	3	77.0843321800			
Medium at 0.05% of antifungal fraction for day4	3	77.2493259700			
Medium at 0.05% of antifungal fraction for day3	3		79.5297225333		
Medium at 0.05% of antifungal fraction for day2	3			80.6908602333	
Medium at 0.05 % of antifungal fraction for day1	3				84.2198344067
Significance		0.914	1.000	1.000	1.000
Medium at 0.1% of antifungal fraction for day5	3	38.7350845967			
Medium at 0.1% of antifungal fraction for day4	3	38.7387530667			
Medium at 0.1% of antifungal fraction for day3	3	38.7436490667			
Medium at 0.1% of antifungal fraction for day2	3		42.5539374833		
Medium at 0.1% of antifungal fraction for day1	3			45.2770163700	
Significance		1.000	1.000	1.000	
Medium at 0.5% of antifungal fraction for day5	3	6.5779239933			
Medium at 0.5% of antifungal fraction for day4	3	6.7810436607			
Medium at 0.5% of antifungal fraction for day3	3		7.5743709903		

Table 3C. Contd.

Medium at 0.5% of antifungal fraction for day2	3			8.5759988667	
Medium at 0.5% of antifungal fraction for day1	3				9.3647047713
Significance		.557	1.000	1.000	1.000
Medium at 1% of antifungal fraction for day5	3	3.5212326953			
Medium at 1% of antifungal fraction for day4	3	3.7308440643			
Medium at 1% of antifungal fraction for day3	3		4.5108440430		
Medium at 1% of antifungal fraction for day2	3			5.5617323067	
Medium at 1% of antifungal fraction for day1	3				6.7300857280
Significance		0.760	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Used harmonic mean sample size = 3.000. Homogeneous subsets; Tukey HSD.

for the identification of the strains of *Aspergillus* used in this study.

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Full Length Research Paper

AmpC beta-lactamase production among *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates at the Komfo Anokye Teaching Hospital, Kumasi, Ghana

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Extended spectrum β-lactamases (ESBLs) are frequently reported in Ghana, but AmpC bacteria reports are scanty. This study determines the prevalence of AmpC *Pseudomonas aeruginosa* and *Proteus mirabilis* clinical isolates at the Komfo Anokye Teaching Hospital (KATH), Kumasi, where ESBL and AmpC are not routinely tested. Non-duplicate 245 isolates comprising 187 (76.3%) *P. aeruginosa* and 58 (23.7%) *P. mirabilis* were tested for AmpC and ESBL production using the modified three-dimensional test method and the double disc synergy test (DDST) methods, respectively. The proportion of the 245 isolates producing AmpC β-lactamase was 93 (38.0%) and 79 (32.2%) produced ESBL. AmpC producers confirmed 49 (52.7%) as inducible and 44 (47.3%) non-inducible AmpC producers. *P. aeruginosa* and *P. mirabilis* that produced AmpC were 84 (44.9%) and 9 (15.5%), respectively. ESBL production was 41 (21.9%) in *P. aeruginosa* and 38 (65.6%) in *P. mirabilis.* Co-producers of AmpC together with ESBL were 7.3%. Both enzyme were detectable in 13 (7.0%) of *P. aeruginosa* and 5 (8.6%) of *P. mirabilis*. AmpC and ESBL are detectable in high proportions among *P. aeruginosa* and *P. mirabilis* isolates at KATH and the Kumasi community. This emphasizes the need to start testing for both enzymes to guide therapeutics.

Key words: AmpC beta-lactamase, extended spectrum beta-lactamase, inducible AmpC beta-lactamase, non-inducible beta-lactamase.

INTRODUCTION

Extended spectrum β-lactamases and AmpC β-lactamases are enzymes encoded by the chromosomal and plasmid genes of many bacteria (Jacoby, 2009). These ESBL and AmpC enzymes are produced as metabolic by-products which have the ability to hydrolyze beta-lactam antibiotics often leading to multiple antibiotic resistances (Black et al., 2005). The enzymes mediate resistance to cephalosporins, penicillins including even β-lactamase inhibitor-β-lactam combinations (Jacoby, 2009). According to Bell et al. (2007), many organisms that produce ESBL sometimes also produce AmpC β-lactamase (Bell). Some bacteria co-produce ESBL and AmpC β-lactamases making the organisms non-suceptible to β-lactam antibiotics including β-lactam and beta-lactam-inhibitor combinations.

ESBL-producer prevalence at the Komfo Anokye Teaching Hospital was reported to be 44.37% for *Escherichia coli* and 55.67% for *Klebsiella pneumoniae* and 42.86% for *Klebsiella* spp. (Adu-Sarkodie, 2010). The only documented study on ESBLs was by Sarkodie (2010) when the ESBL phenotypes of *Klebsiella* and *E. coli* were studied. That study found out that 44% of *E. coli* and 55% of *Klebsiella* sp. (57.8% *K. pneumoniae*) produced ESBL. This ESBL prevalence was found to be high among these enterobacteria and as a result the antibiotic spectrum

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from which to choose to treat severe infections was reduced, creating difficulties with therapeutic options. AmpC β-lactamases are frequency reported worldwide (Coudron et al., 2000) and yet in Ghana, reports on AmpC β-lactamase producing bacteria are scanty. Very few studies have reported on the problem of ESBL in Africa (Ndugulile et al., 2005). There have been reports of ESBL on *K. pneumoniae* in Kenya (Kariuki et al., 2001) South Africa (Essack et al., 2001) and Tanzania (Blomberg et al., 2005). ESBLs have been reported in *Salmonella enterica* and *Salmonella enteritidis* from Mali (Weill et al., 2004) and *S. typhimurium* in Morocco (AitMhand et al., 2002) and in Nigeria (Adeniji and Okesola 2010; Romanus et al., 2009) but in Ghana information on ESBLs and AmpC β-lactamase phenotypes began emerging (Hackman et al., 2013), but information on *Pseudomonas aeruginosa* and *Proteus mirabilis* are still very rare.

Work has been done on most strains of the enterobacteriaceae (*Klebsiellae and Escherichia coli*) in Ghana but not *P. aeruginosa* and the *P. mirabilis*. Meanwhile the hospital laboratory has been recording multidrug resistant *P. aeruginosa* and *P. mirabilis* more recently than the previous years. This study therefore determines the prevalence of AmpC β-lactamase and ESBL production among *P. aeruginosa* and *P. mirabilis* clinical isolates *vis-a-vis* the high antimicrobial resistance being observed at the Komfo Anokye Teaching Hospital.

METHODOLOGY

A total of 5, 859 samples comprising 3,012 blood, 1,794 urine and 1,053 wound samples were taken and analyzed from both in-patients and out-patients from November 2011 to 31 January 2012. The blood cultures were performed using BACTEC 9240 (Becton, Dickinson and company, USA). Clean-catch mid-stream urine samples were cultured on cystein lactose electrolyte deficient (CLED) agar (Oxoid limited, Basingstoke, UK) using a calibrated 2 mm diameter bacteriological loop. Wound samples were collected with sterile cotton wool swabs and transported to the laboratory in Stuart Transport medium. After overnight incubation, a single colony was picked from the growths that occurred and sub-cultured onto nutrient agar slants in test-tubes and stored in the refrigerator. These stored isolates were later identified with biochemical tests and confirmed with API 20E (bioMérieux, Marcy l'Etoile, France) identification system. The biochemical tests employed for the identification of the isolates included oxidase test, citrate utilization test, urease test, indole test and Kligler iron agar test. Antimicrobial susceptibility test of the isolates was performed using the disc diffusion (Kirby-Bauer) test method as recommended by Clinical and Laboratory Standards Institute (CLSI, 2010). The antimicrobial discs tested against the isolates were obtained from Becton, Dickinson and Company, USA.

Screening for AmpC production

The *P. aeruginosa* and *P. mirabilis* isolates were tested for AmpC production using the cefoxitin disc (30 µg) (Becton, Dickinson and Company, USA) as recommended by Upadhyay et al. (2010). In this test, bacterial suspension of 0.5 McFarland density of the test isolate was prepared. Using a sterile cotton-tipped swab the bacterial suspension was inoculated onto the surface of the Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium). The cefoxitin antibiotic disc (Becton, Dickinson and Company, USA) was placed on the inoculated agar using a pair of sterile forceps, and incubated overnight at 37°C. Isolates that yielded zone diameters of less than 18 mm or frank resistance were considered positive for AmpC beta-lactamase production in the screening test (Upadhyay et al., 2010). A previously known AmpC producing *P. aeruginosa* isolate and *E. coli* ATCC 25922 were used as controls.

