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Research Article

The Effect of Alkaloidal Fraction from *Annona squamosa* L. against Pathogenic Bacteria with Antioxidant Activities

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ABSTRACT

Background: *Annona squamosa* is used in different places such as India as a general tonic to enrich blood, relieve vomiting, cancer, as a vermicide, for skin complaints and also applied to wounds and ulcers. The purpose of the study was to evaluate the antibacterial and antioxidant properties from of the alkaloidal fraction of *A. squamosa.*

Methods: Well diffusion assay, minimum inhibitory concentration and the minimum bactericidal concentration (MBC) were used to evaluate antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa,* MRSA and *Helicobacter pylori*. DPPH and SOD assays were used to evaluate antioxidant activity. LC-MS analysis was used to identify alkaloids and scanning electron microscopy studies that revealed mode of action.

Results: Alkaloidal fraction of *A. squamosa* exhibited significant inhibition against the tested bacteria. Extracted alkaloids from the leaves of *A. squamosa* showed high level of antioxidant activities. LC-MS analyses of alkaloids of the plant were identified as corydine, sanjoinine, norlaureline, norcodeine, oxanalobine and aporphine in the leaves of *A. squamosa*. SEM analysis of the interaction of these substances with the bacteria showed morphological changes of cell wall and lysis of the targeted bacterial cells.

Conclusions: It could be concluded that the alkaloids isolated from *A. squamosa* showed good antibacterial and antioxidant activity. The results suggest the alkaloids can be a new source of antimicrobial agents against pathogenic bacteria and antioxidant source.

Introduction

Natural products from different sources such as plants have played an important role in the prevention of infectious diseases and they are of general use in health care. *Annona squamosa* L. was used in this study that belongs to the Annonaceae family. Its common names are *Nona,* According to the literature sugar apple, ata, gishta and sweet sop plant.[1-4] Its specific native range is indefinite because of widespread commercial cultivation but is generally deemed to originate from the Caribbean region.[5] It is a small semi-evergreen tree/shrub, 3-7 m tall, with irregular or crown branches. The leaves are oblong-lanceolate and pale green on both surfaces. The flowers are greenish-yellow and produced in single or short lateral clusters.[6] The petioles are green and 0.6-1.3 cm in length. The fruit of this plant is round, heart shaped, ovate or conical. It is green-yellow in colour initially, but the ripe fruit is white with the sweetly aromatic pulp also white.[2] The seeds are shiny, numerous, and blackish or dark brown in colour.[7]

It is used as a general tonic to enrich blood, relieve of vomiting, cancer treatment, as a vermicide, for skin complaints and also applied to wounds and ulcers. According to the literature, sugar apple contained varied phytochemical compounds which may be extracted from different parts of the plant.[8,9] *A. squamosa* extracts from several solvents possessed antibacterial activity against different strains of bacteria. Padhi, Panda[10] used different solvents in leaf extraction of *A. squamosa* that inhibited Gram-positive bacteria such as *S. aureus, B. subtilis, S. epidermidis* and Gram-negative bacteria including *E. coli, P. aeruginosa, S. typhi, V. alginolyticus* and *V. cholera.* The silver nanoparticles of aqueous extract from the leaf of sweet apple exhibited antibacterial activity against *B. cereus* (NCIM 2703), *B. subtilis* (NCIM 2635), *S. typhimurium* (NCIM 2501), *S. aureus* (NCIM 2654), *P. aeruginosa* (NCIM 5032) and *Proteus vulgaris* (NCIM 2813).[11] The main bioactive compounds

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found in the different parts of the plant were alkaloids such as liriodenine**,** oxoanalobine**,** anonaine, isocorydine, norisocorydine, liriodenine, abenzooxyquinazoline, samoquasine A, anolobine and reticuline.[12-14]

The aim of this study was to determine the antibacterial and antioxidant activities of the extracted alkaloids from *A. squamosa* and also to carry out LC-MS analysis and determine the mode of action of alkaloids using scanning electron microscopy (SEM).