AmpC confirmatory test

The modified three-dimensional test was used to confirm the production of AmpC beta-lactamase. This test was useful in detecting both inducible and non-inducible AmpC beta-lactamases (Upadhyay et al., 2010). Overnight culture on Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium) was transferred to sterile micro-centrifuge tube containing peptone water and then pelleted by centrifugation at 3000 rpm for 15 min. The supernatant was decanted and fresh peptone water was added and the bacterial suspensions were repeatedly frozen and thawed about 10 times to obtain crude AmpC enzyme extract. Mueller-Hinton agar surface was inoculated with *E. coli* ATCC 25922 to produce a lawn culture and cefoxitin (30 µg) disc was placed at the centre of the plate. On the agar, three linear slits about three centimeters (3 cm) long were made three millimeters (3mm) away from the cefoxitin disc using a sterile surgical blade. A small circular well was made on each slits 5 mm from the cefoxitin disc by stabbing the agar with the tip of a sterile Pasteur pipette. One of the wells was filled with the enzyme extracted from the test organism. Each of the remaining wells was filled with extracts from the positive control (previously known *P. aeruginosa* isolate) and the third well filled with the negative control (*E. coli* ATCC 25922). The plates were kept for about 10 min for the surface moisture to dry. They were then incubated at 37°C overnight. After incubation, isolates that showed clear distortion of zone of inhibition around cefoxitin discs were confirmed as AmpC producers. The isolates that produced no zones of distortion were AmpC non-producers (Shoorashetty et al., 2011).

Testing for inducible AmpC

Isolates that were positive in the AmpC test were further tested to determine whether the enzyme was inducible or non-inducible type. The inducible AmpC beta-lactamase was tested for by the disc antagonism test method (Upadhyay et al., 2010). A sterile cotton-tipped swab was used to seed Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium) with the 0.5 McFarland bacterial suspension of the test organism. A disc of cefotaxime (30 µg) and cefoxitin (30 µg) (Becton, Dickinson and Company, USA) were placed 20 mm apart on the seeded Mueller-Hinton agar surface and incubated overnight at 37°C. Isolates that produced blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin discs were taken as inducible AmpC beta-lactamase producers (Upadhyay et al., 2010). A previously known AmpC producing *P. aeruginosa* isolate and *E. coli* ATCC 25922 were used as controls.

Test for ESBLs

The isolates were first screened for ESBL production and those positive in the screening test were later confirmed by the double synergy test. In the screening test, outright resistance of the isolate to third generation cephalosporins namely ceftazidime (30 µg) with < 22 mm zone size, cefotaxime (30 µg) with < 27 mm zone size and

Table 1. General characteristics of the study population stratified by the *P. aeruginosa* and *P. mirabilis* isolates.

Variable	Total (n= 245)	*Pseudomonas aeruginosa* (n=187)	*Proteus mirabilis* (n=58)	P value
Patient demographic data				
Age (years)	36	47	26	
Patient				
Outpatient	99 (40.4%)	71 (38.0%)	28 (48.3%)	0.16
Inpatient	146 (59.6%)	116 (62.0%)	30 (51.7%)	0.16
Sample type				
Urine	70 (28.6%)	43 (23.0%)	27 (46.6%)	< 0.01
Wound	144 (58.7%)	122 (65.2%)	22 (37.9%)	< 0.01
Blood	31 (12.7%)	22 (11.8%)	9 (15.5%)	0.45

Continuous data are presented as mean ± SD and categorical data presented as proportions. Continuous data are compared with each other using unpaired t-test while categorical data are compared with each other using Chi-square analysis. P = comparison between prevalence of *P. aeruginosa* and *P. mirabilis* isolates, P< 0.05 means significant.

ceftriaxone (30 µg) with zone size < 25 mm, as recommended by the CLSI (CLSI 2010) made an isolate an ESBL suspect.

Confirmatory test for ESBL production

ESBL detection was done using the double disc synergy test (DDST). In this test, the test organism (0.5 McFarland's turbidity) was inoculated onto Mueller-Hinton agar surface (Cypress Diagnostics, Langdorp, Belgium) by using a sterile cotton swab. A disc of co-amoxiclav (20 µg amoxycillin + 10 µg clavulanate) was placed at the centre of the inoculated Mueller-Hinton agar. A disc of cefotaxime (30 µg) and another of ceftriaxone (30 µg) were placed at both sides to the co-amoxiclav disc (Metri et al., 2011). The set-up was incubated at 37°C overnight. The enhancement of the zones of inhibition of any of the cephalosporin disc towards the clavulanic acid disc confirms an isolate as an ESBL producer (Metri et al., 2011). The positive and negative control strains used in this test were *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922, respectively.

Pearson X^2 test (P-value < 0.05) was used for data analysis to assess the level of significance of assumed hypothesis using STATA (STATA^{TM} 10, StataCorp., 4905 Lakeway Drive, College Station, Texas 77845 USA).

RESULTS

A total of 245 non-select and non-duplicate isolates were collected for the study. They comprise 187 *P. aeruginosa* and 58 *P. mirabilis*, recording 76.3 and 23.7%, respectively. The ages of patients from whom the isolates were obtained ranged from one day to 82 years with the median age of 36 years. The isolates were obtained from 131 (53.5%) males and 114 (46.5%) females in the study. *P. aeruginosa* was isolated from 95/187 (50.8%) and *P. mirabilis* isolates were obtained from 36/58 (62.1%) male patients. Female patients with *P. aeruginosa* infection were 92/187 (49.2%) and those with *P. mirabilis* infection were obtained from female patients. These were obtained from both in- and out-patients and also from the three sample types studied. Among the sample types there were *P. aeruginosa* isolates of 144 (58.7%), 43 (23.0%) and 31 (12.7) obtained from wound, urine and blood, respect-tively. There were 27/58 (46.6%) *P. mirabilis* obtained from urine, 122 (65.2%) obtained from wound and 22 (11.8%) were obtained from blood as shown in Table 1.

Out of the 245 isolates tested, 93 (38.0%) tested positive for AmpC β-lactamase production. Among the AmpC β-lactamase producers 49 (52.7%) were confirmed inducible AmpC β-lactamase producers while 44 (47.3%) were non-inducible AmpC producers. There were 84/187 (44.9%) *P. aeruginasa* isolates that produced AmpC β-lactamase out of which 49/187 (26.2%) were inducible AmpC β-lactamase producers. There were 9 (15.5%) *P. mirabilis* isolates that produced AmpC β-lactamase, but the enzyme they produced were non-inducible AmpC β-lactamase. Overall results of ESBL prevalence was 79 (32.2%) for both *P. aeruginosa* and *P. mirabilis*. Of the *P. aeruginosa* isolates, 41 (21.9%) were ESBL producers and the 38 (65.5%) *P. mirabilis* isolates were ESBL producers (Table 2). The proportion of *P. aeruginosa* isolates producing only AmpC β-lactamse was 71/187 (38.0%) and 28/187 (15.0%) produced only ESBL while 13 (7.0%) co-produced both AmpC β-lactamse and ESBL. Proportion of the *P. mirabilis* isolates that produced AmpC β-lactamase only was 4 (6.9%), and 33 (56.9%) produced only ESBL, whilst 5 (8.6%) co-produced AmpC β-lactamse and ESBL.

The *P. aeruginosa* isolates had no resistant strains against meropenem but susceptibility levels to aminoglycosides (amikacin and gentamicin) were 94.7 and 79.7%, respectively and to ciprofloxacin was 90.4%. Susceptibility levels to other antibiotics recorded 92.0% to ceftazidme and 60.4% to cefotaxime. The isolates had low susceptibility levels (45.5%) to ceftriaxone and 24.1%

Table 2. Proportion of isolates stratified by susceptibility to antibiotics, ESBL, AmpC and inducible AmpC production.

Variable	Total (n = 245)	*Pseudomonas aeruginosa* (n =187)	*Proteus mirabilis* (n = 58)	P value
Beta-lactam antibiotic susceptibility				
Meropenem	245 (100%)	187 (100%)	58 (100%)	0.379
Cefotaxime	146 (59.6%)	113 (60.4%)	33 (56.9%)	0.632
Ceftazidime	216 (88.2%)	172 (92.0%)	44 (75.9%)	**< 0.001**
Ceftriaxone	108 (44.1%)	85 (45.5%)	23 (39.7%)	0.437
Cefuroxime	69 (28.2%)	45 (24.1%)	24 (41.4%)	**0.0104**
Ampicillin	0 (0.0%)	N/A	0 (0.0%)	N/A
Non-beta-lactam antibiotic susceptibility				
Ciprofloxacin	204 (83.3%)	169 (90.4%)	35 (60.23%)	**< 0.001**
Amikacin	228 (93.1%)	177 (94.7%)	51 (87.9%)	0.078
Gentamicin	193 (78.8%)	149 (79.7%)	44 (75.9%)	0.580
Co-trimoxazole	1 (0.4%)	N/A	1 (1.7%)	N/A
Chloramphenicol	0 (0.0%)	N/A	0 (0.0%)	N/A
ESBL	79 (32.2%)	41 (21.9%)	38 (65.5%)	**< 0.001**
AmpC	93 (38.0%)	84 (44.9%)	9 (15.5%)	**< 0.001**
Inducible AmpC	49 (20.0%)	49 (26.2%)	0 (0.0%)	N/A
Non-inducible AmpC	44 (18.0%)	35 (18.7%)	9 (15.5%)	0.580
ESBL only	61 (24.9%)	28 (15.0%)	33 (56.9%)	**< 0.001**
AmpC only	75 (30.6%)	71 (38.0%)	4 (6.9)	**< 0.001**
Both ESBL and AmpC	18 (7.3%)	13 (7.0%)	5 (8.6%)	0.670

Data are presented as proportions. ESBL = Extended spectrum beta-lactamase, AmpC = AmpC beta-lactamase, N/A = Not-applicable, P = Comparison between *P. aeruginosa* and *P. mirabilis* isolates. P< 0.05 means significant (bolded).

to cefuroxime. The *P. mirabilis* isolates had no resistant strains to meropenem but had susceptibility proportion of 87.9% to amikacin, 75.9% to gentamicin and 60.2% to ciprofloxacin and other antimicrobials registering lower susceptibility values as shown in Table 2.

AmpC producing *P. aeruginosa* and *P. mirabilis* were obtained from 93 patients with the mean age of 35.41 years (SD = 23.89) and males yielding 51 (54.8%) females 42 (45.2%). Among the AmpC producing isolates, 49 (52.7%) were inducible AmpC and 44 (47.3%) were non-inducible AmpC producers. There were 61 (24.9%) isolates that produced only ESBLs while 18 (7.3%) of the isolates co-produced AmpC and ESBLs. There were 58 *P. mirabilis* isolates, of which 15.52% produced AmpC and 56.89% produced ESBL. There were 187 *P. aeruginosa* isolates involved in the study and 84 (44.92%) of them tested positive for AmpC. Among the 84 AmpC producing *P. aeruginosa* isolates 49 (58.33%) of them were positive for inducible AmpC. There were no *P. mirabilis* isolates that yielded inducible AmpC enzymes.

By sample type, most of the AmpC producers 66 (71.0%) were found in wound followed by 19 (20.4%) obtained from urine and then 8 (8.6%) obtained from blood. Most of the non-AmpC-producing isolates were obtained from wound 78 (51.3%), followed by urine 51 (33.6%) and 23 (15.1%) were obtained from blood. The details of these results are presented in Table 3.

Antimicrobial susceptibility of the isolates in relation to β-lactamase production

The AmpC producing isolates had no resistant strains to meropenem. Susceptibility to amikacin was 92.5 and 77.4% to gentamicin. Similar results were obtained for the non-AmpC producing isolates where there were no resistant strains against meropenem but susceptibility to amikacin was 92.8%. Proportions of isolates susceptible to other antibiotics, though high, were varied recording 86.0% for ciprofloxacin. Susceptible proportions of the isolates to cephalosporins were generally low with only 32.3% being susceptible to cefotaxime and 10.8% to ceftriaxone but were high to ceftazidme (83.9%). All the AmpC producing isolates were resistant to cefuroxime, ampicillin, co-trimoxazole and chloramphenicol.

There were however high proportions, about 90.8% being susceptible to ceftazidime, 81.6% to ciprofloxacin, gentamicin 79.6%, cefotaxime 76.3%, ceftriaxone 64.5% and cefuroxime 45.4%. The non-AmpC producing isolates had very low proportions susceptible to co-trimoxazole, cefuroxime, ampicillin and chloramphenicol.

Table 3. Patient socio-demographic characteristic, sample type and antibiotic susceptibility level of the isolates stratified by AmpC and ESBL phenotypes.

Variable	AmpC (n= 93)	Non-AmpC (n= 152)	Inducible AmpC (n= 49)	Non-inducible AmpC (n= 44)	ESBL Only (n= 61)	ESBL and AmpC (n= 18)	P value[a]	P value[b]	P value[c]
Socio-demographic data									
Age (years)	35.41 ± 23.89	32.9 6 ± 2 3.50	16.48 ±10.84	56.50 ± 15.03	33.89 ± 22.36	43.83 ± 22.69	0.432	**< 0.001**	**< 0.001**
Male	51 (54.8%)	83 (54.6%)	21 (42.9%)	30 (68.2%)	37 (60.7%)	9 (50.0%)	0.972	**0.014**	0.421
Female	42 (45.2%)	69 (45.4%)	28 (57.1%)	14 (31.8%)	24 (39.3%)	9 (50.0%)	0.972	**0.014**	0.421
Patient									
Outpatient	31 (33.3%)	58 (38.2%)	14 (28.6%)	17 (38.6%)	18 (29.5%)	6 (33.3%)	0.446	0.304	0.757
Inpatient	62 (66.7%)	94 (61.8%)	35 (71.4%)	27 (61.4%)	43 (70.5%)	12 (66,.7%)	0.446	0.304	0.757
Sample type									
Urine	19 (20.4%)	51 (33.6%)	11 (22.4%)	8 (18.2%)	23 (37.7%)	7 (38.9%)	**0.027**	0.610	0.928
Wound	66 (71.0%)	78 (51.3%)	38 (77.6%)	28 (63.6%)	27 (44.3%)	9 (50.0%)	**0.002**	0.140	0.668
Blood	8 (8.6%)	23 (15.1%)	0 (0.0%)	8 (18.2%)	11 (18.0%)	2 (11.1%)	0.136	N/A	0.487
Beta-lactam antibiotic susceptibility									
Meropenem	93(100%)	152 (100%)	49 (100%)	44 (100%)	61 (100%)	18 (100%)	0.725	0.939	0.353
Cefotaxime	30 (32.3%)	116 (76.3%)	12 (24.5%)	18 (40.9%)	41 (67.2%)	6 (33.3%)	**< 0.001**	0.091	**0.010**
Ceftazidime	78 (83.9%)	138 (90.8%)	38 (77.6%)	39 (88.6%)	50 (82.0%)	14 (77.8%)	0.104	0.157	0.691
Ceftriaxone	10 (10.8%)	98 (64.5%)	1 (2.0%)	9 (20.5%)	32 (52.5%)	3 (16.7%)	**< 0.001**	**0.004**	0.070
Cefuroxime	0 (0.0%)	69 (45.4%)	0 (0.0%)	0 (0.0%)	26 (42.6%)	0 (0.0%)	N/A	N/A	N/A
Ampicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A
Non-beta-lactam antibiotic susceptibility									
Ciprofloxacin	80 (86.0%)	124 (81.6%)	41 (83.7%)	38 (86.4%)	41 (67.2%)	13 (72.2%)	0.366	0.717	0.688
Amikacin	86 (92.5%)	141 (92.8%)	45 (91.8%)	41 (93.2%)	56 (91.8%)	16 (88.9%)	0.933	0.806	0.702
Gentamicin	72 (77.4%)	121 (79.6%)	39 (79.6%)	33 (75.0%)	45 (73.8%)	10 (55.6%)	0.802	0.597	0.140
Co-trimoxazole	0 (0.0%)	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A
Chloramphenicol	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A

Continuous data are presented as mean ± SD and categorical data presented as proportions. Continuous data are compared to each other using unpaired t-test while categorical data are compared with each other using Chi-square analysis. AmpC = AmpC beta-lactamase, ESBL = extended spectrum beta-lactamase, N/A = non-applicable. P value[a] = AmpC vrs Non- AmpC, P value[b] = Inducible AmpC vrs non- inducible AmpC, P value[c] = ESBL only vs. ESBL and AmpC. P = Comparison between two phenotypes. P< 0.05; means significant, P> 0.05; means not significant.