Materials and Methods

Plant collection

A. squamosa leaves were collected in November, 2010, from Juasseh, Kuala Pilah. This plant was identified at University of Malaya herbarium and given the voucher number KLU 047368. All samples leaves were washed under tap water and dried in an oven at 40ºC for 3 days. The dried plant materials were pulverized using a grinder with mesh size of 2 mm.

Alkaloid reach fraction from A. squamosa leaves

Two hundred and fifty grams of grounded plant material were immersed in 100% cold distilled methanol, thoroughly mixed and blended for 30 minutes. The extract was filtered with a Whatman No.1 filter paper and methanol was removed at 40ºC using a rotary evaporator (Heidolph WB2000, Germany). The extract was acidified with 5% acetic acid. The liquid was then extracted with dichloromethane and the aqueous layer was made alkaline with 10% sodium carbonate (pH 10). Further fractionation of the extract was conducted with dichloromethane. The obtained fraction was concentrated under reduced vacuum at 40ºC to yield approximately 0.04 g of alkaloids fraction which was dissolved in 1 ml of 5% dimethyl sulfoxide (DMSO) and diluted to give 100, 50 and 25 mg/ml solutions. The applied method of extraction explained above was based on the procedure of Hadi and Bremner[15] with some modifications. The modifications included varying the quantity of the used samples and the incubation period of extraction. The qualitative analysis of the alkaloidal fraction was conducted using Dragendorff's reagent where a red precipitate indicated presence alkaloids.

Determination of antimicrobial activities

For this study, four species of bacteria were used *S. aureus* (RF 122), *E. coli* (UT181), *B. cereus* (ATCC 14579), and *P. aeruginosa* (PA7) were procured from cultures maintained at the Fermentation Technology Laboratory in the Microbiology Division, Institute of Biological Sciences, University of Malaya, Malaysia in same suspension for number of bacteria. Other strains that were used in this study included methicillin-resistant *Staphylococcus aureus* (MRSA-ATCC-BA-43) and *Helicobacter pylori* ATCC 43504. Antibacterial activities were measured using well diffusion assay. The used positive control was 10 mg/ml of tetracycline, while the negative control was 5% DMSO. The MIC values for the sample were determined using a standard protocol.[16] Nutrient broth (Difco, Detroit, MI, USA) was used as the medium to culture bacteria. One ml of this broth was added to the numbered tubes 1-9. One ml of the stock culture was added to tube 1 and successively diluted until tube number 7. The last 1 ml of tube 7 was discarded. Tube number 8 was used as a negative control and the tube 9 as a positive control. The bacterial inoculum was cultured in nutrient broth and incubated overnight, but *H. pylori* was inoculated into tryptic soy broth with 5% fetal calf serum (Sigma, Aldrich GmbdH, Germany) and incubated at 37°C under microaerophilic conditions. All the tubes were inoculated with 1 ml of the test bacteria media, except tube number 8, and incubated for 24 hrs at 37ºC. MIC values were determined based on the tube which showed no growth. MBC values were determined by sub-culturing the MIC assay tubes onto Muller-Hinton agar (Difco, Detroit, MI, USA), and represent the dilution at which growth was detected.

Determination of antioxidant activity

DPPH radical scavenging assay

Free radical scavenging activity was determined using the method of Bozin, Mimica-Dukic[17] The reagent of the assay was 2,2-diphenyl-1-picrylhydrazyl solution (Sigma Aldrich GmbdH, Germany) (950 µl) that was added to 50 µl of the extract (10 mg/ml) and the volumes of the solutions made up to 4 ml by adding 95% ethanol. This mixture was shaken vigorously and incubated at room temperature for two hours in the dark. All samples were measured at 515 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The percentage of DPPH radical scavenging activity of the resulting solutions was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity(\%) = [(A_{control} - A_{sample})/A_{control}] * 100 \quad \text{Eq.(1)}$$

Ascorbic acid (10 mg/ml) was used as a positive control of the assay.

IC_{50} was calculated using linear regression plots. The IC_{50} value represents the concentrations of the sample that was required to scavenge for 50% of DPPH free radicals.

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was determined using a SOD Assay Kit-WST (Dojindo Molecular Technologies, Gaithersburg). The protocol that was used in this study was modified

from Sakudo et al.[18] The Included modifications were varying the amount of the used samples and the incubation period. The alkaloidal fraction of *A. squamosa* (20 µl) was mixed with the reaction mixture in the kit. Then, the mixture was gently shaken and incubated at 37ºC for 20 min. Antioxidant activity was measured at 450 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The positive control was ascorbic acid (10 mg/ml). The negative control to measure inhibition rates of SOD-like activity used were the sample mixture without the alkaloidal fraction of *A. squamosa.*

LC-MS analysis

Alkaloidal fraction of from *A. squamosa* was analyzed with the Agilent 6530 quadrupole time-of-flight liquid chromatography mass spectrometer (Agilent Technologies, USA) with binary pump and automatic sampler, while the data were analysed by Agilent MassHunter Workstation Software B.01.03.

Scanning electron microscopy of the bacterial cells

Bacterial culture (*B. cereus*) was inoculated into nutrient broth and then incubated overnight at 37°C. This culture (1 ml) was added to one milliliter of alkaloidal fraction of *A. squamosa.* All treated and untreated samples were kept for 4 hr at 37°C. This mixture was then centrifuged at 6500 g at 4°C for 10 min. The pellet was washed twice with 50 mM sodium phosphate buffer (pH 7). The bacterial cells were re-suspended with buffer and 1 µl of suspension deposited on a membrane filter. Bacterial cells were fixed with 8% glutaraldehyde for 1 hr. The fixed cells were washed with buffer in distilled water in a ratio of 1:3 for 15 min. The bacterial cells were dehydrated in ascending concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100%) with a 15 min exposure period for each concentration. The bacterial cells were further dehydrated in different ratios of ethanol: acetone (3:1, 1:1 and 1:3) for 20 min for each mixture and then washed with pure acetone four times each for 20 min. These bacterial cells were subjected to critical point drying using liquid CO_2 and the cells mounted on a stub. The cells were then coated with gold and examined through a scanning electron microscope (Model: JEOL JBM 7001F, UK).

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were carried out using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with the control (Different letters a, b and C). A P value < 0.05 was considered significant. Each treatment was duplicated thrice and each experiment was repeated at least twice.

Results

Antibacterial activity

The zones of inhibition for the alkaloidal fraction of *A. squamosa* were as 12.66 mm for *S. aureus*, 12.33 mm for *E. coli*, 14.66 mm for *B. cereus*, 9.66 mm for *P. aeruginosa*, 16.33 mm for MRSA and 16.66 mm for *H. pylori* at a high concentration of this extract (100 mg/ml). (Figure 1).

Table 1 shows MIC and MBC values alkaloidal fraction of *A. squamosa*, exhibiting inhibition at 25 mg/ml for *S. aureus*, *E. coli*, MRSA and *H. pylori* while *B. cereus* exhibited the value of 12.5 mg/ml. However, for *P. aeruginosa* was only achieved at 50 mg/ ml.