The proportions of non-AmpC producing isolates that were susceptible to cefotaxime was significantly higher than the AmpC producing isolates that were susceptible to cefotaxime ($p < 0.001$). There was also a significantly higher proportion of ceftria-xone- susceptible strains among non-AmpC producing isolates than AmpC producing isolates ($p < 0.001$). The detailed results are shown in Table 3.

Antimicrobial susceptibility patterns of AmpC- and non-AmpC- producing isolates

The inducible AmpC-producing isolates had no resistant strains to meropenem but proportions susceptible to amikacin, ciprofloxacin, gentamicin and ceftazidime were 91.8, 83.7, 79.6 and 77.6%, respectively. The isolates had very low proportions (24.5 and 2.0%) susceptible to cefotaxime and ceftriaxone. All the isolates were resistant to cefuroxime, ampicillin, co-trimoxazole and chloramphenicol, where no susceptible strains were recorded as shown in Table 3.

The isolates that produced only ESBL had no resistant strains to meropenem but their susceptibility levels varied recording 91.8, 82.0 and 73.8% to amikacin, ceftazidime and gentamicin, respectively. Their susceptibility levels to ciprofloxacin, ceftriaxone and cefotaxime were 67.2, 52.5 and 42.6%, respectively, whilst only 27.3% were susceptible to cefuroxime.

DISCUSSION

This was a prospective study involving the AmpC β-lactamase production among *P. aeruginosa* and *P. mirabilis* clinical isolates at the Komfo Anokye Teaching Hospital, Kumasi, Ghana. There was a high prevalence of AmpC production among the isolates (38.0%) as compared to other studies in which AmpC prevalence of about 20.7% was recorded in Delhi, India (Manchanda and Singh, 2003). Another study undertaken among hospitalized patients in Kolkata, India reported 17.3% (Arora and Bal, 2005) but among Gram negative isolates obtained from ICU patients in Bombay again in India showed only 5.4% prevalence (Oberoi et al., 2012). These differences in prevalence may be due to differences in the geographical distribution as a result of degree of antibiotic usage in the different localities (Canton et al., 2008). AmpC β-lactamase-producing isolates were obtained from male and female patients (Rand et al., 2011) of all ages, but there were no significant differences between gender and age of affected patients with AmpC enzymes.

AmpC beta-lactamase phenotypes were isolated from all the sample types tested in this study (urine, wound and blood), just as was reported that urine, blood, wounds, sputum, and stool were reported as sources of positive cultures for AmpC phenotypes (Jacoby, 2009). Wound was the commonest source of the isolates obtained in the study producing majority (65.2%) of the *P. aeruginosa* and 37.9% of the *P. mirabilis* isolates whilst blood sample produced the least proportion (12.7%) of the isolates.

The differences in prevalence may also be due to the different methods of selection of isolates, the variation in an ability to produce AmpC beta-lactamases among different Gram-negative bacteria (Arora and Bal, 2005). The high prevalence of AmpC producing isolates may be indicative of the threatening trends of more isolates acquiring resistance and mechanisms to render antibiotic therapy ineffective (Bell et al., 2007).

AmpC phenotypes were significantly higher among the *P. aeruginosa* isolates than the *P. mirabilis* isolates. *Pseudomonas aeruginosa* produces both inducible chromosomal AmpC and non-inducible plasmid mediated AmpC (Lister et al., 2009) whilst *P. mirabilis* produces only non-inducible plasmid mediated AmpC (Parveen et al., 2012). The *P. aeruginosa* isolates obtained in the study produced both inducible and non-inducible AmpC whereas the *P. mirabilis* isolates produced only inducible AmpC (Upadhyay et al., 2010), the very reason for which treatment of *P. aeruginosa* infections are more challenging than *P. mirabilis* infections (Lister et al., 2009).

In this study, AmpC phenotypes were obtained from both outpatients and inpatients but with the majority (66.7%) of the AmpC phenotypes isolated from inpatients, an indication that the enzymes have spread from the hospitals where antimicrobials are heavily used in the Kumasi community, as observed in England by the Infection Control Committee of Royal Devon and Exeter in England (2010). A similar study reported that AmpC beta-lactamases have been isolated from patients after several days of hospitalization (Jacoby, 2009), confirming the association of antibiotics intake of antibiotics. Co-production of AmpC and ESBL was detected in 7.3% of the isolates. This finding is comparable to the 8.0 and 6.59% reported in separate studies India by Sinha et al. (2008) and Oberoi et al. (2012), respectively. The existence of AmpC and ESBL co-production isolate especially *P. aeruginosa* is of serious concern because the organisms already has intrinsic resistance to wide range of clinically important antimicrobials (Lister et al., 2009) and acquisition of ESBLs make the organisms become resistance not only to β-lactam antibiotics but also to other non-β-lactam drugs such as the quinolones and the aminoglycosides. These drugs are also the drugs of choice for the treatment of infections caused by the *P. aeruginosa* and *P. mirabilis* (Hackman et al., 2013). Besides, co-production of both AmpC and ESBL by an organism makes detection of ESBL difficult by laboratory methods. So such strains often give false negative tests results in the detection of ESBLs (Sinha et al., 2008). The resistance proportions of ESBL-producing *P. mirabilis* strains to cefotaxime and ceftazidime were 43.1 and 24.1%, respectively, values considerably lower than the 56.9% ESBL *P. mirabilis* isolates recorded. This observation may be as a result of differences in β-lactamase expression or outer membrane permeability. For example, decreased expression of outer membrane porins that are channels for β-lactam entry can considerably add to or increase β-lactamase action to cause more resistance. In addition, *P. mirabilis* lacks intrinsic chromosomal β-lactamase genes, so, are dependent upon acquisition of different β-lactamase mechanisms to express a β-lacta-

mase- mediated resistance phenotype (Livermore, 1995). It has been reported in a study that about 85 to 97% of ESBL-producing *P. mirabilis* strains appeared to be susceptible to ceftazi-dime, cefepime and aztreonam (Wang et al., 2011). An ESBL producing *P. mirabilis* strain may be susceptible to higher derivatives of a β-lactam antibiotic because its production may have been induced by lower one, eg ESBL induced by cefuroxime would tend to be resistant to ceftriaxone and/or cefotaxime (Jacoby, 2009). It is gratifying to observe that all the isolates, including AmpC and ESBL producing isolates were susceptible to meropenem which recorded no resistant strains against it. This observation supports the report that carbapenems are usually the drugs of choice against AmpC- and ESBL-producing bacteria (Lister et al., 2009). The AmpC producing isolates also had high proportions susceptible to aminoglycosides (amikacin 92.5% and gentamicin 77.4%), quinolone (ciprofloxacin, 86.0%) cephalosporin (ceftazidime, 83.9%), but care must be taken not to abuse these antimicrobials. The isolates recorded low susceptible proportions to some of the cephalosporins where cefotaxime had 32.3%, ceftriaxone had 10.8% and none of the isolates at all tested sensitive to cefuroxime a situation attributed to inducible AmpC. Introduction of cephalosporins antibiotic into a hospital triggers production of inducible AmpC in bacteria causing them to hyperproduce the inducible AmpC enzyme in that hospital (Thomson, 2001). β-Lactam antibiotic exposure confers resistance to the β-lactam antibiotics (Cruz et al., 2013).

It has been found that the organisms tested have the ability to co-produce ESBL and AmpC. Also, the drugs intended to treat infections caused by these *P. aeruginosa* induce the production of inducible AmpC. These mechanisms reduces the very narrow already treatment options available for treating the AmpC-producing bacteria (Rodriguez-Martinez et al., 2003). The approach now is to adopt good diagnostic laboratory procedures and institute good prevention strategies as a matter of priority in controlling the development and spread of these strains.