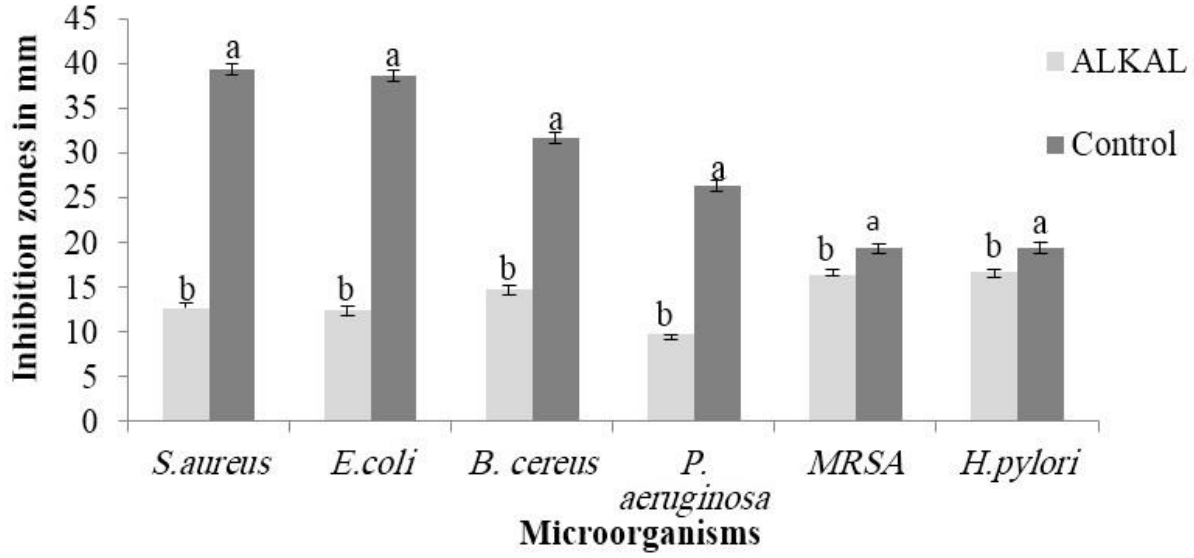

Figure 1. The inhibition zones of alkaloidal fraction of *A. squamosa* leaves (ALKAL) on selected test microorganisms. The positive control (10 mg of Tetracycline).

Table 1. MIC and MBC of alkaloidal fraction of *A. squamosa* on selected microorganisms.

	Plant extracts (mg/ml)	
Bacteria	**MIC**	**MBC**
S. aureus	25	12.5
E. coli	25	12.5
B. cereus	12.5	6.5
P. aeruginosa	50	25
MRSA	>25	12.5
H. pylori	< 25	12.5

Antioxidant activity

The results of the DPPH radical scavenging activity of alkaloidal fraction of *A. squamosa* was obtained with IC50 of 5.51 mg/ml compared to ascorbic acid as a positive control at (IC_{50} 5.18 mg/ml) (Fig. 2). While the inhibition rate of SOD-like activity of alkaloidal fraction of *A. squamosa* was at 90.69% ± 0.68 for its leaves (Figure 2).

LC-MS analysis

Figure 3 showed the LC chromatograms and MS data of the major alkaloid extract from the leaves of *A. squamosa*. The peak at the retention time of 1.562 min exhibiting an $[M + H]^+$ at *m/z* 342.1366 was identified as corydine. The peak at retention time 1.741 min exhibiting an $[M + H]^+$ at *m/z* 328.1750 was identified as sanjoinine. The peak at retention time 1.896 min exhibiting an $[M + H]^+$ at *m/z* 296.1270 was identified as norlaureline. The peak at retention time 41.688 min exhibiting an $[M + H]^+$ at *m/z* 286.2870 was identified as norcodeine. The peak at retention time 43.700 min exhibiting an $[M + H]^+$ at *m/z* 293.1047 was identified as oxanalobine. The peak at retention time 45.569 min exhibiting an $[M + H]^+$ at *m/z* 236.0960 was identified as aporphine.

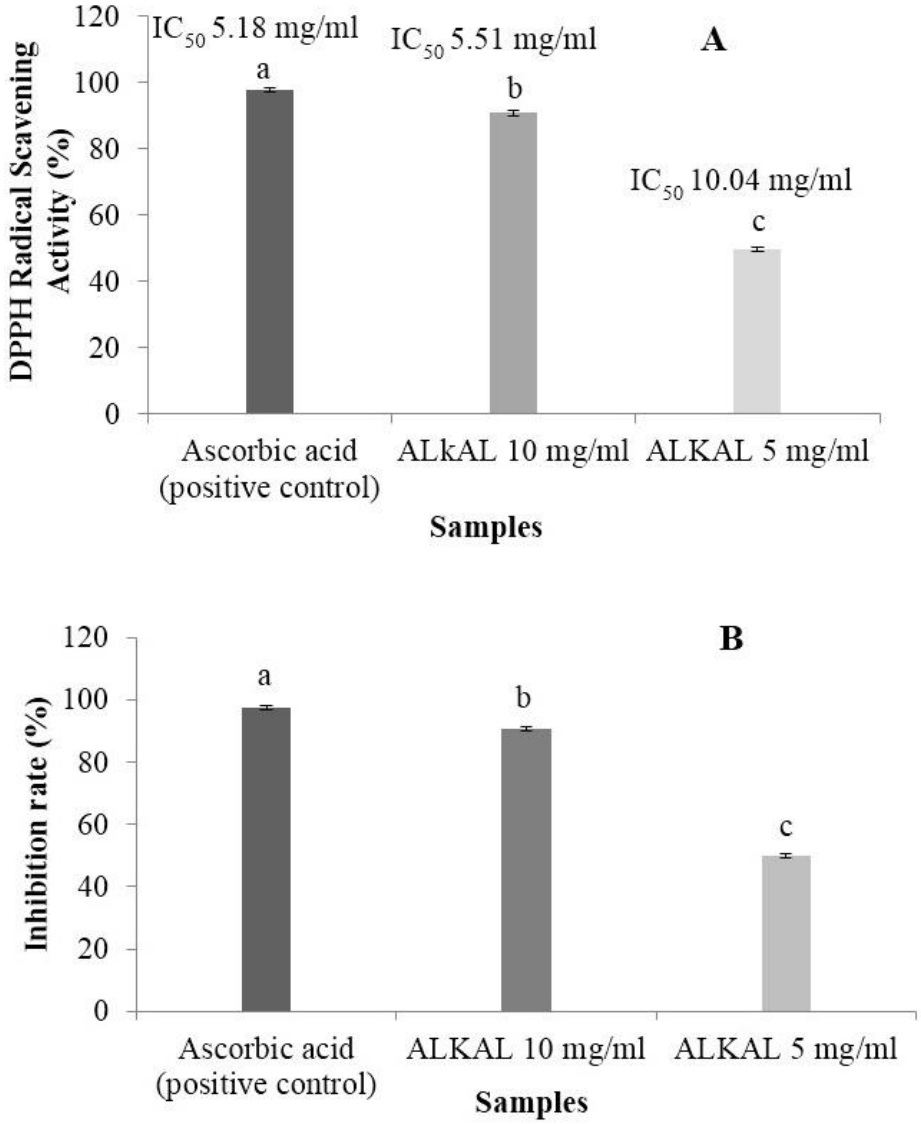

Figure 2. (A) DPPH scavenging activity with IC_{50} of alkaloidal fraction of *A. squamosa* leaves (ALKAL**)** (B) Inhibition rate of SOD activity of alkaloidal fraction of *A. squamosa* leaves (ALKAL**)**.