Conclusion

This study has demonstrated that there is high prevalence of AmpC- and ESBL- producing *P. aeruginosa* and *P. mirabilis* strains circulating in the Komfo Anokye Teaching Hospital and in the community with higher antimicrobial resistance than the non AmpC and ESBL strains. Proportion of isolates that co-produced both enzymes was more resistant than those that possess either ESBL or AmpC only.

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Investigation of the antimicrobial and hemolytic activity of venom of some Egyptian scorpion

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***Leuirus quinquestriatus, Androctonus amoreuxi* and *Androctonus australis* are venomous scorpion in Egypt. Their venom is complex mixture of salts, neurotoxins, peptides and proteins which has therapeutic applications, and can rapidly kill a broad range of microbes. Estimations of total proteins of the venom indicated that *L. quinquestriatus* had the highest value than the others (10.64 ± 0.04). Hemolytic activity of human erythrocytes was detected and showed that all concentrations (20, 8 and 5 mg/ml) of tested scorpion species crude venom have hemolytic activity against human erythrocytes. The present study was conducted to evaluate the antimicrobial activity of the three Egyptian scorpion's venom against four Gram-positive and negative bacterial strains (*Bacillus cereus*, *Bacillus subtillis, Citrobacter freundi* and *Klibsella pneumonia)* in addition to one fungus species *Candida albicans.* The results show that *L. quinquestriatus* venom has a significant antibacterial effect against *B. subtillis* and *C. freundi*. In contrast, *A. amoreuxi* and *A. australis* venoms do not have a noticeable effect on tested microbes. So, the aim of the present study was to investigate the antimicrobial activity of crude venom against Gram-negative, as well as Gram-positive bacteria, fungi and to evaluate the hemolytic activity of the three investigated species on human erythrocytes.**

Key words: Crude venom, *Leuirus quinquestriatus, Androctonus amoreuxi, Androctonus australis,* hemolytic activity, antibacterial activity.

INTRODUCTION

Scorpions are venomous arthropod animals belonging to the class Arachnida (Gouge et al., 2001). All scorpions are venomous but approximately 50 of them have enough poison to kill a person (Osnaya-Romero et al., 2001; Isbister et al., 2003). Scorpions use venoms for immobilization of prey and protection against predators, and these venoms consist of a complex of several toxins that exhibit a wide range of biological properties and actions (Petricevich, 2010).

Natural products are an important source of medicinal compounds. A wide variety of organisms produce such bioactive compounds and some of these natural substances have been shown to be able to kill bacteria (Shittu et al., 2007). Scorpion venoms contain a great variety of biologically active low molecular weight proteins responsible for various pathological effects as, neurotoxins, enzyme inhibitors, hemolytic toxins (Possani et al., 1999, 2002). In recent years, venoms and venom components from different venomous animals have shown potential antimicrobial activity, this includes snake (Perumal Samy et al., 2007; Shittu et al., 2007; Al-Ahmadi et al., 2010), scorpion (Conde et al., 2000, Torres-Larios et al., 2002) and spider venom (Kozlov et al., 2006; Kuhn-Nentwig et al., 2002a, b). Many studies detected the toxicity of the three scorpion species used in the present study. *Leuirus quinquestriatus* is a venomous species which are incremented in most stings in Saudi Arabia (Antoplosky et al., 2009). While, *Androctonus australis* is one of the

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most dangerous scorpions in the world, with very potent venom. This species are medically important, and cause several deaths each year (Gaban, 1997). *Androctonus amoreuxi* venom caused teratogenic effect on rats (Ismail et al., 1983).

Hemolysis due to envenomation by scorpion is so common that when a patient is suspected of been stung by a scorpion, the first clinical test is to check the presence of hemoglobin in the patient's urine (Radmanesh, 1990). First reports on the hemolytic activity of scorpion venom have been available since 1918 (Balozet, 1971). Starting in 1996, possible pore-forming peptides from the venom of *Scorpio maurus palmatus* were assumed to be responsible for the induction of leak currents in *Xenopus laevis* oocytes (Debont et al., 1996) and cardiac cells of the rat (De Plessis et al., 1999). Scorpion sting cause severe hemolysis which fluctuated according to scorpion species and venom concentration. So, the present study was designed to characterize the antimicrobial activity against one fungus species, Gram positive and negative bacteria, as well as hemolytic activity of three Egyptian scorpion crude venom: *L. quaniquestriatus*, *A. australis* and *A. amoreuxi.*

MATERIALS AND METHODS

Sites of collection

Scorpions were collected by professional hunters during the period of April to October 2012. Three species of scorpion, *L. quaniquestriatus*, *A. australis* and *A. amoreuxi* were collected from Aswan, Borg El Arab and Baltim in Egypt. Then determination of scorpion age and sex as female adult scorpion was according to pectin, body length and color. Their length range between 9-9.5, 9.5-10, and 8.5-10, respectively, and they were kept them in glass containers according to the geographical areas. Then, the scorpions were anesthetized to collect the venom samples immediately at collection to avoid stress.

Venom samples

Twenty telsons from each collected species of scorpions were used. Venom solution was prepared using the maceration method in which telsons were removed from anesthetized scorpions at the point of their articulation with the last caudal segment (Ozkan and Filazi, 2004; Ozkan et al., 2006). All telsons were weighed, ground to a fine powder and dissolved in physiologic saline solution (PSS; 0.9% w/v NaCl) and kept at 4°C for 72 h. The venom solution was centrifuged at 10000 g for 10 min at 4°C. Supernatant was removed and immediately lyophilized and stored at -20°C until use.

Protein assay

Protein concentrations were determined by using a commercial kit (Biomed Diagnistics, 30175 Hannover, Germany) using bovine serum albumin as a standard.

Hemolytic assay

Hemolytic activity of the crude venom was assayed with heparinized human red blood cells rinsed three times in 5 mL phosphate buffered saline (PBS - 50 mM $_{NaH2PO4}$ and 150 mM NaCl, pH 7.2) and centrifuged for 5 min at 3,000 rpm. Red blood cells were then incubated at room temperature for 1 h in deionized water as positive control (100% hemolysis), in PBS as negative control or blank (0% hemolysis), or with venoms at three concentrations (5, 8 and 20 mg/mL) in PBS. Three replicas of the samples were centrifuged at 12,000 rpm for 5 min. The supernatant was separated from the pellet, and its absorbance was measured at 570 nm and its standard deviation (SD) was calculated. The percentage of hemolysis was calculated using the following equation:

Absorbance of sample **/** Absorbance of control (100% hemolysis) x 100

Gram-positive and negative bacterial strains

Different Gram-positive and negative bacteria in addition to a fungus were used as indicator organisms in this study. *Citrobacter freundi*, *Klibsella pneumoniae, Bacillus cereus* and *Bacillus subtillis*, as well as *Candida albicans* were used. These microorganisms were kindly provided by Bacteriology Unit, Botany Department, Faculty of Science, Tanta University. The indicator organisms were grown on nutrient agar plates at 37°C for 24 h.

Preparation of venom dilution for antimicrobial activity

Two concentrations were used from each scorpion's crude venom (20 and 10 mg/ml). The extracted venom from each scorpion species were diluted with PBS.

Evaluation of antimicrobial activity

Overnight culture of microorganisms was prepared and 5×10^5 cfu ml^{-1} in 100 µl Potassium-Sodium-Phosphate buffer, (pH 7) were spread onto nutrient agar plates. 50 µl of each prepared concentration of the crude venom was loaded into well made in the center of inoculated plates. The plates were incubated at suitable temperature (37°C) overnight. The antimicrobial activity was determined by the agar well diffusion method (Galvez et al., 1986). The largest inhibition zone of the indicator bacterium indicates the most antimicrobial-producing test organisms. Nutrient broth or agar (Lapage et al., 1970): Peptone, 5 g; Beaf extract, 3 g; sodium chloride, 5 g; distilled water up to 1000 ml and pH was adjusted to 7.

Statistical analysis

Data were statistically processed three times for scorpion species, and standard variations (SD) were estimated, also coefficients were calculated between species using Pearson correlation (r2).

RESULTS

Total protein contents

Total protein contents is shown in Table 1 and Figure 1 show that the highest value of protein concentration was in *L. quinquestriatus* (10.64 ± 0.04 mg/ml), then *A. amoreuxi* which was 9.18± 0.13 mg/ml, while the lowest value was in *A. australis* (8.6 ± 0.053).