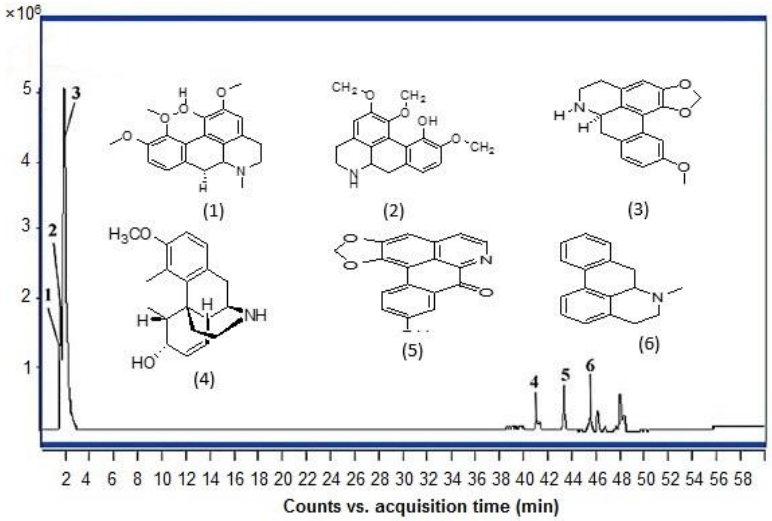

Figure 3. LC chromatograms of the major alkaloidal fraction of from the leaves of *A. squamosa* (1) corydine, (2) sanjoinine, (3) norlaureline, (4) norcodeine, (5) oxaanalobine and (6) aporphine.

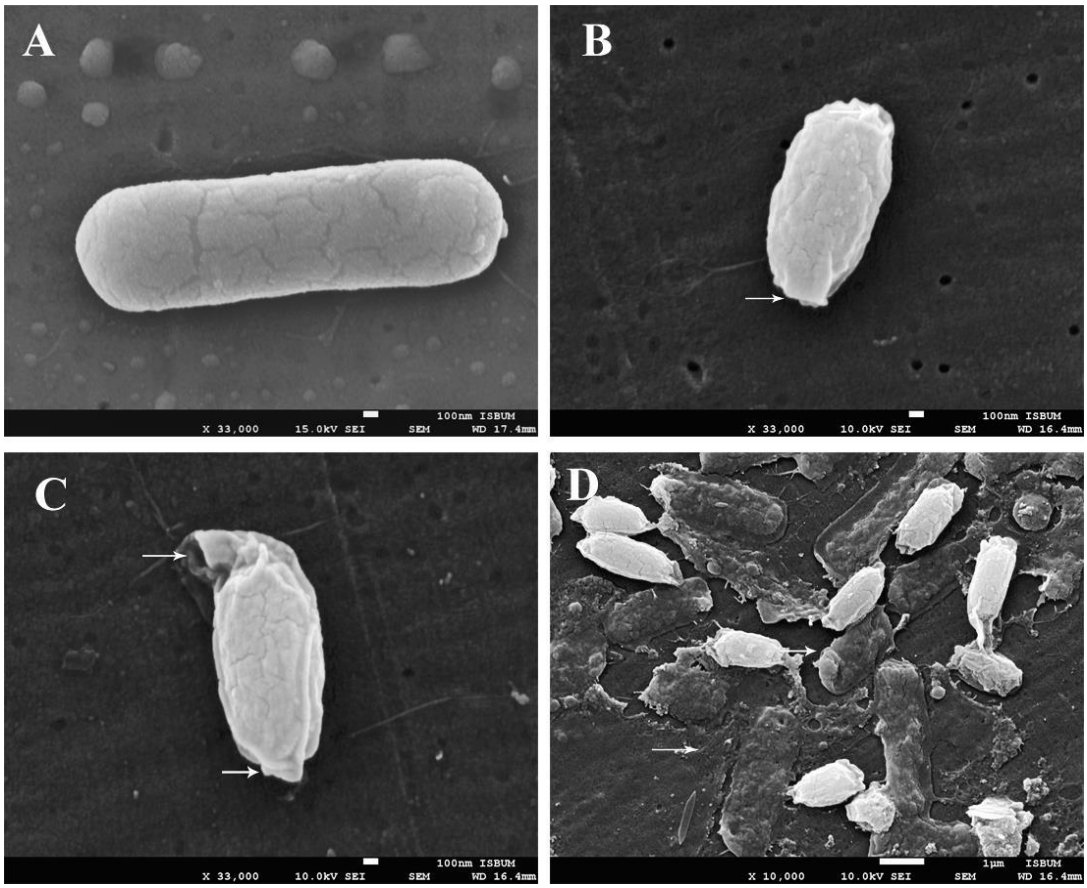

Figure 4. Effect of alkaloidal fraction of *A. squamosa* by scanning electron microscope. (A) Control: *B. cereus*. (B), (C) and (D) *B. cereus* treated with alkaloids. B) showed changes in morphology with swelling (C) rupture in cell walls; (D) showed buildup of cell debris and cell death.

Effect of alkaloids extracts from A. squamosa leaves examined by scanning electron microscopy

The effects on bacterial cells treated with alkaloidal fraction of *A. squamosa* leaves were observed using scanning electron microscope (Figure 4). The morphological changes included swelling, rupture in cell walls and cell lysis which eventually culminates in the death of the cell. Untreated cells exhibited an unchanged shape.

Discussion

Alkaloidal fraction of *A. squamosa* showed antibacterial activity against all test bacterial strains including MRSA and *H. pylori*. The plant was

known to produce alkaloids as secondary metabolites and these had antibacterial properties.[8,19,20] Costa, Pinheiro[21] reported that alkaloids *O*-methylmoschatoline, lysicamine and liriodenine from the bark of *Guatteria hispida* (Annoaceae) had antibacterial activity against *S. epidermidis*. Yang, Zuo[22] reported that the new indole alkaloids from the twigs of *Kopsia* hainanensis (Apocyanaceae) exhibited antibacterial properties against *S. aureus*. The above plants belong to the same family of Annonaceae. MIC and MBC values of alkaloidal fraction of from *A. squamosa* leaves showed significant antibacterial effects against all test bacterial strains with *B. cereus* being most sensitive.

Antioxidant activity of Alkaloidal fraction of from *A. squamosa* leaves exhibited higher DPPH radical scavenging activity with significant IC_{50} values. In addition, alkaloidal fraction of *A. squamosa* had a higher level of inhibition of SOD-like activity. Past studies revealed that the leaves of this plant were rich in alkaloids such as anonaine.[23] These compounds had antioxidant activity and showed inhibition of microsomal lipid peroxidation induced by $Fe2^{+}$/ ascorbate, CCl4/NADPH or $Fe3^{+}$ ADP/NADPH. Furthermore, these alkaloids increased deoxyribose degradation by the hydroxyl radical and increased antioxidant capacity.[24]

The major Alkaloidal fraction of from the leaves of *A. squamosa* was identified as corydine, sanjoinine, norlaureline, norcodeine, oxanalobine and aporphine. Earlier studies isolated other compounds from the leaves of this plant and identified them as liriodenine**,** oxoanalobine**,** anonaine, isocorydine, norisocorydine, liriodenine, abenzooxyquinazoline, annosquamosin A**,** anolobine and reticuline.[12,13] In the present study, some of these compounds were not detected because of the differences in the methods of extraction for isolation and identification of the alkaloids.

The effects of alkaloidal of from the leaves of *A. squamosa* showed changes in morphology of *B. cereus* such as swelling of entire cell, rupture in cell wall, cell lysis and apoptosis. Past studies reported that the alkaloids, berberine and piperine intercalate in cell wall and or DNA.[25] Obiang-Obounou, Kang[26] found that the sanguinarine from the root of *Singuinarina canadensis* cause distorted septa in MRSA with rare discerned separation and cell lysis of treated cells by using transmission electron microscopy.

Conclusion

In conclusion, this is the first report on the study of antibacterial activity antioxidant capacity and mode of action with LC-MS analysis in on the alkaloidal fraction of *A. squamosa.* Alkaloids of *A. squamosa* leaves had antibacterial activity against all strains of tested bacteria including MRSA and *H. pylori*. Alkaloidal fraction of *A. squamosa* revealed have a high level of antioxidant activity with significant IC 50 value of 5.51 mg/ml. SEM observation of the mode of action of Alkaloidal on bacterial cells showed changes in cell morphology such as swelling of cells, rupture in cell wall and cell lysis. LC-MS analysis of alkaloidal fraction of *A. squamosa* identified important compounds which may be used to develop biopharmaceuticals against infectious diseases and antioxidants source in future.

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Conflict of interests

The author claims that there is no conflict of interest.

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