Table 1. Total protein contents of the three scorpion species.

Specimen	Total protein content (mg/ml)
Leiurus quinquestriatus	10.64 ± 0.04
Androctonus amoreuxi	9.18 ± 0.13
Androctonus australis	8.6 ± 0.053

Hemolytic activity

Different concentrations of scorpion venom disrupted human erythrocytes. Hemolytic activity of scorpion venom is dose dependent for each species as shown in Table 2 and Figure 2. Hemolytic activity of *L. quinquestriatus* (Lq) show high value range between 98.18 and 28.03%. *A. amoreuxi* (Ax) concentrations show high values close to each other ranging between 95.4 and 89.5 %, while the lowest values obtained for the concentrations of *A. australis* venom (As) ranged between 65.91 and 10.03%, which showed the highest coefficient between different concentrations ($r^2 = 0.98$).

Antimicrobial effect of scorpion venom

Antimicrobial effect of venom of three species of scorpion using two concentrations (20 and 10 mg/ml) were tested on Gram-positive bacteria (*B. cereus* and *B. subtillis*), Gram-negative bacteria (*C. freundi* and *K. pneumoniae*) and one species of fungus (*C. albicans*) as shown in Figures 3 to 7. All venom concentrations of both *A. amoreuxi* and *A. australis* have no effect on all species of bacteria and fungus. While, *L. quinqestriatus* venom inhibited two bacterial strains; *B. subtillis* and *C. freundi*.

Inhibitory zone of different venom concentrations of *L. quinquestriatus* is shown in Table 3 and Figures 3 and 4, which illustrated that, the higher the concentration, the higher the inhibitory zone. The most effective results were observed on *B. subtilis*, it showed inhibitory zone of 19.66 ± 0.95 and 17 ± 0.127 with both concentrations of *L. quinquestriatus* venom. While, it showed inhibitory zone of 17.66 ± 0.255 and 15.33 ± 0.337 on *C. freundi.*

DISCUSSION

Scorpions are soil animals exposed to microorganisms such as bacteria and fungi that inhabit the soil. Thus, it is anticipated to produce some antibacterial substance for predation and self -protection. As described in the literature, venom consists of many different substances like proteins and enzymes, which are responsible for its biological activities (Petricevich, 2010). So, the present study measured total protein of scorpion crude venom. First reports on the hemolytic activity of scorpion venom have been available since 1918. Starting from 1996, possible pore forming peptides from the venom of *Scorpio maurus palmatus* and *Opistophthalmus carinatus* (both Scorpionidae) were assumed to be responsible for the induction of leak currents in *Xenopus laevis* oocytes and cardiac cells of the rat (Du Plessis et al., 1999).

Hemolysis can be induced by several protein toxins from animals, plants and microbes, particularly marine animals (Parker and Feil, 2005). Some of these venoms affect biological membranes by inducing the formation of pores or channels in natural and model bilayer lipid membranes (García-Sáez et al., 2011; Savva et al., 2013). Thus, hemolytic activity induced by protein toxins has been used as a sensitive toxicological tool to investigate the targeting and attachment of proteins to cell membranes (Sabirov et al., 1993).

In the present study, three concentrations of different venom of investigated samples were used to estimate percentage of hemolysis of human erythrocytes. The study found that the percentage of erythrocyte hemolysis was concentration dependent. The highest percentage of hemolysis was recorded with high venom concentration (20 mg/ml) which reached 98.18% in *L. quinquestriatus*. While, hemolysis percentage of 8 mg/ml of *A. amoreuxi* venom (94.45%) is more than that represented by the same concentration of *L. quinquestriatus* venom (78.7%). Thus, we assumed that the hydrophilic pores in erythrocyte cell membrane induced by scorpion venom caused colloid osmotic burst that resulted in erythrocyte lysis. In addition to pore-forming mechanisms, lipid peroxidation of erythrocyte membranes plays an important role in the hemolysis induced by hemolytic protein toxins, resulting in cell membrane disorder (Parker and Feil, 2005; García-Sáez et al., 2011). Valavi and Alemzadeh-Ansari (2008), reported that *Hemiscorpius lepturus* scorpion has hemolytic activities. Also, crude venom of wolf spider *Lycosa singoriensis* has hemolytic effect on human erythrocytes in a dose-dependent manner (Liu et al., 2009). On the other hand, some toxins in both scorpion venom as hadrurin and in centipedes as scolopendrin I showed hemolysis activity at different concentrations, and it reached 75% hemolysis under the effect of hadrurin from scorpion (Wenhua et al., 2006).

The present study shows also the antimicrobial effect of scorpion species venom, because scorpion species often use to spray venom on their own bodies to disinfect them from possible saprophytic organisms including bacteria and fungi, showing that venom of these scorpions could contain some sort of antibiotic potential (Torres-Larios et al., 2002). Scorpion venom contains peptides which exhibit anti-microbial properties (Gordon et al., 2005; Brown and Hancock, 2006). In this study, the sensitivity of five microbial strains was tested against the crude venom of three scorpion species. Four Gram-negative and positive bacteria and fungal strains were studied.

From the three scorpion species, only *Leiurus quinquestriatus* crude venom with its two concentrations were effective only against *Bacillus subtillis* and *Citrobacter freundi* with inhibition zone ranging between

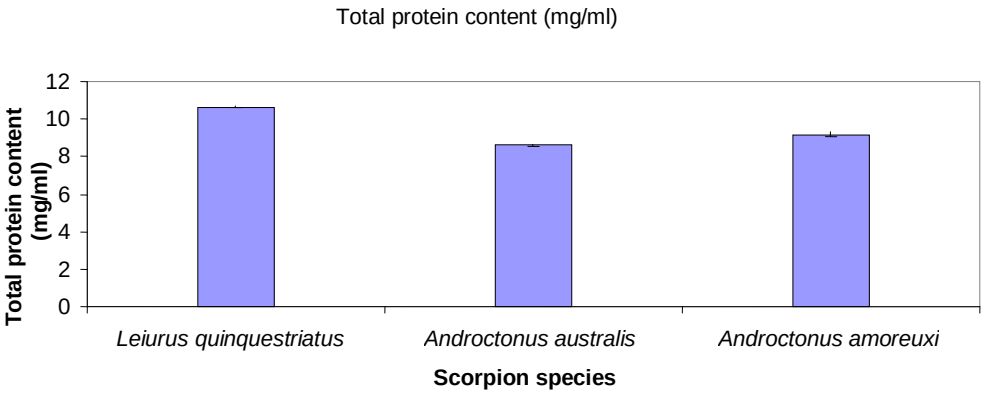

Figure 1. Total protein contents (mg/ml) of scorpion species.

Table 2. Absorbance (560 nm) and percentage of hemolysis (%) due to different concentrations of scorpion venom.

Specimen/concentration	Absorbance at 560 nm	Hemolysis (%)
***Leiurus quinquestriatus* (Lq)**		
20 mg/ml (Lq 1)	1.996 ± 0.05	98.18
8 mg/ml (Lq 2)	1.6 ± 0.028	78.7
5 mg/ml (Lq 3)	0.57 ± 0.022	28.03
r2	0.695	
***Androctonus amoreuxi* (Ax)**		
20 mg/ml (Ax 1)	1.94 ± 0.050	95.4
8 mg/ml (Ax 2)	1.9 ± 0.066	93.45
5 mg/ml (Ax 3)	1.82 ± 0.041	89.5
r2	0.747	
***Androctonus australis* (As)**		
20 mg/ml (As 1)	1.34 ± 0.0317	65.91
8 mg/ml (As 2)	0.314 ± 0.094	15.44
5 mg/ml (As 3)	0.204 ± 0.148	10.03
r2	0.989	

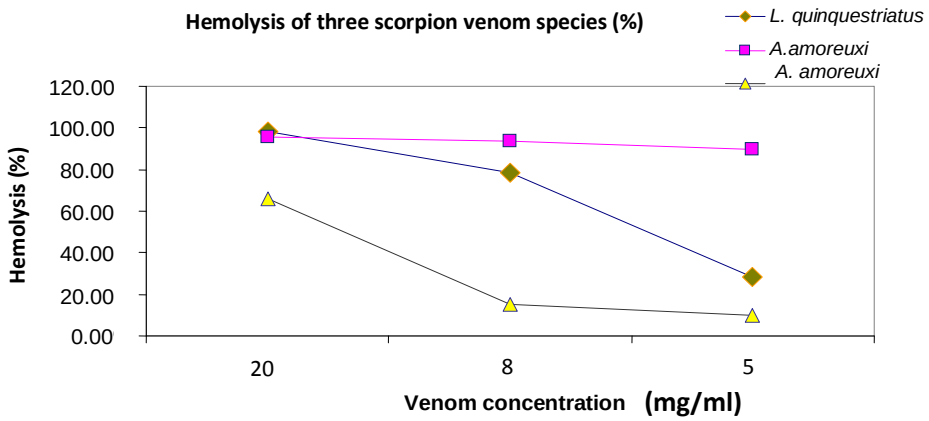

Figure 2. Percentage (%) of hemolysis of three scorpion venom.

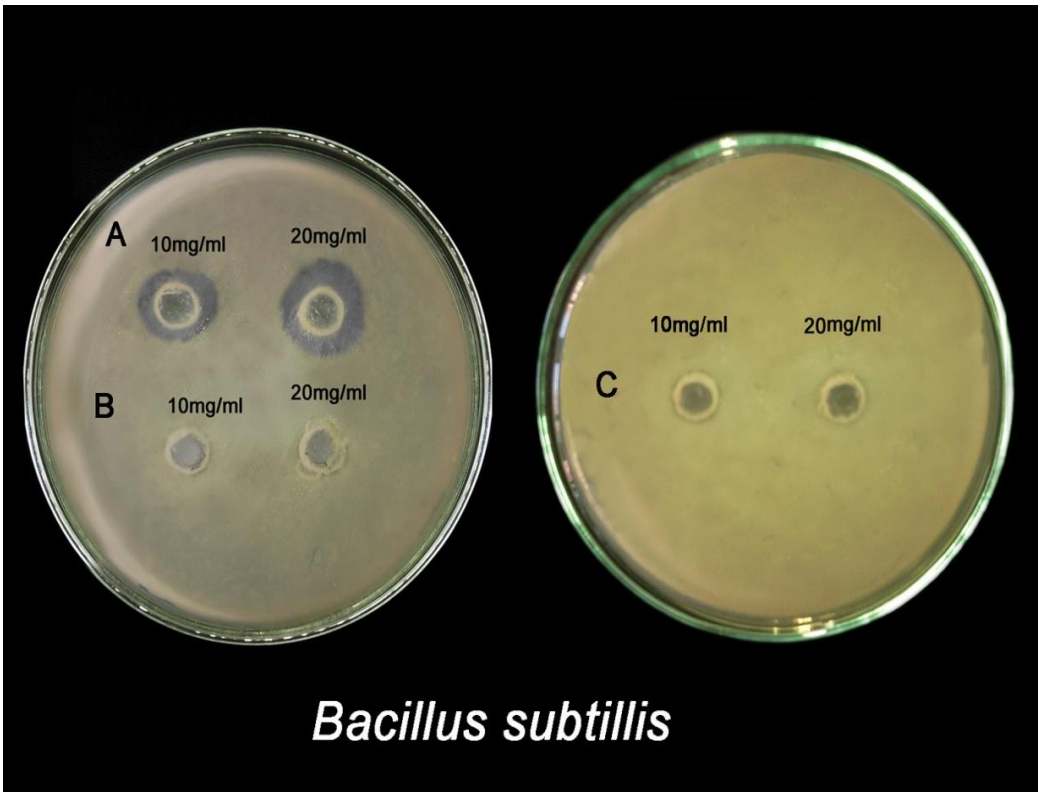

Figure 3. Antibacterial activity of different concentration of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *B. subtillis.*

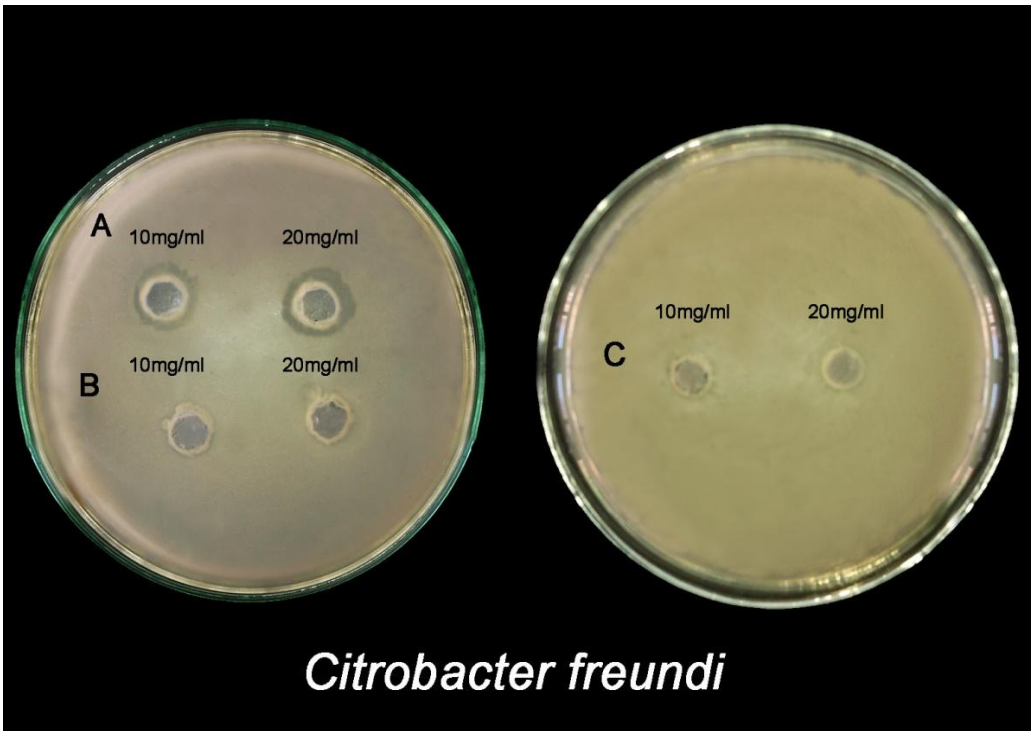

Figure 4. Antibacterial activity of different concentration of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *C. freundi.*

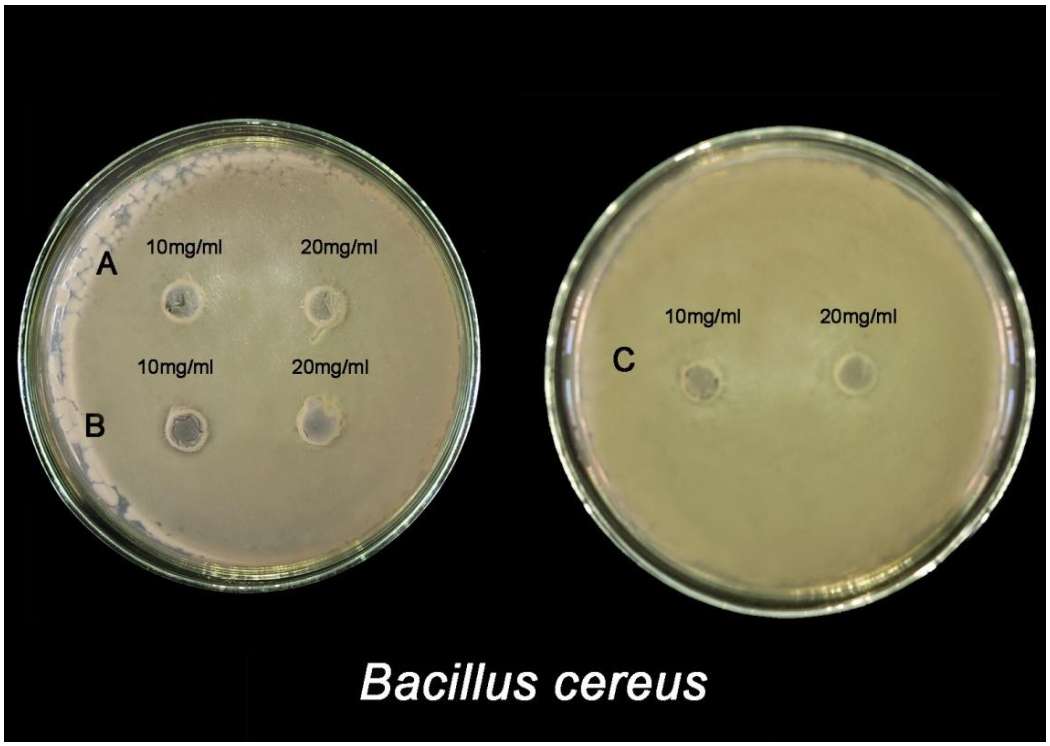

Figure 5. Antibacterial activity of different concentrations of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *B. cereus.*

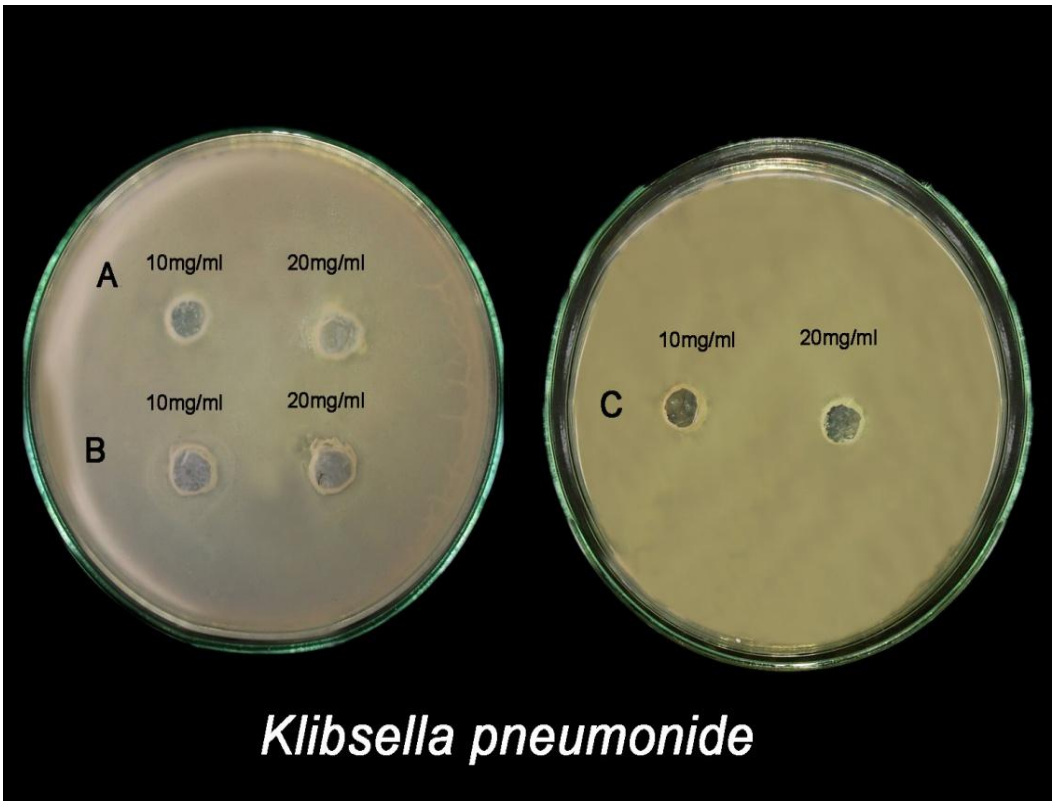

Figure 6. Antibacterial activity of different concentrations of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *K. pneumoniae.*

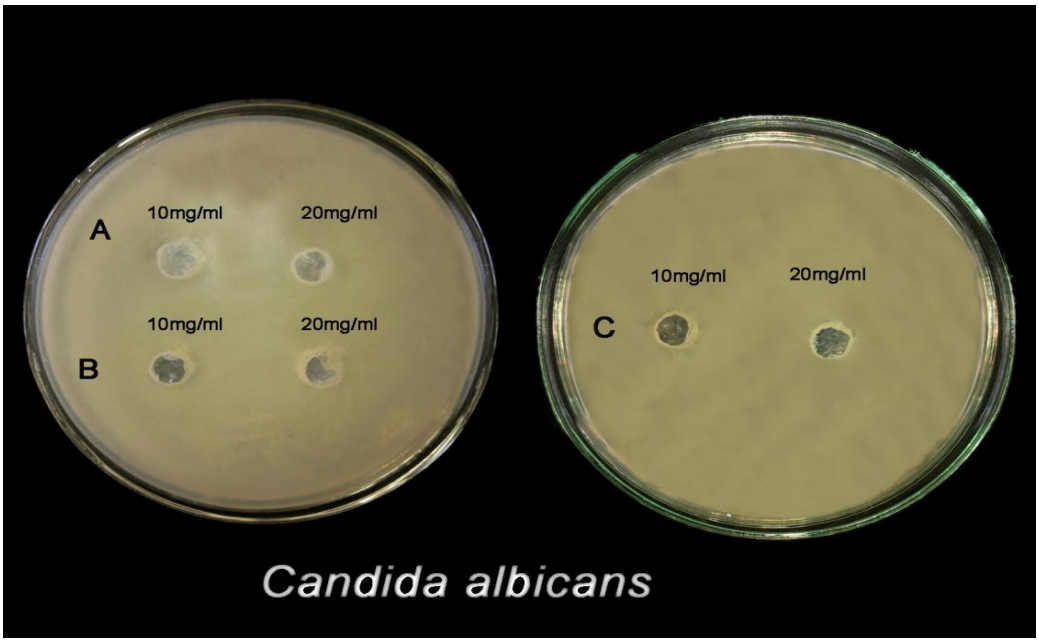

Figure 7. Antimicrobial activity of different concentrations of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *C. albicans.*

Table 3. Inhibitory zone (mm) of *L. quinquestriatus* against two bacterial strains.

Bacteria strain	Concentration	
	Leiurus quinquestriatus	
	20 mg/ml	**10 mg/ml**
Bacillus subtillis	19.66 ± 0.95	17 ± 0.127
Citrobacter freundi	17.66 ± 0.255	15.33 ± 0.337

19.66 and 15.33 mm. While, all the bacterial strain (*B. cereus*, *K. pneumoniae)*, and fungus (*C. albicans*) were resistant to this crude venom. These results consistent with that of Ahmed et al. (2012) which found that scorpion *Heterometrus xanopus* has antibacterial effect on *B. subtillis* with inhibition zone of 30 mm, while, *Escherichia coli* showed resistance against the same venom. This study was compatible with Liu et al. (2009) study on wolf spider *Lycosa singoriensis* which was effective only on *B. subtillis* under very low concentration (3 mg/ml) and has weak effect on *C. albicans* fungus. Interestingly, *Buthus martensii* venom was detected only against Gram-positive bacteria but not against Gram-negative one (Gao et al., 2007). Because venom consists of many different substances like proteins and enzymes which are responsible for its biological activities, therefore, these compounds may interact with specific molecules of some bacteria while not affecting other strains. Herein, we believed that the venom of *A. amoreuxi* and *A. australis* may lack effective proteins responsible for its antimicrobial activity for all strains; while *L. quinquestriatus* venom may be have effective proteins which affect some microbial strain (*B. subtillis and C. freundi*). Moreover, *L. quinquestriatus*, possesses venom that is the most toxic of all scorpions. This venom contains "histamines, enzymes, enzyme inhibitors, and the potent neurotoxins, chlorotoxin and charbydotoxin (Neurophysiologywordpress.com).

Interstingly, *L. quinquestriatus* showed the highest protein content, and this elucidated the increase in its hemolytic and antimicrobial activity. Further studies are needed using a wider spectrum of Gram-positive and negative bacteria to determine the *L. quinquestriatus, A. amoreuxi,* and *A. australis* venom for detection of their active components.

Finally, in the present study, all venom concentrations of three scorpion species showed hemolytic activity and the hemolysis percentage depending on the protein contents of their crude venom. *A. australis* venom showed the lowest hemolytic activity as compared to the other species. However, not all of them showed antimicrobial activity on the above mentioned strains. As

a result, we recommended separating the antibacterial polypeptides from *L. quinquestriatus* venom to avoid hemolytic activity of its crude venom, using other microbial strains. Furthermore, there should be application of lower concentration of *A. australis* venom on wide range of microbes, due to its lower hemolytic activity.

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